Signaling mechanism of DNAM-1, a natural killer cell cytotoxicity receptor

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December 2013

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science

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ABSTRACT

DNAM-1 (CD226) was discovered in 1996 and first defined as an adhesion molecule expressed by lymphocytes. For the past few years, DNAM-1 has attracted more and more attention, and its critical roles in immunosurveillance of certain tumors, mediation of autoimmune diseases and acute graft-versus-host disease (GVHD) have been reported. Nonetheless, the signal transduction mechanism by which DNAM-1 exerts its functions in NK cells remains poorly understood.

With various biochemistry and genetic approaches, we have demonstrated that DNAM-1 is an authentic activating NK cell receptor that is capable of enhancing NK cell-mediated cytotoxicity when it is engaged by its ligands. Stimulation of DNAM-1 with monoclonal antibody could induce activating biochemical signals, leading to intracellular calcium fluxes and phosphorylation of Erk and Akt. Further investigations revealed that phosphorylation of tyrosine 319 (Y319) instead of serine 326 (S326) in the C-terminal tail of DNAM-1 is critical for DNAM-1-mediated functions. After phosphorylation at Y319, DNAM-1 recruited Grb2, Vav-1 and p85, which helped initiating activating signal cascades. Grb2 plays an essential role in the signal transduction of DNAM-1. In support of this, mutation of asparagine 321 (N321), which is another residue critical for Grb2 binding, completely abolished DNAM-1-mediated cytotoxicity. NK cells expressing DNAM-1 with mutation at Y319 or N321 showed a considerable defect in granule polarization after DNAM-1 engagement, which might explain the failure of these derivatives to promote cytotoxicity. We also tried to figure out which kinase is responsible for the

phosphorylation of DNAM-1 *in vivo*. Although it has been proposed that DNAM-1 can be phosphorylated by Fyn, NK cells deficient in Fyn could still transduce activating signals through DNAM-1, suggesting that DNAM-1 can be phosphorylated by other kinases in the absence of Fyn.

RÉSUMÉ

DNAM-1 (CD226) a été découverte en 1996 et définie dans un premier temps comme étant une molecule d'adhésion exprimée par les lymphocytes. Au cours des dernières années, de nombreuses études ont permis de mettre en évidence les rôles importants de DNAM-1, notamment dans l'immunosurveillance anti-tumorale, la médiation des maladies autoimmunes et de la maladie aiguë du greffon contre l'hôte (GVHD). Cependant, le(s) mécanisme(s) de transduction du signal par le(s)quel(s) DNAM-1 exerce(nt) ses fonctions dans les cellules NK reste(nt) à clarifier.

Par différentes approches biochimiques et génétiques, nous avons démontré que DNAM-1 est un recepteur activateur des cellules NK, capable d'augmenter la fonction de cytotoxicité de ces cellules effectrices, lors de son engagement par ses ligands (CD155 et CD122). L'utilisation d'un anticorps monoclonal agoniste de DNAM-1, pour la stimulation des cellules NK, a permis de mettre en évidence l'induction de signaux d'activation, conduisant aux flux calciques intracellulaires et à la phosphorylation des kinases ERK et Akt. D'autres études, nous ont permis de révéler que la phosphorylation de la tyrosine 319 (Y319), au lieu de la phosphorylation de la sérine 326 (S326), dans la portion carboxy-terminale de DNAM-1 est importante pour la mise en place de ses fonctions. Après phosphorylation de la Y319, DNAM-1 recrute Grb-2, Vav-1 et p85, lesquels permettent d'initier les cascades de signalisations activatrices de DNAM-1. En effet, Grb2 joue un role essentiel dans la transduction du signal de DNAM-1, car une mutation de DNAM-1 à l'asparagine 321 (N321), résidu important pour la liaison de Grb-2, abolit la fonction cytotoxicique des NK. Les

cellules NK exprimant DNAM-1 ayant une mutation Y319 ou N321 ont montré un défaut dans la polarisation des granules après engagement de DNAM-1, lequel pourrait expliquer l'échec de ces formes mutées à induire la cytotoxicité. Nous avons également cherché à determiner la (les) kinase(s) responsable(s) de la phosphorylation de DNAM-1 *in vivo*. Bien qu'il ait été proposé que DNAM-1 peut être phosphorylé par Fyn, les cellules NK déficientes pour Fyn peuvent toujours transduire les signaux activateurs *via* DNAM-1, suggérant que DNAM-1 peut être phosphorylé par d'autres kinases en absence de Fyn.

ACKNOWLEDGEMENT

First of all, I would like to thank Dr. Andre Veillette for the supervision of this work. He gave me the opportunity to work on this project in his laboratory. It's the brilliant ideas and suggestions provided by Dr. Veillette that made it possible for me to carry out this project.

I would also like to thank Dr. Marco Colonna from Washington University for generously providing DNAM-1-deficient mice and monoclonal antibodies to human and mouse DNAM-1. These reagents are critical for this project.

Finally, I would like to thank all my colleagues in Dr. Veillette's lab for all the support and encouragement in the past 3 years. In particular, I would like to thank Dr. Dominique Davidson for her unwavering support throughout the years and for her critical reading of this thesis.

INTRODUCTION AND LITERATURE REVIEW

1.1 Natural Killer Cells

Natural killer (NK) cells are effector lymphocytes that play important roles in the innate immune responses against cancer cells and pathogens. They are capable of recognizing and destroying target cells that have been stressed, transformed by oncogenes, or infected by virus [1, 2]. Unlike T and B cells, whose differentiation and function are mainly mediated by antigen-specific receptors expressed at the surface (T cell receptor (TCR) and B cell receptor (BCR), respectively), NK cells exert their function by expressing a sophisticated repertoire of cell surface receptors that do not require somatic recombination [3]. Activation or tolerance of NK cells is determined by the balance between the signals delivered by these receptors. As NK cells can eliminate target cells without pre-immunization, they react faster than T and B cells to the challenge of virus infection or cell transformation. It has been demonstrated that several mechanisms are involved in the function of NK cells. The primary mechanism, termed “natural cytotoxicity”, is mediated by the capacity of NK cells to release cytolytic granules (i.e., perforin and granzymes) that can induce the death of target cells directly. Furthermore, NK cells can facilitate the recruitment and activation of other immune cells by secreting cytokines such as interferon-γ (IFN-γ), thus amplifying the subsequent immune reaction [4, 5].

1.1.1 Development of NK cells

NK cells, like T and B cells, are derived from hematopoietic stem cells (HSCs); they are the three major lineages of lymphocytes. While T cells and B cells constitute the major cellular components of the adaptive immune response, NK cells are considered to be an indispensible part of the innate immune system [6, 7]. It has been demonstrated that NK cells, like B cells and myeloid-lineage cells, develop primarily in the bone marrow, and that an intact bone marrow microenvironment is critical for the maturation and function of NK cells [8]. In contrast, thymus seems dispensable for NK cell development, since athymic nude mice do not show any defect in development and maintenance of NK cells [9, 10].

In the last several decades, technological advances have made it possible for researchers to investigate the detailed development events of NK cells in depth. A model for NK cell development has been proposed [6] (**Figure 1.1**). Although NK cell development is obviously a continuous process, people have operationally divided it into several different stages: NK precursor (NKP), immature NK cell (iNK) and mature NK cell (mNK). NKPs, characterized by expression of CD122 and NKG2D but no other NK cell markers, are derived from HSCs. In mice, most NKPs exist in the bone marrow, but some NKPs have also been identified in the thymus, spleen and lymph node (LN) [11, 12]. Although the signals that regulates the transition of HSCs to NKPs are poorly understood, this process likely involves interactions between HSCs and stromal cells [13]. Cytokine signals, generated by Flt3, c-kit, or γ_c–dependent receptors, could also influence NK cell commitment [14-16]. Additionally, generation of NKPs from HSCs is tightly controlled by the expression of

several critical transcriptional factors (TFs), such as the Ets family members PU.1 and Ets-1 and members of the Ikaros zinc-finger family, including Ikaros, Helios, and Aiolos [17]. Under the influence of Ikaros, PU.1 and E2A (a transcriptional factor belonging to E-protein family), HSCs commit to the lymphocyte lineage and differentiate into early lymphoid progenitors (ELPs) and common lymphoid progenitors (CLPs). Expression of Id2 (Inhibitor of DNA binding protein 2) can promote NK development from HSC, ELP and CLP [18, 19].

After committed to the NK cell lineage, NKPs continue to develop to become mature NK cells. The development intermediate (i.e., iNKs) between NKPs and mature NK cells has been characterized and defined by expression of NK1.1, but not DX5, Ly49 receptors or CD11b [20]. As iNKs can be found in the bone marrow as well as in the liver, it is likely that the liver represents an important site for iNKs to finish their maturation [21].

Maturation of iNKs in the bone marrow and in the liver is coordinated by several critical factors, including cytokines (e.g., IL-15 produced and presented by stromal cells [22]), signaling pathways (e.g., signals emanating from receptors of the Axl protein tyrosine kinase (PTK) family) and several TFs (e.g., Gata-3, IRF-2, T-bet). Mature NK cells are characterized by CD49b (DX5) and Ly49 receptor expression. CD11b and CD43 are also late markers expressed by mature NK cells. Once NK cells complete the differentiation process, some of them will leave the bone marrow and redistribute in the peripheral organs, including spleen, liver, LNs, lung, omentum and uterus during gestation. Mature NK cells can also be found in the bone marrow and

blood. Homeostasis of these peripheral NK cells is tightly controlled by cell-intrinsic mechanisms (via TFs) and by extrinsic signals, including the cytokine IL-15 and the transforming growth factor (TGF)-β [23, 24].

1.1.2 NK cell function

Natural killer (NK) cells were named because of their constitutive capacity to rapidly eliminate susceptible cells without pre-immunization. It has been demonstrated, both in humans and mice, that NK cells play an important role in the early control of virus replication (especially herpesvirus) and tumor development [25, 26]. NK cells are equipped with a variety of methods to destroy target cells. Firstly, NK cells can kill susceptible cells directly through exocytosis of perforin- and granzymes-containing cytoplasmic granules. Secondly, NK cells express Fas ligand and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL). Through binding to their receptors on target cells, Fas ligand and TRAIL can induce the activation of caspase-8 in target cells. Caspase-8 is a caspase protein that is able to trigger the onset of apoptosis, a process leading to death of target cells. Furthermore, when the antigens are recognized and coated by antibodies, they are more susceptible to NK cell-mediated cytotoxicity through interactions between the antibody Fc domain and CD16 (i.e. FcγRIII) expressed on NK cell surface, a process named antibody-dependent cell-mediated cytotoxicity (ADCC).

In addition to their direct killing capacity, NK cells can shape innate and adaptive

immune responses in several different ways (**Figure 1.2**). Firstly, target cell debris produced by NK cell-mediated cytotoxicity can promote antigen cross-presentation to $CD8^+$ cytotoxic T cells, thus decreasing the antigenic load [27, 28]. Moreover, in many physiological and pathological conditions, NK cells will secrete large amounts of inflammatory cytokines such as interferon-γ (IFN-γ), which can shape T cells responses in lymph nodes either by a direct impact on naive T cells or by an indirect effect through dendritic cells (DCs) [29]. NK cells also produce tumor necrosis factor-α (TNF-α), which is a proinflammatory cytokine. However, in the situations of chronic or systemic inflammation, NK cells are biased to produce IL-10 instead, thus dampening macrophage and T cell responses. Additionally, it was demonstrated that NK cells also secrete some growth factors such as granulocyte macrophage colony-stimulating factor (GM-CSF) and many chemokines, including CCL2, CCL3, CCL4 [30].

The cross-talk between NK cells and DCs, as mentioned previously, has also attracted much attention in the last few years. Recent work has revealed that NK cells serve to regulate DC function in several ways, including direct killing of immature DCs and promotion of DC maturation by NK cell-derived IFN-γ or TNF-α. As DCs are the major antigen-presenting cells (APCs), the latter effect leads to enhanced T cell response, [29].

Through all these biological activities, NK cells play a critical role in the innate immune response and they also participate in the conditioning of subsequent adaptive immune response.

1.1.3 NK cell receptors

Unlike T and B cells, whose functions are mainly determined by antigen-specific receptors expressed at cell surface (TCR and BCR, respectively), NK cells express a repertoire of activating and inhibitory receptors that is calibrated to efficiently eliminate stressed cells infected by viruses or transformed by oncogenes while sparing healthy tissues to avoid autoimmunity. The balance of inhibitory and stimulatory signals received by a natural killer cell determines the outcome of interactions with target cells. In addition, NK cells also express a variety of chemotactic receptors, cytokine receptors and adhesion receptors (**Figure 1.3**).

Activating receptors

Critical activating NK cell receptors include NKG2D, CD16 (FcγRIII), signaling lymphocytic activation molecule (SLAM) family receptors, DNAM-1, as well as natural cytotoxicity receptors (NKp30, NKp44, and NKp46). Most of these receptors transduce activating signals through immunoreceptor tyrosine-based activation motifs (ITAMs), which are characterized as Tyr-x-x-Leu/Ile (where x donates any amino acid). The ITAM motifs are located in the cytoplasmic tails of activating receptors and typically repeated twice separated by between 6 and 8 amino acids. Tyrosine residues within these motifs can be phosphorylated after receptor engagement, forming docking sites for other proteins that transduce activating signals to cells.

NKG2D: NKG2D is probably the most-studied activating receptor on NK cells. It is

expressed at the surface of all murine and human NK cells as well as some T cell subsets (e.g., γδTCR$^+$ T cells) [31]. As an activating receptor on immune cells, it exerts an important role in immunosurveillance and cytokine-mediated tumor therapies [32, 33]. Moreover, it is a critical factor in the mediation of graft rejection in transplantation and pathogenesis in some autoimmune diseases [34]. NKG2D recognizes stress-induced ligands with structural homology to major histocompatibility complex (MHC) class I, such as MICA (in humans) and Rae-1 (in mice) [35, 36]. The intracellular domains of NKG2D are too short to transduce signals; therefore, they have to associate with specific adaptor proteins to be functional. Two isoforms of NKG2D have been described: long isoform (NKG2D-L) and short isoform (NKG2D-S). NKG2D-L exclusively associates with the DAP10 adaptor protein, whereas NKG2D-S is able to bind both DAP10 and DAP12 [37, 38]. The cytoplasmic domain of DAP12 contains an ITAM, which after tyrosine phosphorylation activates the Syk and ZAP70 tyrosine kinases, initiating the downstream activating signaling cascades [39]. In contrast, DAP10 does not have an ITAM but instead contains a YINM motif (where Y is tyrosine, I is isoleucine, N is asparagine and M is methionine) in its cytoplasmic domain that upon tyrosine phosphorylation recruits Grb2-Vav-1 complex and p85 subunit of phosphatidylinositol-3 kinase (PI3K), leading to intracellular calcium release and enhanced cytotoxicity against target cells [40].

The SLAM family receptors: The SLAM family is a group of hematopoietic cell-specific receptors including SLAM (CD150), 2B4 (CD244), Ly-9 (CD229), CD84,

NTB-A (Ly108 in mice) and CRACC (CD319) [41]. Through their cytoplasmic domains, SLAM receptors associate in an inducible fashion with the SLAM-associated protein (SAP) family of adaptors to transduce signals. The SAP family encompasses SAP, Ewing's sarcoma-activated transcript-2 (EAT-2), and EAT-2-related transducer (ERT). SAP transduces signals by recruiting the protein tyrosine kinase (PTK) Fyn that in turn activates Vav-1 and by preventing coupling of SLAM family receptors to SH2 domain-containing inositol 5'-phosphatase-1 (SHIP-1), an inhibitor of Ca^{2+} fluxes [42]. EAT-2 and ERT regulate SLAM receptor function in an alternative way that is less well characterized. Among the SLAM receptors, 2B4 is most studied. It is present on all human and murine NK cells, most γδTCR$^+$ T cells, and CD8$^+$ T cells. Unlike other SLAM receptors, which are "self-ligands" (that is, they are their own ligands), 2B4 recognizes as ligand CD48, a member of the CD2 family also expressed solely on hematopoietic cells. It has been demonstrated that in human NK cells, engagement of 2B4 with CD48 can augment the NK cell-mediated cytolysis of certain tumors and trigger the production of IFN-γ by NK cells [43].

Other activating receptors: Some other activating receptors are also involved in the regulation of NK function. Among them CD16, a low-affinity Fc receptor of IgG that is responsible for ADCC, is recognized as an activating receptor that is important for NK cell-mediated cytotoxicity [44]. DNAX accessory molecular-1 (DNAM-1, or CD226) is an activating receptor that has been overlooked for a long time. Its structure and function will be discussed in detail in the next section. Natural cytotoxicity receptors, including NKp30, NKp44, NKp46, belong to an important family of orphan

receptors that is implicated in the antitumor and antiviral functions of NK cells; however, their ligand specificity and signaling properties are still poorly understood [45-47].

Inhibitory receptors

NK cells express a repertoire of inhibitory receptors to antagonize signals from activating receptors and avoid attacking self-tissues. Critical inhibitory receptors on NK cells include killer cell immunoglobulin-like receptors (KIRs), leukocyte immunoglobulin-like receptors (LILRs), Ly49, CD94/NKG2A receptors, and T cell immunoreceptor with Ig and ITIM domains (TIGIT). Most of these receptors (e.g., Ly49, KIRs, LILRs, and CD94/NKG2A) specifically recognize MHC class I molecules; all these receptors share a common immunoreceptor tyrosine-based inhibitory motif (ITIM) in their cytoplasmic region, which can transduce inhibitory signals in a tyrosine-dependent manner by recruiting lipid phosphatase SHIP-1 or tyrosine phosphatase SHP-1 or SHP-2 [48]. SHIP-1 can degrade phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P_3) to phosphatidylinositol-3,4-bisphosphate (PtdIns(3,4)P_2) to terminate the PI3K signaling pathway, whereas SHP-1 and SHP-2 exert their inhibitory functions by dephosphorylating intracellular signaling proteins, such as Syk, ZAP70, PLCγ and Vav-1, thus terminating Ca^{2+} fluxes, degranulation, cytokine production and proliferation of NK cells [49-51].

Although most of the inhibitory receptors expressed by NK cells are MHC class I-specific, NK cells also express some inhibitory receptors that recognize other ligands. Among them TIGIT has attracted much attention in the past few years and will be

discussed in detail in the next section.

1.1.4 NK cell regulation

NK cell function is delicately conditioned by the activating or inhibitory signals transduced by the various receptors at the surface. Target cells commonly upregulate expression of ligands for NK cell activating receptors in conditions of cellular stress. For example, the ligands for NKG2D (e.g., MICA, Rae-1) are normally expressed at low levels in most tissues. However, in the case of cellular stress, such as DNA damage response, their expression will be upregulated, leading to better recognition and elimination by NK cells through NKG2D [52]. In addition, B7-H6, a ligand for NKp30, can only be detected in certain tumor cells but not in healthy cells [53]. Upregulation of ligands for activating receptors renders target cells susceptibility to NK cell-mediated cytotoxicity through engagement of specific activating receptors. It should be pointed out that engagement of more than two classes of activating receptors seems to be more efficient in the elimination of target cells. For example, NKG2D can work in synergy with SLAM receptors or NCRs to generate stronger activating signals.

Another mechanism named “missing self” also contributes to the full activation of NK cells. It has been shown that stressed target cells, such as cancer cells and virus-infected cells, frequently downregulate MHC class I expression at their surface. This leads to decreased triggering of inhibitory NK cell receptors that recognize MHC

class I, thus resulting in NK cell activation and eradication of these target cells [54]. Therefore, through these two mechanisms, NK cells apt to spare healthy cells that express self-MHC class I molecules and low levels of stress-induced molecules, and selectively destroy target cells that downregulate MHC class I expression and/or upregulate stress-induced molecules [52].

However, like T and B cells, NK cells also have a potential for autoreactivity. This is because the set of receptors expressed by every individual NK cell seems to be determined by a process that is largely random, and MHC class I genes are inherited independently of NK cell receptor genes [55]. Therefore, some NK cells lack inhibitory receptors that recognize self-MHC class I molecules, while some NK cells express activating receptors that interact with self ligands, including MHC molecules. However, these potentially autoreactive NK cells acquire a state of hyporesponsiveness to stimulation of activating receptors and are prevented from attacking the host cells. It has been reported that in mice or humans that lack MHC class I molecules, NK cells are unresponsive to self cells. Moreover, these self-tolerant NK cells also fail to exert effective responses to various other stimuli, including MHC class I-deficient tumor cells or crosslinking antibodies specific for activating receptors [56]. Likewise, in normal mice and humans, the NK cells expressing activating receptors that recognize self MHC are unable to react against neighboring normal cells. But these NK cells showed higher cytotoxicity against target cells *in vitro* [57]. Several models have been proposed to explain NK cell tolerance, but much attention has been focused on the role of “licensing”. According to this model, during

maturation of NK cells, their inhibitory receptors have to specifically bind to a self MHC class I molecule to induce NK cell cytotoxic potential and cytokine-production ability [58]. However, the molecular mechanisms that govern this licensing process remain unsettled.

1.2 DNAM-1

DNAX accessory molecule-1 (DNAM-1, also known as CD226) is a member of immunoglobulin superfamily encoded by a gene located on chromosome 18. DNAM-1 is constitutively expressed on the majority of T cells, NK cells and macrophages in humans. However, in mice, while most of T cells also express DNAM-1, only some of splenic NK cells and macrophages have DNAM-1 expression [59]. Since its discovery in 1996, DNAM-1 was identified as an adhesion molecule that facilitates the cytolytic function of T cells and NK cells [60]; however, subsequent work has indicated that DNAM-1 probably has more functions than just promoting cell-cell adhesion.

1.2.1 Structure of DNAM-1

DNAM-1 is a ~65 kiloDalton (kDa) type I transmembrane glycoprotein containing two Ig-like domains. The structure of DNAM-1 has been deciphered both in humans

and in mice (**Figure 1.4**). Human DNAM-1 (hDNAM-1) is composed of an 18-amino acid (aa) leader sequence, a 230-aa extracellular domain, a 28-aa transmembrane domain, and a 60-aa cytoplasmic region, whereas mouse DNAM-1 (mDNAM-1) is composed of an 18-aa leader sequence, a 233-aa extracellular domain, a 23-aa transmembrane domain, and a 59-aa cytoplasmic region [59, 60]. The amino acid sequence of mDNAM-1 demonstrates 53% homology with hDNAM-1; specifically, two critical residues (tyrosine 322 and serine 329 in humans, or tyrosine 319 and serine 326 in mice) that can be phosphorylated are conserved in these two species. These two residues have been demonstrated to exert important roles in DNAM-1-mediated signaling in lymphocyte activation and intercellular adhesion [61, 62]. Moreover, both human and mouse DNAM-1 harbor a tyrosine-valine-asparagine-tyrosine (YVNY) motif in the cytoplasmic domain, which is a typical immunoglobulin tail tyrosine (ITT); when the first tyrosine is phosphorylated, this motif constitutes a typical docking site for the Src homology 2 (SH2) domain of Grb2, an adaptor protein that is involved in the signal transduction of various activating receptors. ITTs can be found in many critical receptors that determine the development and activation of lymphocytes, such as IgG- and IgE-containing BCR in B cells, CD28 and ICOS in T cells, and DAP10 in NK cells, suggesting that DNAM-1 may play an important role in NK cell activation [40, 63, 64] (**Table 1.1**).

1.2.2 DNAM-1 “family”

The ligands for DNAM-1 have been identified both in humans and in mice, namely the poliovirus receptor (PVR, or CD155) and its family member PVR-related receptor 2 (PRR2, CD112, or Nectin-2) [59, 65]. Interaction of DNAM-1 with its ligands induces NK cell- and $CD8^+$ T cell-mediated cytotoxicity and cytokine secretion [66]. Interestingly, the ligands for DNAM-1 are shared by two other receptors: T cell-activated increased late expression (Tactile, or CD96), and TIGIT.

CD96 is a single pass transmembrane glycoprotein expressed on NK cells and T cells. The major ligand for CD96 is CD155, while CD111 (nectin-1) also binds to CD96 in mice. Although CD96 contains an ITIM-like motif in the cytoplasmic domain, it is not an inhibitory receptor. In fact, CD96 is able to promote NK cell adhesion to target cells expressing CD155, and trigger cytolysis ability of activated NK cells [67]. Thus, CD96 and DNAM-1 may compose a dual receptor system that recognizes CD155 on target cells, leading to the formation of a mature immunological synapse between NK and target cells. However, the underlying mechanism of the activating signals mediated by CD96 remains unclear.

TIGIT was identified as an inhibitory receptor that is mainly expressed on NK cells, activated $CD4^+$ and $CD8^+$ T cells [68]. It binds to the same set of ligands (CD155 and CD112) as DNAM-1, and engagement of TIGIT with its ligands can inhibit NK cell-mediated cytotoxicity [69]. Moreover, TIGIT serves to suppress T cell activation, either directly acting on T cells by attenuating TCR-driven activation signals or in an indirect way by enhancing IL-10 production by DCs [68, 70]. The inhibitory mechanism of TIGIT has been elucidated recently. TIGIT contains an

ITT-like motif followed by an ITIM motif in the cytoplasmic domain. The ITT-like motif of TIGIT seems to play a major role in its negative signaling. Proposed by this paper, engagement of TIGIT by CD155 induces the phosphorylation of Tyr225 in the ITT-like motif, leading to the binding of adaptor Grb2, which in turn recruits SHIP-1. SHIP-1 serves to terminate PI3K and MAPK signaling pathways, thus attenuating NK cell activation [71]. However, this work is partially in disagreement with previous studies on other ITT motif-containing receptors (e.g., IgE, Igμ, DAP10), which harbor the similar motifs, recruit the same adaptor molecule (Grb2) but induce activating signals in lymphocytes [40, 63].

In summary, DNAM-1, CD96 and TIGIT are immunoglobulin superfamily members that interact with the same set of ligands (CD155 and CD112 for DNAM-1 and TIGIT, CD155 for CD96). While DNAM-1 and CD96 are positive regulators of lymphocyte responses, TIGIT serves to inhibit them. Therefore, this set of receptors is strikingly analogous to the well-known co-stimulatory ICOS/CD28/CTLA-4 pathway, through which T-cell responses can be enhanced by co-stimulatory signals provided by ICOS and CD28 and attenuated by up-regulating CTLA-4 expression. This DNAM-1 "family", containing DNAM-1, CD96 and TIGIT, represents a novel signal regulation mechanism for lymphocytes and may play a critical role in their development and function.

1.2.3 DNAM-1 function

As already mentioned, DNAM-1 was initially defined as an adhesion molecule that promotes the intercellular conjugation of NK cells or cytotoxic T lymphocytes (CTLs) with target cells. Interaction of DNAM-1 with its ligands boosts cytolysis of target cells and DNAM-1-specific antibody can efficiently block this enhancement [60, 66]. It has been reported that DNAM-1 is involved in the surveillance and elimination of tumor cells such as melanoma cells, rhabdomyosarcoma cells and Ewing's sarcoma cells [72, 73]. After treatment with certain chemical carcinogens, DNAM-1-deficient mice are more susceptible to carcinogen-induced fibrosarcoma and papilloma than wild-type (WT) mice [74]. In addition, DNAM-1 is engaged in the surveillance of virus-infected normal cells. For example, recognition and elimination of hepatitis C virus (HCV)-infected hepatoma cells by NK cells relies on DNAM-1 [75]; DNAM-1 on NK cells is also involved in the eradication of human cytomegalovirus (HCMV)-infected monocytes-derived DCs (moDCs), which bear high levels of DNAM-1 ligands [76]. Moreover, mutations in the *DNAM-1* gene have been shown to be associated with multiple autoimmune diseases such as type 1 diabetes (T1D), multiple sclerosis (MS), and rheumatoid arthritis (RA) [77, 78].

DNAM-1 is also involved in the regulation of various immune cells. During T cell development, DNAM-1-mediated co-stimulatory signals may direct the differentiation of naïve T cells into the Th1 pathway by promoting IFN-γ production [79]. Macrophages and DCs, the two major antigen-presenting cell (APCs) types, express DNAM-1 ligands (CD155 and CD112) on the surface. It has been demonstrated that NK cells apt to eliminate DCs and macrophages that are exposed to

microbial stimuli in a way that relies on DNAM-1 [80, 81]. Additionally, DNAM-1 promotes the monocyte transendothelial process via its interaction with CD155 expressed on endothelial cells [82].

Recently, more interests have been focused on the role of DNAM-1 in acute graft-versus-host disease (GVHD). GVHD is a mortal complication following bone marrow transplantation. Experiments conducted on mice demonstrated that DNAM-1 on donor $CD8^+$ T cells plays a pivotal role in the development of GVHD. Compared with $CD8^+$ T cells from WT mice, donor $CD8^+$ T cells lacking DNAM-1 displayed significantly less proliferation, infiltration and cytokine production, causing milder GVHD and lower mortality in recipient mice. Moreover, GVHD could be ameliorated by administration of antibody to DNAM-1, providing a possible therapeutic approach to GVHD [83].

1.2.4 Signaling mechanism of DNAM-1

Although much work has demonstrated the important role of DNAM-1 in immune responses, little is known regarding the mechanism by which DNAM-1 exerts its functions. DNAM-1 was initially believed to function as an adhesion molecule based on the fact that engagement of DNAM-1 with its ligands promotes intercellular adhesion [60]. The association of DNAM-1with the integrin, LFA-1, was suggested to be important for the function of DNAM-1, as NK cells deficient in LFA-1 failed to exert effective responses against target cells through anti-DNAM-1 antibody-mediated

ADCC. Further investigation showed that DNAM-1 physically associates with LFA-1 in NK cells and in anti-CD3 antibody-stimulated T cells. Crosslinking CD3 and LFA-1 in human naive $CD4^+$ T cells was shown to induce tyrosine phosphorylation of WT but not of the Y322 mutated DNAM-1 (see **Figure 1.4**). The tyrosine phosphorylation of DNAM-1 on Y322 has been shown to be mediated by Fyn. The other critical residue, S329 can be phosphorylated by the protein kinase C (PKC), which is important for the association of DNAM-1 with LFA-1 [61, 62]. However, as most of these experiments were carried out on cell lines, it remains to be established whether DNAM-1 function is dependent on LFA-1 *in vivo*. Experiments performed on T cells suggested that DNAM-1 could be phosphorylated after engagement of CD3 and LFA-1 [79]. However, as CD3 is not expressed on the surface of NK cells, how DNAM-1 functions in the absence of CD3 remains enigmatic. Moreover, little is known regarding the downstream signaling molecules that are responsible for DNAM-1 signal transduction.

1.3 Research Objectives and Hypothesis

Prior to our study, there was no solid evidence to settle the question whether DNAM-1 transduces authentic intracellular signals or not and little was known about the signaling mechanism by which DNAM-1 mediates its functions. In this study, we try to elucidate the signaling mechanism of DNAM-1 in NK cells, and in particular demonstrate the capability of DNAM-1 to transduce biochemical signals.

Our hypothesis is that DNAM-1 is an activating NK cell receptor, rather than an adhesion molecule. As the cytoplasmic domain of DNAM-1 harbors an ITT motif that can be phosphorylated after stimulation, we propose that DNAM-1 promotes NK cell activation largely through its interaction with the adaptor protein Grb2.

It should be pointed out that DNAM-1 was considered as an adhesion molecule in most of previous papers. From the results we got, DNAM-1 is indeed capable of promoting adhesion between NK cells and targets; however, our work also indicates that DNAM-1 apt to mediate stimulating signals like other activating receptors, which means it should be categorized as an activating receptor instead of an adhesion molecule.

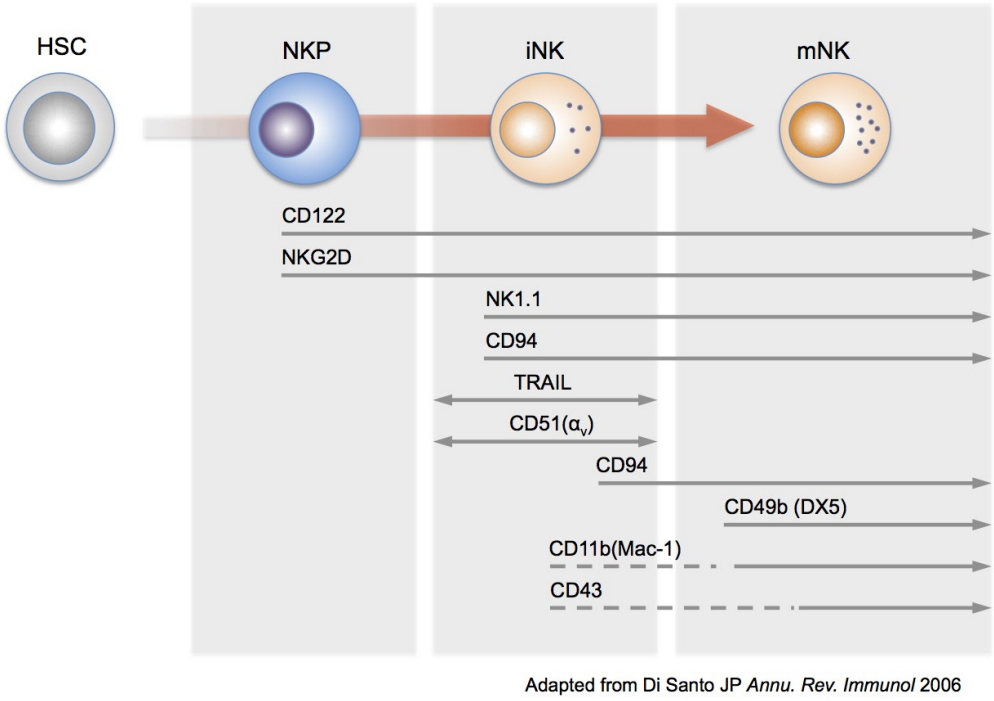

Figure 1.1 Phenotypic markers of mouse NK cells in different differentiation stages. NK precursors (NKPs) are characterized by expression of CD122 and NKG2D but no other NK cell markers. NK1.1 and CD94 are upregulated at the stage of immature NK cells (iNKs), while TRAIL and CD51 are transiently expressed in this period. Ly49 receptor repertoires and DX5 are expressed by mature NK cells at the late stage of NK cell differentiation. Expression of CD11b and CD43 increases during NK cell differentiation, although their functions in NK cell differentiation remain unknown.

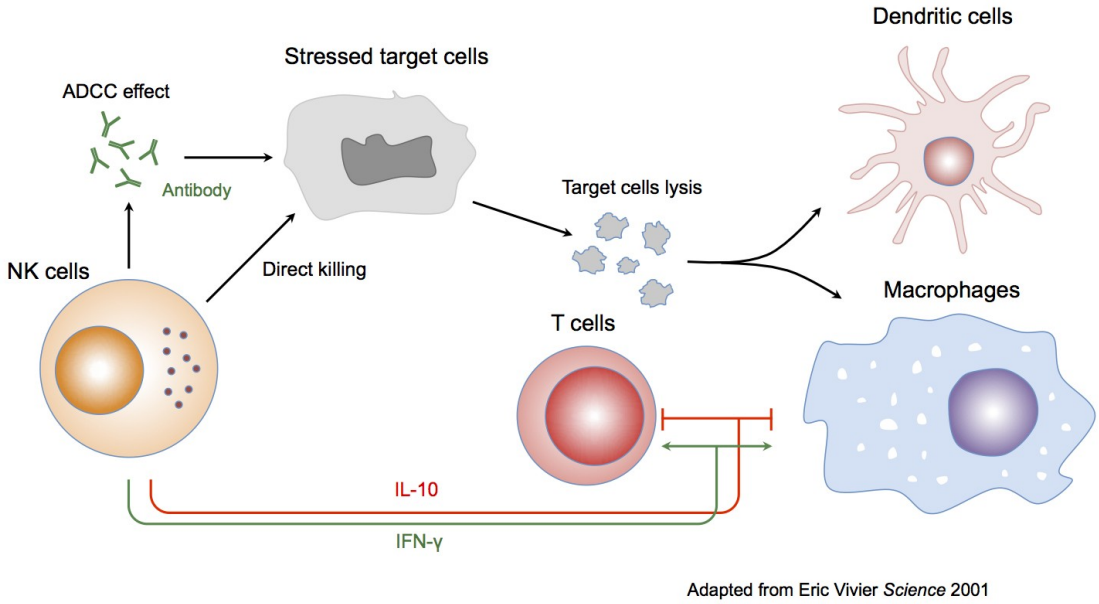

Figure 1.2 The biological functions of NK cells.

NK cells can efficiently eliminate target cells directly, i.e. without the help of antibodies; they can also recognize and destroy target cells coated with antibodies (ADCC effect). The products of the lysis of target cells will be captured by antigen-presenting cells (e.g. dendritic cells, macrophages, etc.) and presented to T cells, leading to onset of subsequent adaptive immune responses. Moreover, NK cells can secrete large amount of cytokines. In most conditions, NK cells are biased to produce IFN-γ (green arrow), which will boost macrophage and T cell responses. However, during chronic or systemic inflammation, NK cells will secrete the inhibitory cytokine IL-10 (red line), resulting in the attenuation of immune response.

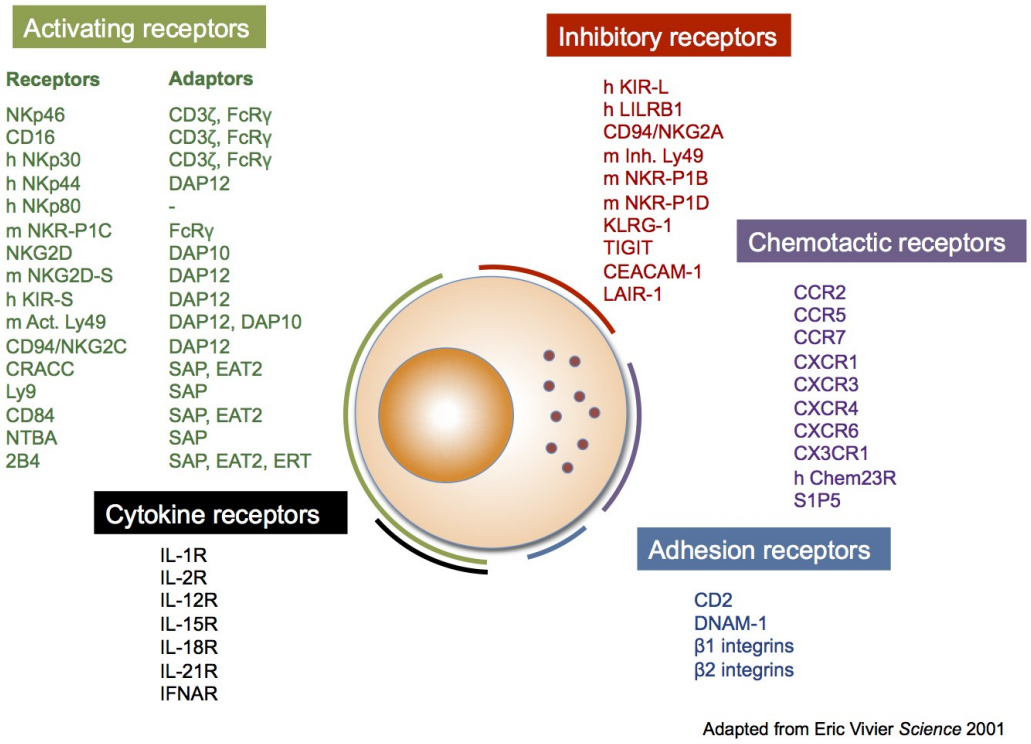

Figure 1.3 NK cell receptors.

The receptors expressed on the NK cell surface can be grouped into activating (green), inhibitory (red), adhesion (blue), chemotactic (purple) and cytokine receptors (black). The specific adaptor molecules involved in the signal transduction of the activating receptors (green) are also indicated.

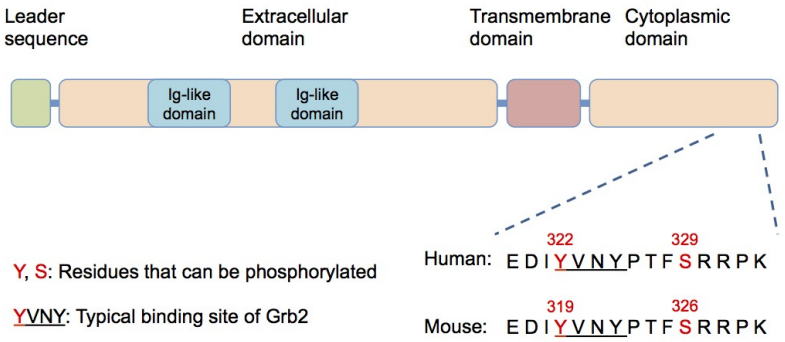

Figure 1.4 Structure of DNAM-1.

DNAM-1 is composed of a short leader sequence, an extracellular region with two Ig-like domains, a transmembrane domain, and a cytoplasmic domain. The cytoplasmic domain of DNAM-1 harbors a sequence that is highly conserved in humans and in mice; Y322 and S329 in humans, or Y319 and S326 in mice (residues marked in red) can be phosphorylated. pYVNY (the motif underlined) is a typical docking site for Grb2.

Receptor	ITT-like motif	Expression
IgG1-BCR	TIIPDYRNMI	B cells
IgE-BCR	QTFQDYANIL	B cells
Igμ	PTGPDYKNVL	B cells
CD28	LLHSDYMNMT	T cells
ICOS	DPNGEYMFMR	T cells
CD7	AACVVYEDMS	NK, T cells
JAML	DSEATYMTMH	γδT cells, CTL
DAP10	EDGKVYINMP	NK cells, CTL
DNAM-1	TREDIYVNYP	NK, T cells

Adapted from Niklas Engels *Current Opinion in Immunology* 2011

Table 1.1 ITT-like costimulatory motifs.

These motifs are conserved in several structurally unrelated costimulatory receptors on NK, T and B cells. They are characterized by asparagine and/or methionine residues at the C-terminal positions +2 and +3 relative to the phosphotyrosine, allowing for recruitment of Grb2 and p85, respectively. Conserved amino acids are marked in red. Negatively charged residues required for the kinase-substrate reaction are underlined.

MATERIALS AND METHODS

Mice: DNAM-1 KO mice were generous gifts from Dr. Marco Colonna. LFA-1 KO mice were from The Jackson Laboratory. Mice lacking Fyn or Lck were obtained from The Jackson Laboratory, and backcrossed to the B6 background at least 18 generations when the experiments were performed. Wild-type littermates were used as controls in all experiments. Animal experimentation was approved by the IRCM Animal Care Committee and was performed as defined by the Canadian Council of Animal Care.

Antibodies and reagents: Anti-mouse DNAM-1 polyclonal antibody was generated by immunizing rabbits with the mouse DNAM-1 cytoplasmic domain fused to GST. Rabbit serum was collected and its specificity towards mouse DNAM-1 was confirmed. Monoclonal antibody anti-human DNAM-1 (11A8) and mouse DNAM-1 (480.2) were generous gifts from Dr. Marco Colonna. Fluorescence conjugated-anti-CD3e (145-2C11), anti-NK1.1 (PE136), anti-CD11a (M1714), anti-mDNAM-1 (10E5), anti-mCD155 (TX56) and isotype-matched control antibodies were from eBioscience. Anti-mCD112 (MAB3387) was from Abnova. Anti-Erk (9102), anti-Akt (9272) and antibodies to phosphorylated Erk (E10) and phosphorylated Akt (9271) were from Cell Signaling. Polyclonal antibodies to SHIP-1 (165.1), Vav-1 (911), c-Cbl (989) and Fyn (97.5), and monoclonal antibodies to mouse SLAM (12F12) and 2B4 (C1.7) were generated in the Veillette laboratory. Goat anti-rat (112-005-143) was from Jackson ImmunoResearch. Anti-Grb2 (C-23) was from Santa Cruz. Anti-GST

(AB3282) was from Millipore. Anti-p85 (06-497) was from Upstate. Fluorescence conjugated-anti-hNKp46 (9E2) and anti-perforin (dG9) were from BioLegend. Anti-tubulin (236-10501) and phalloidin (A22283; binds actin) were from Invitrogen.

Primary NK cells: Fresh NK cells were obtained after intraperitoneal injection of mice with 200 μg poly(I:C) (Sigma-Aldrich). 36 hours later, spleen NK cells were isolated by positive selection (StemCell Technologies). For the generation of IL-2-activated NK cells, spleen NK cells were enriched using the negative selection kit (StemCell Technologies) and then cultured *in vitro* for 4-5 days with 1000 U/ml mouse IL-2.

cDNA and constructs: cDNAs encoding mouse DNAM-1 were obtained from Thermo Scientific. To generate site-specific mutants, *mouse DNAM-1* cDNAs were cloned into vector pBluescript II SK. Mutagenesis primers containing a codon for phenylalanine (TTT) instead of tyrosine 319 (TAT) or tyrosine 322 (TAT), or glutamine (CAA) instead of asparagine 321 (AAC), or alanine (GCT) instead of serine 326 (TCT) were designed. Mutagenesis was conducted with Site-Directed Mutagenesis Kit (Quickchange) according to the instructions of manufacture. The resulting cDNAs were fully sequenced to make certain that they carried no undesired mutations. Then the cDNAs encoding WT or mutant mDNAM-1 were cloned into vector pSRα-Puro or PA-Puro, which contain puromycin-resistance. cDNAs encoding mCD155 were cloned into vector pSRα-Puro or the retroviral vector pFB-GFP, which

encodes green fluorescent protein (GFP) for selection by cell sorting. cDNAs encoding Grb2, p85 and Fyn ΔSH2 were cloned into vector pXM139 for expression in COS-1 cells.

Cell lines: YT-S is a human NK cell line expressing moderate amounts of human DNAM-1. DT40 is a chicken B cell line. COS-1 is a fibroblast-like cell line derived from monkey kidney tissue. RMA-S is a mouse lymphoma cell line with decreased cell surface expression of MHC class I. B16 is a mouse melanoma cell line. YAC-1 is a mouse T cell lymphoma. CMT-93 is a mouse colon carcinoma cell line. K562 is a human myelogenous leukemia cell line. Hela is a human cervical cancer cell line. YT-S cells expressing WT or mutant mDNAM-1 and K562 expressing mCD155 were generated by electroporation, and cells successfully transfected with the pSRα-Puro vectors were selected by puromycin (1 μg/ml). RMA-S cells expressing mCD155 were generated by retroviral infection, and GFP^+ cells were selected by sorting. DT40 cells expressing mDNAM-1 were generated by electroporation with PA-Puro plasmids encoding mDNAM-1 and selected by puromycin. COS-1 cells were transfected with plasmids encoding mDNAM-1, Grb2, p85 and Fyn ΔSH2 with the help of Lipofectamine 2000 (Life Technologies). Ectopic expressions of all these molecules were verified by flow cytometry analysis or immunoblot analysis. All the non-adherent cells were maintained in RPMI 1640 with 10% fetal bovine serum, penicillin, streptomycin and glutamine (Life Technologies). The adherent cells (i.e. COS-1, B16 and Hela) were maintained in Dulbecco's modified Eagle's medium

supplemented with 10% fetal bovine serum, penicillin, streptomycin and glutamine (Life Technologies). Cells were cultured at 37ºC in a 5% CO_2 humidified incubator.

Retroviral Infection: The cell line Phoenix was used for packaging retroviruses. pFB-GFP encoding mCD155 was introduced into Phoenix cells together with VSV-G (an envelope vector) under the assistance of Lipofectamine 2000 (Life Technologies). After 24 hours, culture medium of Phoenix cells containing viable retrovirus was collected and incubated with RMA-S cells in the presence of polybrene (5ug/ml). The infection process was repeated the next day. Five days later GFP^+ cells were purified by sorting and the expression of mCD155 on these GFP^+ RMA-S cells was verified by flow cytometry analysis.

Cytotoxicity assays: Target cells (~1 million) were labeled with ^{51}Cr for one hour at 37ºC. Then target cells (3000 per well) were mixed with NK cells at various effector/target ratios in duplicate. After incubation for 6 hours at 37ºC, supernatant of the mixture was collected and release of ^{51}Cr into the supernatant was measured with a γ counter.

Antibody-mediated cell stimulation: YT-S, DT40 or IL-2-activated NK cells were collected and washed for two times with RPMI without serum. Then cells were counted and cell concentrations were adjusted. After incubation with 480.2 (anti-mDNAM-1), 11A8 (anti-hDNAM-1), or C1.7 (anti-2B4) for 10 minutes at room

temperature, cells were washed once and resuspended in RPMI containing secondary antibody. Afterwards cells were immediately incubated for indicated time at 37ºC, and stimulations were terminated by addition of equal amount of 2×TNE buffer containing various phosphatase inhibitors. Unstimulated cells were processed in the same manner, except that no primary antibody was added. After lysis, cell debris was eliminated after centrifuge and the supernatant was recovered for the following immunoprecipitation and immunoblot analysis.

Immunoprecipitations (IPs) and re-immunoprecipitations (re-IPs): Protein concentrations in total cell lysates were measured with Protein Assay Dye Reagent (Bio-Rad) and standardized to same level. For IPs, polyclonal antibodies derived from rabbit were added to cell lysates and incubated at 4ºC for 1h with subtle agitation, followed by addition of *Staphylococcus aureus* protein A (Staph A, EMD Bioscience Inc.) and incubated at 4ºC for another 1 hour. Staph A was then extensively washed and the associated proteins were eluted by loading buffer for polyacrylamide gel and ready for standard immunoblot analysis. For re-IP of Vav-1, eluted proteins from immunoprecipitation of mDNAM-1 were diluted with 1×TNE buffer supplemented with phosphatase inhibitors and incubated with anti-Vav-1 (or normal rabbit serum (NRS) as control) and Staph A same as in the first IP. Proteins precipitated by anti-Vav-1 were eluted and ready for immunoblot analysis.

Immunoblot analysis: Samples from immunoprecipitations or directly from cell

lysates were resolved in loading buffer, denatured at 105ºC for 5 minutes and loaded onto 8% or 10% polyacrylamide gel. After electrophoresis, proteins were transferred onto polyvinylidene difluoride (PVDF) membrane using electroblotting system in wet conditions. Then the membranes bearing proteins were blocked with 5% milk or 5% bovine serum albumin (BSA) for 1 hour, incubated with primary antibodies for 1.5 hours, washed 3 times, incubated with horseradish peroxidase (HRP) or ^{125}I-coupled secondary antibodies for 1h and washed 5 times afterwards. Protein bands specifically recognized by primary antibodies were revealed by ECL detection reagents (GE Healthcare Biosciences) or radioactivity emitted by ^{125}I.

Calcium fluxes: YT-S cells (2×10^6 per sample) or DT40 cells (3×10^6 per sample) were loaded with Indo-1 (10 μM; Invitrogen) for 20 minutes at 37ºC. After washing, 480.2 (antibody to mDNAM-1) was added to cells (1 μg per sample) and incubated at 37ºC for 4 minutes. Then cells were loaded to a BD LSR (BD Biosciences) and measured for 30 seconds to get a baseline curve. Goat anti-rat (3 μg per sample) was added and changes in intracellular calcium (calculated as Fluo-4/Fluo-5) over time were monitored for another 4.5 minutes.

Peptide binding assays: Biotinylated peptides encompassing segments of the cytoplasmic domain of mDNAM-1 (KEDIYVNYPTFSRRPKPRLK) were synthesized, with Y319 phosphorylated or not, by the W.M. Keck Facility (Yale University, New Haven, CT). Peptides were first coupled to agarose-avidin beads

(Neutravidin; Pierce Biotechnology). Peptide-coupled beads were then incubated with lysates of indicated cell line. After 1.5 hours, beads were washed extensively to remove unbound proteins and associated proteins were detected by silver staining or immunoblot analysis.

Silver staining: Proteins associated with mDNAM-1 peptides were eluted and separated through polyacrylamide gel electrophoresis. The gel was fixed in 50% methanol, 10% acetic acid for 30 minutes. After rinsed, the gel was reduced by sodium thiosulfate (0.2 g/L) for 2 minutes and then incubated in silver nitrate (2 g/L) for 30 minutes in darkness. Afterwards, the gel was developed to desired intensity with the developing solution containing sodium carbonate (30 g/L), formaldehyde (0.05%) and sodium thiosulfate (10 mg/L). The reaction was stopped by incubating the gel in 1% acetic acid for 30 minutes. The protein band indicated in Fig 6a was excised and sent to IRCM Proteomic Discovery Platform for mass spectrometry analysis.

Conjugation assays: YT-S and RMA-S cells were collected and washed with RPMI without serum. Then YT-S cells were stained with Brilliant Violet 421-conjugated antibody to hNKp46, while RMA-S cells were stained with Alexa 647-conjugated antibody to mSLAM. After incubation with antibodies for 30 minutes on ice, cells were washed and cell concentrations were adjusted to 2×10^6/ml. 50 μl YT-S cells were mixed with same volume of RMA-S cells and the mixtures were immediately

incubated at 37ºC for indicated time. The reactions were terminated by addition of 100 µl 4% formaldehyde. The conjugates (characterized as $hNKp46^{+}mSLAM^{+}$) were detected by flow cytometry.

Confocal microscopy imaging: YT-S and RMA-S cells were collected and cell concentrations were adjusted to 2×10^{6}/ml. 100 µl YT-S cells were mixed with 100 µl RMA-S cells and incubated at 37ºC for 30 minutes. After incubation, cell mixtures were loaded onto poly-l-lysine-coated slides. Afterwards, cells were fixed and permeabilized by PBS solution containing 4% formaldehyde, 0.1% saponin and 0.1% Triton X-100. Cells were then blocked with 5% mouse serum and stained with fluorescence-conjugated phalloidin, which constitutively binds actin, and antibodies to tubulin and perforin.. The confocal images were taken with LSM 710 confocal microscopy (Zeiss). More than 50 conjugates were pictured for each condition.

RESULTS

Engagement of DNAM-1 with its ligands enhances NK cell-mediated cytotoxicity

DNAM-1 has been reported to be able to promote killing of target cells by NK cells and Cytotoxic T Lymphocytes (CTLs) [74, 84]. Therefore we performed ^{51}Cr-release assay to ascertain the impact of DNAM-1 engagement on NK cell-mediated cytotoxicity. Firstly, expression of mouse DNAM-1 ligands (mCD155 and mCD112) on target cells RMA-S (a mouse lymphoma cell line) and B16 (a mouse melanoma cell line) was examined. While RMA-S expressed neither of the ligands, both of them were detected on B16 (**Fig. 2.1a**). In wild-type (WT) mice, DNAM-1 was only expressed by about half of splenic NK cells. DNAM-1 KO mice have been generated and provided by Marco Colonna [84]; expression of DNAM-1 in these mice was completely abolished (**Fig. 2.1b**). Compared to WT NK cells, DNAM-1-deficient NK cells demonstrated severely decreased killing of B16, while no defect was seen toward RMA-S (**Fig. 2.1c**). These findings were consistent with the notion that DNAM-1 was required for the elimination of melanoma cells [72]. To better understand the effect of DNAM-1 engagement, we expressed the ligand for mouse DNAM-1 (mCD155) on K562 human myelogenous leukemia cells and investigated its ability to promote cytolysis by NK cells (**Fig. 2.1d**). Ectopic expression of mCD155 enhanced killing of K562 by WT but not by DNAM-1-deficient NK cells. Similar results were obtained with polyinosinic-polycytidylic acid (poly (I:C))-activated NK cells (**Fig. 2.1e**) and IL-2-activated NK cells (**Fig. 2.1f**).

Next we verified DNAM-1 function in YT-S, a human NK cell line that has been widely used for the investigation of NK cell function. YT-S constitutively expressed a

moderate amount of human DNAM-1 (hDNAM-1) which is not recognized by the anti-mouse DNAM-1 (mDNAM-1) antibody (**Fig. 2.1g**). To unravel the signaling mechanism of mouse DNAM-1, we expressed mDNAM-1 on YT-S cells and expressed mCD155 on RMA-S (**Fig. 2.1h, i**). As YT-S is a human cell line while B16 and RMA-S were derived from mice, parental YT-S cells showed no killing toward these two target cells. However, ectopic expression of mouse DNAM-1 on YT-S significantly enhanced killing of B16 as well as RMA-S that expressed mCD155, but exhibited no effect on the control RMA-S-GFP cells (**Fig. 2.1j**). These findings, together with the results obtained with mouse primary NK cells, confirmed that engagement of DNAM-1 with its ligands was sufficient to promote NK cell cytotoxicity.

Stimulation of DNAM-1 induced phosphorylation of Erk and Akt both in IL-2-activated NK cells and transfected YT-S cells

To address whether DNAM-1 engagement could trigger activating signals in NK cells, we stimulated IL-2-activated NK cells with a monoclonal antibody 480.2 anti-mDNAM-1, which was generated and generously provided by Marco Colonna [84]. Cell lysate proteins were separated by polyacrylamide gel electrophoresis and phosphorylated proteins was detected by specific antibodies. We found that DNAM-1 engagement resulted in phosphorylation of Erk and Akt (**Fig. 2.2a**), which are two important regulators governing the activation and proliferation of lymphocytes [85, 86]. As mouse NK cells express high levels of CD16, a Fc receptor for IgG, it was

possible that the biochemical signals we detected were triggered by engagement of CD16. To exclude this possibility, we performed the same experiment with DNAM-1-deficient NK cells, and found that the same treatment failed to induce any biochemical signal, indicating that DNAM-1 was responsible for the phosphorylation of Erk and Akt (**Fig. 2.2b**). We also investigated the biochemical signals in a cell line by stimulating YT-S cells expressing mouse DNAM-1 with antibodies to 2B4, hDNAM-1 or mDNAM-1. In YT-S cells, engagement of 2B4, an activating receptor belonging to the SLAM family receptors, led to phosphorylation of Erk and Akt. While activation of human DNAM-1 evoked the phosphorylation of Akt but not Erk, mouse DNAM-1 engagement induced phosphorylation of both, indicating that compared to human DNAM-1, mouse DNAM-1 might be able to trigger a broader range of signals (**Fig. 2.2c**).

DNAM-1-mediated cytotoxicity and signals are independent of LFA-1

LFA-1 was reported to physically associate with DNAM-1 in NK cells and to play a critical role in DNAM-1 function [61]. However, it remains unclear about whether LFA-1 is indeed involved in DNAM-1 signal transduction or acts as an adhesion molecule to facilitate cell-cell contact. To address this question, we assessed the natural cytotoxicity of NK cells from LFA-1 KO mice, which were bought from Jackson Laboratory. LFA-1-deficient NK cells showed a complete failure to kill RMA-S and exhibited severely diminished cytotoxicity toward YAC-1 (a mouse thymoma cell line that expresses ligands for DNAM-1 and NKG2D). Surprisingly, the

capacity of NK cells to destroy B16 was preserved in the absence of LFA-1 (**Fig. 2.3a**). Combined with the notion that NK cells lacking DNAM-1 showed defective killing of B16 (**Fig. 2.1c**), the ability of LFA-1-deficient NK cells to kill B16 suggested that LFA-1 was not required for DNAM-1-triggered cytotoxicity. LFA-1-deficient NK cells also exhibited ability, although to a less extent compared to WT NK cells, to kill K562 cells expressing or not mCD155 (**Fig. 2.3b**). Indeed, expression of mCD155 on K562 was still capable of promoting killing by LFA-1-deficient NK cells. When DNAM-1 was engaged on LFA-1-deficient NK cells, cytotoxicity was promoted by ~120% compared to cells on which it was not engaged, an activating effect slightly higher than that obtained with WT NK cells (**Fig. 2.3c, d**). Moreover, DNAM-1-evoked phosphorylation of Erk and Akt was not affected by LFA-1 deficiency (**Fig. 2.3d**). From these findings, we postulate that the requirement of LFA-1 for killing by NK cells is largely due to its capacity to promote adhesion between effector and target cells, and that LFA-1 is dispensable for the signal transduction of DNAM-1.

Tyrosine 319 (Y319) but not serine 326 (S326) is critical for DNAM-1-mediated cytotoxicity and DNAM-1-induced signals

Analysis of the amino acid sequence of DNAM-1 revealed that DNAM-1 is highly conserved among humans, gibbons, monkeys and mice and the sequence of 15 amino acids close to the C-terminal is identical in these species (**Fig. 2.4a**). Y319 and S326 (in mice, or Y322 and S329 in humans), which have been reported to be

phosphorylated after stimulation [61, 62], are conserved among the different species, suggesting that they have important evolutionary biological functions (**Fig. 2.4a**). To investigate the role of these two residues in DNAM-1 function, we mutated Y319 to phenylalanine (Y319F mutation), S326 to alanine (S326A mutation), and expressed these DNAM-1 mutants in YT-S cells (**Fig. 2.4b**). Compared to WT DNAM-1, engagement of Y319F DNAM-1 with its ligand showed a complete failure to boost killing of target cells. No defect was observed with DNAM-1 harboring the S326A mutation, indicating that Y319 rather than S326 is critical for DNAM-1-mediated cytotoxicity (**Fig. 2.4c**).

To ascertain how Y319 affects DNAM-1 function, we tested the capacity of the Y319F mutant of DNAM-1 to transduce biochemical signals. YT-S cells expressing WT or Y319F DNAM-1 were stimulated with 480.2 (an anti-mDNAM-1 monoclonal antibody), and phosphorylation of Erk and Akt was assessed. While engagement of WT DNAM-1 induced a pronounced phosphorylation of Erk and Akt, engagement of Y319F DNAM-1 resulted in a complete failure to induce this phosphorylation (**Fig. 2.5a**). In agreement with previous reports [61, 79], we found that DNAM-1 could be phosphorylated after stimulation, and that its phosphorylation was also abolished when Y319 was mutated (**Fig. 2.5b**), suggesting that Y319 is the residue responsible for phosphorylation and signal transduction of DNAM-1. Next we analyzed the tyrosine phosphorylation of several molecules that might be involved in DNAM-1 signal transduction. We found that SH2 domain-containing inositol 5'-phosphatase-1 (SHIP-1), Vav-1 and c-Cbl were phosphorylated after DNAM-1 stimulation, in a

Y319-dependent manner (**Fig. 2.5c**). Vav-1 is a guanine nucleotide exchange factor promoting multiple functions in immune cells, including receptor clustering, cytoskeletal reorganization and Ca^{2+} fluxes [87]. SHIP-1 is a lipid phosphatase that inhibits immune cell activation by terminating Ca^{2+} fluxes [88], and c-Cbl functions as an E3 ubiquitin-protein ligase that promotes ubiquitination and degradation of receptor tyrosine kinases [89]. SHIP-1 and c-Cbl probably act to antagonize the activating signals by DNAM-1 to prevent immune cells from over-activation. After WT DNAM-1 engagement, we noted that there was one molecule (~95-kDa) which was phosphorylated and co-precipitated with the DNAM-1 complex (**Fig. 2.5b**). By re-immunoprecipitation assays, we ascertained that this 95-kDa phosphorylated protein was Vav-1 (**Fig. 2.5d**). As both Vav-1 and SHIP-1 are prominent regulators of Ca^{2+} fluxes, we examined the Ca^{2+} fluxes in YT-S cells after DNAM-1 engagement. Stimulation of DNAM-1 alone was able to trigger intracellular Ca^{2+} flux in YT-S cells, and this response was also dependent on Y319, as revealed by the absence of response obtained with the Y319F DNAM-1 mutant (**Fig. 2.5e**). These findings support the idea that DNAM-1 signaling is initiated by phosphorylation of Y319, followed by tyrosine phosphorylation and recruitment of Vav-1, which in turn triggers intracellular Ca^{2+} fluxes.

Deficiency of Fyn or Lck does not abolish DNAM-1 function

We have shown that mouse DNAM-1 could be phosphorylated at Y319 after stimulation (**Fig. 2.5b**), and that this process was critical for the functions of DNAM-1.

However, which tyrosine kinase is responsible for the phosphorylation of DNAM-1 remained enigmatic. Pioneering work claimed that the tyrosine kinase Fyn was able to phosphorylate Y319 of DNAM-1 in an *in vitro* model [61], but the significance of Fyn for DNAM-1 function has never been examined *in vivo*. To probe the role of Fyn in DNAM-1 function, we investigated the natural cytotoxicity of NK cells from Fyn knockout mice. Fyn-deficient NK cells showed an overall defect in destroying hematopoietic and non-hematopoietic target cells (**Fig. 2.6a**). However, in the absence of Fyn, engagement of DNAM-1 with its ligand still enhanced killing of K562. Surprisingly, this enhancement was significantly stronger than the response obtained with WT NK cells (**Fig. 2.6b, c**). Moreover, phosphorylation of Erk and Akt induced by DNAM-1 engagement was not affected by the deficiency of Fyn (**Fig. 2.6d**), suggesting that Fyn is not the only kinase that is implicated in the phosphorylation and signal transduction of DNAM-1. We also tested the role of another Src family kinase, Lck, in the natural cytotoxicity of NK cells. Lck-deficient NK cells exhibited same killing ability as WT NK cells toward RMA-S, YAC-1 and B16. Their cytotoxicity towards K562 could be boosted by engagement of DNAM-1 (**Fig. 2.6e, f**). These findings indicate that even if Fyn is responsible for the phosphorylation of DNAM-1, its function can be compensated by at least one other kinase *in vivo*.

Signal transduction of DNAM-1 requires Grb2

To identify the molecules that were implicated in DNAM-1 signal transduction, we synthesized peptides corresponding to a portion of DNAM-1 cytoplasmic domain [59],

containing Y319 unphosphorylated (Y319) or phosphorylated (pY319). We also synthesized Y319 phosphorylated peptides with asparagine 321 replaced by glutamine (N321Q) or tyrosine 322 replaced by phenylalanine (Y322F). YT-S cells expressing WT mDNAM-1 were lysed and precipitated with these peptides, and then binding proteins were detected with silver staining (**Fig. 2.7a**). We noted that one small protein (~25-kDa) could only be precipitated by the pY319 but not the Y319 DNAM-1 peptides, which interaction is dependent on N321 but not Y322. Mass spectrometry analysis ascertained that this protein was Grb2, an adaptor protein that has been shown to play important roles in the signal transduction of many activating receptors [90]. Blot of the proteins pulled down by DNAM-1 peptides with a monoclonal antibody to Grb2 showed the same band, confirming that this associated protein was Grb2 (**Fig. 2.7b**). As reported, phosphorylated tyrosine, together with asparagine at C-terminal position +2, constitutes a typical immunoglobulin tail tyrosine (ITT)-like motif that is capable of binding to the SH2 domain of Grb2 [91]. To address whether DNAM-1 peptides can directly associate with the SH2 domain of Grb2, we precipitated Grb2 SH2 domain-glutathione S-transferase (GST) fusion proteins with DNAM-1 peptides and detected binding proteins with antibody to GST. As expected, the pY319 but not the Y319 DNAM-1 peptides could bind to the Grb2 SH2 domain. While mutation of N321 completely abolished this interaction, mutation of tyrosine 322 (Y322F) showed no effect (**Fig. 2.7c**). The interaction of Grb2 with DNAM-1 was also confirmed in physiological systems. In YT-S cells, Grb2 was indeed recruited to DNAM-1 after stimulation with 480.2, in a manner that is dependent on the phosphorylation of Y319

(**Fig. 2.7d**).

As N321 is critical for the association of Grb2 with DNAM-1, we generated YT-S derivatives expressing DNAM-1 variants harboring N321Q or Y322F mutation (**Fig. 2.7e**). Cytotoxicity assays were performed with these YT-S cells, and we found that compared to WT DNAM-1, engagement of the N321Q mutant of DNAM-1, as indeed was the case for the Y319F mutant (**Fig. 2.4c**), showed a complete failure to enhance killing of B16 and RMA-S expressing mCD155. In contrast, no defect was observed for the Y322F mutant of DNAM-1, correlating with its ability to recruit Grb2 (**Fig. 2.7f**).

To determine the importance of Grb2 for DNAM-1 function, we used DT40, a chicken B cell line for which a variant lacking Grb2 is available. We expressed mouse DNAM-1 in WT or Grb2-deficient DT40 cells, and stimulated these cells with an antibody to mouse DNAM-1. As DT40 lacks PTEN, a phosphatase that regulates the phosphoinositide 3-kinase (PI3K) pathway, phosphorylation of Akt could not be used as an activation marker in this system ([92], data not shown). However, stimulation of DNAM-1 was capable of inducing Erk phosphorylation and intracellular Ca^{2+} fluxes in WT but not Grb2-deficient DT40 cells, suggesting that Grb2 is responsible for the signal transduction of DNAM-1 (**Fig. 2.7g, h**). Grb2 has been reported to associate with Vav-1 [40, 93]. Together with our previous findings regarding the activation of Vav-1, we hypothesize that DNAM-1 is phosphorylated at Y319 after stimulation, enabling it to recruit the Grb2-Vav-1 complex, thus initiating the signaling cascade that leads to intracellular Ca^{2+} release and phosphorylation of Erk and Akt.

P85 binds to DNAM-1 in a Grb2-dependent and a Grb2-independent manner

As DNAM-1 engagement induced phosphorylation of Akt (**Fig. 2.2**) and as Akt is known as a critical component of PI3K-signaling pathway (ref), we decided to assess whether PI3K was involved in signal transduction of DNAM-1. We observed that, in YT-S cells, p85 (the regulatory subunit of PI3K) was also recruited to DNAM-1 after DNAM-1 engagement. Moreover, as with Grb2, the association of p85 with DNAM-1 required the phosphorylation of DNAM-1 at Y319 (**Fig. 2.8a**). Given that DNAM-1 lacks a typical binding motif for p85 and that Grb2 has been reported to associate directly with p85 [94], it is possible that recruitment of p85 to the DNAM-1 complex can be mediated by the adaptor protein Grb2. To address this possibility, we transiently expressed WT DNAM-1 or DNAM-1 mutants with or without Grb2 in the monkey kidney cell line COS-1. Fyn without SH2 domain (Fyn ΔSH2) was also expressed to phosphorylate DNAM-1. Mouse DNAM-1 was immunoprecipitated and the presence of p85 was examined by immunoblotting with an antibody to p85. We found that p85 was associated with DNAM-1 even in the absence of Grb2. While mutation of Y319 and N321 nearly abolished the binding of P85, mutation of Y322 did not seem to affect this interaction (**Fig. 2.8b**). The direct interaction of p85 with DNAM-1 was also confirmed by the capability of Y319-phosphorylated DNAM-1 peptides to precipitate the p85 SH2 domain-GST fusion protein (**Fig. 2.8c**). While mutation of N321 completely abolished association of DNAM-1 with Grb2 (**Fig. 2.7c**), it only reduced binding of DNAM-1 to p85. The dispensability of Grb2 for p85

recruitment was further ascertained by the observation that pY319 DNAM-1 peptides could pull down p85 from cell lysates of Grb2-deficient DT40 cells (**Fig. 2.8d**). Taken together these results indicate that association of p85 with DNAM-1 could be achieved without Grb2, but do not exclude the possibility that recruitment of p85 to DNAM-1 requires the help of Grb2 *in vivo*.

Residues Y319 and N321 of DNAM-1 are critical for perforin polarization but not conjugate formation

DNAM-1 is known as an adhesion molecule because of its ability to promote conjugate formation between effector and target cells [60]. As mutation of Y319 or N321 completely abrogated cytolysis of target cells mediated by DNAM-1 (**Fig. 2.7f**), we suspected that this loss of function was due to the incapability of these mutants to induce conjugate formation. Conjugation assays were performed to address this question. YT-S cells transfected with mouse DNAM-1 were labeled with Brilliant Violet-conjugated antibody to human NKp46, whereas RMA-S cells expressing mCD155 were labeled with Alexa 647-conjugated antibody to mouse SLAM. Then YT-S and RMA-S cells with equal numbers were mixed together and incubated for up to 10 minutes, and the presence of conjugates was detected by flow cytometry (**Fig. 2.9a, b, c**). As reported [60], engagement of DNAM-1 with its ligand could dramatically increase conjugate formation between NK and target cells. Interestingly, this ability to form conjugates was not disrupted by the mutation of Y319, N321, Y322, or S326 in the DNAM-1 cytoplasmic domain, of which Y319 and N321 have

been shown to be critical for the function and signal transduction of DNAM-1. This finding indicates that the two essential functions of DNAM-1, namely conjugation promotion and signal transduction, are separately regulated. While the latter requires tyrosine phosphorylation and Grb2 recruitment, the former does not. After conjugate formation, perforin in the NK cell must be polarized to the edge of the synapse to be released to destroy target cells [95]. We performed confocal microscopy to assess the polarization of perforin in YT-S cells after conjugate formation. While Y319F and N321Q DNAM-1 were capable of enhancing conjugate formation, they showed a significant defect in promoting perforin polarization compared with WT DNAM-1 (**Fig. 2.9d**). This result might explain the failure of these mutants to mediate killing of target cells (**Fig. 2.7f**).

DISCUSSION

Using genetic and biochemical approaches, we have shown here that DNAM-1 is a true activating NK cell receptor that serves a critical function in NK cell-mediated killing. Previous reports have defined DNAM-1 as an adhesion receptor in NK cells or a co-stimulatory receptor in T cells [60, 91, 96]. Although the ability of DNAM-1 to promote NK cell-mediated cytotoxicity has been noted by some groups [66, 84], it was believed that in this process DNAM-1 acted to promote intercellular adhesion rather than induce activating signals. In fact, one group has reported that engagement of DNAM-1 failed to activate either Erk or JNK signaling pathway in NKL cell line [97], which further supported the idea that DNAM-1 is just an adhesion molecule without any signaling function. However, our experiments demonstrated that engagement of DNAM-1 by its ligands was sufficient to trigger NK cell-mediated cytotoxicity. Moreover, stimulation of DNAM-1 alone by monoclonal antibody was capable of conveying activating biochemical signals, both in primary NK cells and in cell lines, leading to the phosphorylation of Erk and Akt, which is, to our knowledge, the first evidence of DNAM-1-induced biochemical signals. Therefore, we believe that DNAM-1 should be categorized as an activating NK cell receptor instead of an adhesion molecule.

One published report has suggested that LFA-1, a major adhesion receptor in immune cells, physically associates with DNAM-1 in NK cells and the association with LFA-1 is critical for DNAM-1 function in T cells [61]. However, the role of LFA-1 in the DNAM-1 function in NK cells has never been investigated. With genetically manipulated mice, we found that while LFA-1-deficient NK cells showed

a considerable defect to kill some hematopoietic targets (RMA-S and YAC-1), they exhibited normal killing ability toward B16, whose elimination is mainly mediated by DNAM-1 [72]. Moreover, engagement of DNAM-1, either by its ligand or a monoclonal antibody, could still enhance cytotoxicity or induce activating biochemical signals in the absence of LFA-1. These data suggested that DNAM-1 acts as an independent activating receptor whose function does not require engagement of LFA-1. In keeping with this hypothesis, mutation of S326 of DNAM-1, which was claimed to be critical for the association of DNAM-1 with LFA-1 [61], showed no effect on DNAM-1 function. The requirement of LFA-1 for NK cell-mediated elimination of some hematopoietic targets is probably because LFA-1 is the major adhesion receptor that is involved in the conjugate formation between NK cells and these targets. Lack of LFA-1 will prevent NK cells from forming solid conjugates with these target cells, thus disrupting the formation of mature synapses and the release of granules afterwards. Although the mechanism by which NK cells recognize and adhere to B16 is unclear now, it is plausible that the interaction of NK cells and B16 is mediated by another unknown adhesion molecule other than LFA-1.

Two highly conserved residues in the C-terminal of DNAM-1 cytoplasmic domain, Y319 and S326, could be phosphorylated after DNAM-1 engagement. S326 was believed to play a critical role in the association of DNAM-1 with LFA-1 [61]. Nonetheless, the importance of these two residues for DNAM-1 function has not been fully understood. Herein, we showed that while mutation of S326 exhibited no effect on DNAM-1-mediated cytotoxicity, mutation of Y319 completely abolished it,

suggesting that Y319, rather than S326, plays a major role in DNAM-1/CD155-mediated cytolysis. Further investigations revealed that the phosphorylation of Y319 was critical for DNAM-1-initiated signaling cascades. Phosphorylated Y319, together with N321, enables the recruitment of Grb2. The constitutive association of Grb2 and Vav-1 has been reported by different groups [40, 98], which might explain how Vav-1 is translocated to the DNAM-1 complex and gets phosphorylated. Activated Vav-1 in turn induces intracellular Ca^{2+} fluxes and activates the Ras/Erk pathway. The recruitment of Grb2 seems to be critical for the signal transduction of DNAM-1, as the site-directed mutation of Y319 or N321 completely abolished DNAM-1-mediated cytolysis of target cells. Interestingly, the signaling mechanism of DNAM-1 is quite similar to that of NKG2D, another important activating receptor expressed by NK cells. Same as in DNAM-1 signaling pathway, the Grb2-Vav1 intermediate is necessary for NKG2D-mediated cytotoxicity and calcium fluxes [40]. Therefore, it is possible that these two receptors act in a synergic or compensatory way in NK cells, which might explain the fact that NK cells from NKG2D or DNAM-1 KO mice did not show a severe deficiency of function as expected. Therefore, we postulate that NK cells deficient in both NKG2D and DNAM-1 will exhibit a much more striking defect in the elimination of various tumor cells.

As phosphorylation of Y319 is the first step of DNAM-1 signaling pathway, identifying the kinase responsible for DNAM-1 phosphorylation is important for a better understanding of DNAM-1 activation. Our experiments with YT-S cells treated

with the inhibitor of Src family kinases (pp2) have demonstrated that Src family kinases are responsible for the phosphorylation of DNAM-1 (data not shown). Src family kinases include Fyn, Lyn, Src, Lck etc. and a published report has showed that Y319 of DNAM-1 can be phosphorylated by Fyn [61]. However, our experiments with genetically modified mice showed that Fyn deficiency did not preclude DNAM-1–mediated cytotoxicity or biochemical signals. Similarly, NK cells lacking another Src family kinase, Lck, also failed to exhibit any significant defect of DNAM-1 function. Nonetheless, in Lyn-deficient DT40 cells, engagement of DNAM-1 could not induce either Erk phosphorylation or intracellular Ca^{2+} fluxes (data not shown), indicating that Lyn plays a major role in the phosphorylation of DNAM-1 in this chicken B cell line. Taken together these results indicate that Src family kinases are involved in the phosphorylation of DNAM-1, and their activities can be compensated by others in the case of gene knockout.

Given that DNAM-1 engagement could induce phosphorylation of Akt, the major component of PI3K signaling pathway, it was not a surprise that p85 subunit of PI3K was also recruited to phosphorylated DNAM-1. However, the mechanism by which p85 is associated with DNAM-1 needs clarification. DNAM-1 cytoplasmic domain harbors no canonical binding site for p85 (YxxM; where 'x' is any amino acid). It is possible that binding of DNAM-1 with p85 is mediated by Grb2, as Grb2 has been shown to be able to associate directly with both DNAM-1 (**Figure. 2.7**) and p85 [94]. The fact that recruitment of p85 requires Y319 and N321, the same residues also critical for Grb2 binding, supported this hypothesis to some extent. Alternatively, p85

may associate with DNAM-1 in a direct way. This assumption is supported by our data showing that DNAM-1 is able to bind to p85 in the absence of Grb2. In this case, based on our results, association of p85 with DNAM-1 is dependent on phosphorylated Y319 and at least partially dependent on N321, which is controversial with a previous study showing that methionine at the "+3" position, instead of asparagine at the "+2" position, is critical for binding of p85 to DAP10 [99]. More evidences are needed to address this question.

The capacity of DNAM-1 to promote intercellular adhesion has been observed by another group [60]. However, here we have shown that DNAM-1 can also promote cytotoxicity and induce activating signals. Moreover, it seems that the signaling function of DNAM-1 is not required for the adhesion mediated by DNAM-1, as site directed mutations that completely abrogated DNAM-1 signals did not preclude DNAM-1-mediated conjugate formation. On the basis of these data, we propose that DNAM-1 is an activating receptor with the capability to promote intercellular adhesion. Association of the extracellular domain of DNAM-1 with its ligands is probably enough to promote conjugate formation, whereas an intact binding motif for Grb2 in the cytoplasmic domain of DNAM-1 is indispensable for the transduction of activating signals. Although the Y319F and N321Q mutants of DNAM-1 showed no defect in the conjugate formation assay, their ability to promote perforin polarization was significantly impaired, which probably explains at least in part why they failed to promote cytotoxicity against target cells expressing DNAM-1 ligands. While the mechanism by which DNAM-1 promotes perforin polarization is unclear, it is possible

that activation of PI3K signaling pathway after DNAM-1 engagement is involved in this process. This hypothesis is supported by the fact that treatment of NK cells with PI3K inhibitor would abrogate the cytotoxicity enhanced by engagement of DNAM-1 (data not shown).

Although several details need further clarification, we have demonstrated here a rather clear signal transduction pathway of DNAM-1. After DNAM-1 engagement, either by ligands or antibody, Y319 will be phosphorylated by Src family kinases. Phosphorylated Y319, together with N321, is responsible for the recruitment of Grb2-Vav-1 complex and p85. While Grb2-Vav-1 complex is able to trigger intracellular Ca^{2+} fluxes and activate Ras/Erk signaling pathway, p85 will induce phosphorylation and activation of Akt, leading to perforin polarization and granule release. More work should be done to verify this signaling mechanism *in vivo* in the future.

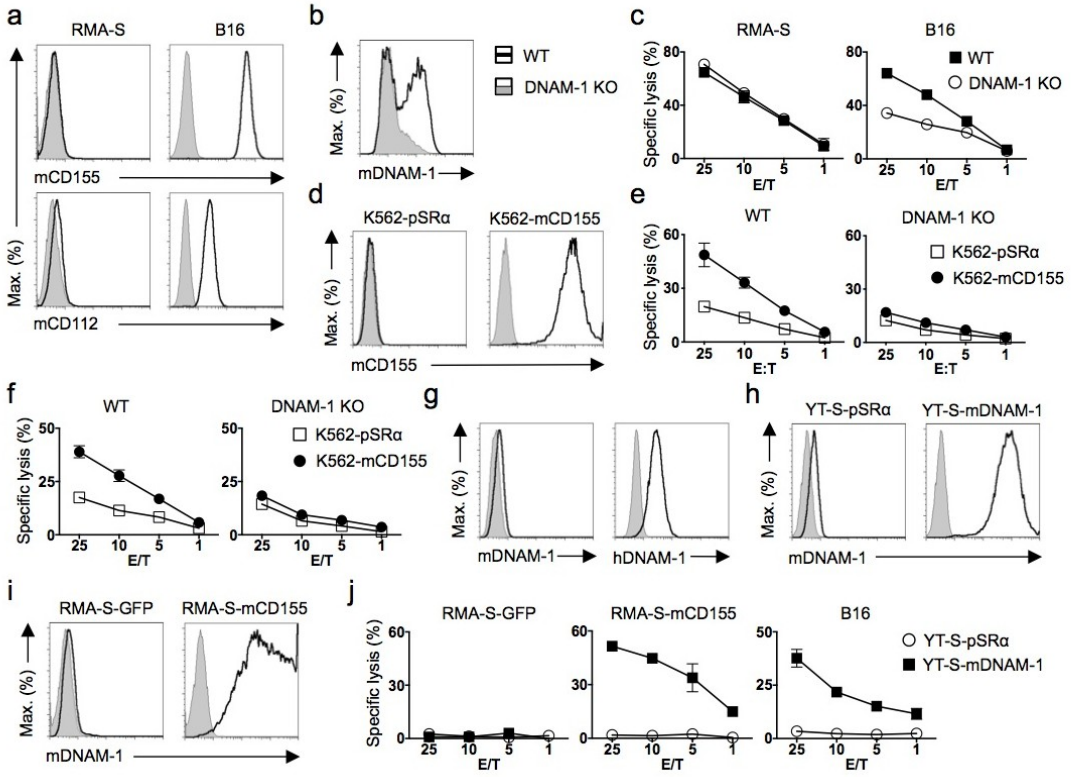

Figure 2.1 Engagement of DNAM-1 with its ligands enhances NK cell-mediated cytotoxicity.

(a) Flow cytometry analysis of expression of mouse CD155 (mCD155) and mouse CD112 (mCD112) on RMA-S and B16 cells. Open histograms represent staining with the respective antibody, whereas filled histograms showed isotype staining. (b) Flow cytometry analysis of expression of mouse DNAM-1 (mDNAM-1) on spleen NK cells. Splenocytes were isolated from wild-type (WT, open histogram) and DNAM-1 KO (filled histogram) mice and gated on $CD3^{-}NK1.1^{+}$ population. (c) ^{51}Cr-release assay of the ability of IL-2-activated NK cells from WT and DNAM-1 KO mice to kill RMA-S and B16. NK cells were cultured *in vitro* for 4 days in the presence of IL-2 (1000U/ml) before cytotoxicity assay. Each data point is the mean ±SEM of values in duplicate for each effector/target ratio. (d) Flow cytometry analysis of expression of mCD155 on

K562 cells transfected with mCD155 (K562-mCD155) or blank vector as control (K562-pSRα). (e) ^{51}Cr-release assay of the ability of NK cells from WT and DNAM-1 KO mice to kill K562 cells expressing or not mCD155. Mice were injected with polyinosinic-polycytidylic acid (poly(I:C)) 36 hours before cytotoxicity assay. (f) ^{51}Cr-release assay of the capacity of IL-2-activated NK cells to kill K562 cells expressing or not mCD155. (g) Flow cytometry analysis of expression of mouse and human DNAM-1 on YT-S cells. (h) Flow cytometry analysis of ectopic expression of mouse DNAM-1 (mDNAM-1) on YT-S cells transfected with mDNAM-1 (YT-S-mDNAM-1) or blank vector as control (YT-S-pSRα). (i) Flow cytometry analysis of ectopic expression of mCD155 on RMA-S cells transduced with mCD155 (RMA-S-mCD155) or blank vector as control (RMA-S-GFP). (j) ^{51}Cr-release assay of the ability of YT-S cells with or without mDNAM-1 to destroy B16 and RMA-S expressing or not mCD155. Data are representative of at least three independent experiments.

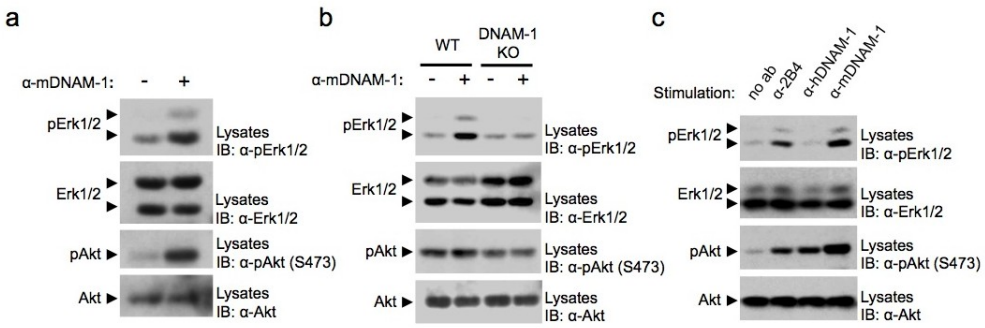

Figure 2.2 Stimulation of DNAM-1 induces phosphorylation of Erk and Akt both in IL-2-activated NK cells and transfected YT-S cells.

(a and b) Immunoblot analysis of the activation of Erk and Akt in total cell lysates of IL-2-activated NK cells stimulated with monoclonal antibody 480.2 (rat anti-mDNAM-1), followed by goat anti-rat (2 min at 37 °C), or exposed to goat anti-rat alone (unstimulated; -). (a) NK cells were from C57BL/6 mice. (b) NK cells were from WT or DNAM-1 KO mice. (c) Immunoblot analysis of the phosphorylation of Erk and Akt in total cell lysates of YT-S cells expressing mDNAM-1 stimulated with antibody to 2B4, hDNAM-1, mDNAM-1 or no antibody as control (no ab), followed by secondary antibody (2 min at 37 °C). Data are representative of three (a), two (c), or one (b) independent experiments.

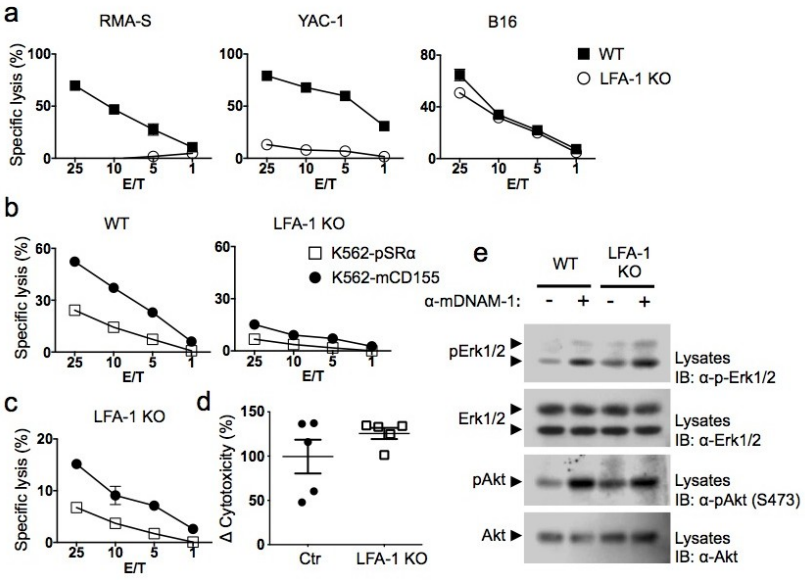

Figure 2.3 DNAM-1-mediated cytotoxicity and signals are independent of LFA-1.

(a and b) ^{51}Cr-release assay of the ability of IL-2-activated NK cells from WT or LFA-1 KO mice to kill RMA-S, YAC-1, B16 (a) and K562 expressing or not mCD155 (b). (c) An expanded view of the data on the right panel of (b) with the range of vertical axis changed from 60 to 20 to better display the difference between the two lines. (d) The difference in cytotoxicity (Δ cytotoxicity) toward K562 expressing mCD155 versus K562 lacking mCD155 was calculated at the E/T ratio 25. Each dot represents one mouse. (e) Immunoblot analysis of the phosphorylation of Erk and Akt in total cell lysates of IL-2-activated NK cells from WT or LFA-1 KO mice. NK cells were stimulated with 480.2, followed by goat anti-rat (2 min at 37 °C), or treated by goat anti-rat alone (unstimulated; -). Data are representative of three (a, b, c) or two (d) independent experiments.

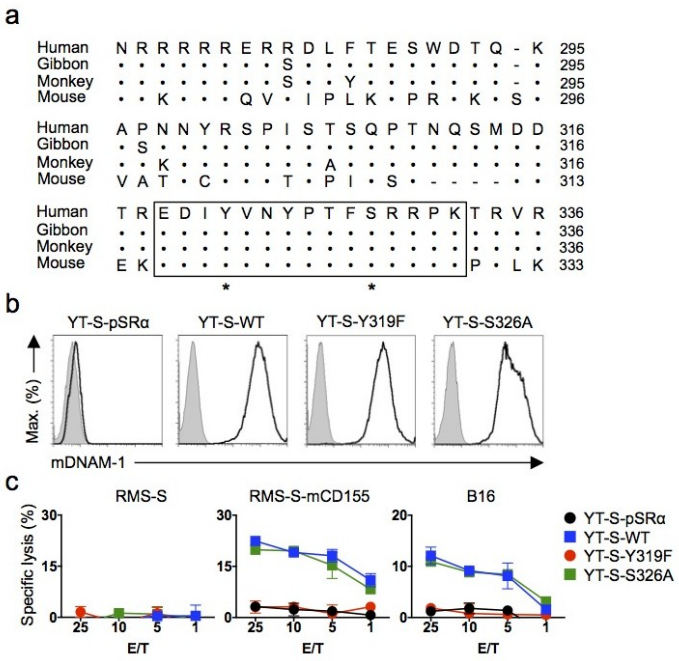

Figure 2.4 Y319 but not S326 is critical for DNAM-1-mediated cytotoxicity

(a) Amino acid sequence analysis of the cytoplasmic domain of DNAM-1 from human, gibbon, monkey and mouse. The residues that can be phosphorylated are marked by stars. The region that is highly conserved between different species is boxed. (b) Flow cytometry analysis of expression of mDNAM-1 on YT-S cells transfected with WT, Y319F, or S326A mDNAM-1, or transfected with blank vector (YT-S-pSRα). Open histograms represent staining with anti-mDNAM-1, whereas filled histograms showed isotype staining. (c) ^{51}Cr-release assay of the ability of YT-S derivatives to kill B16 and RMA-S expressing or not mCD155. Data are representative of three independent experiments.

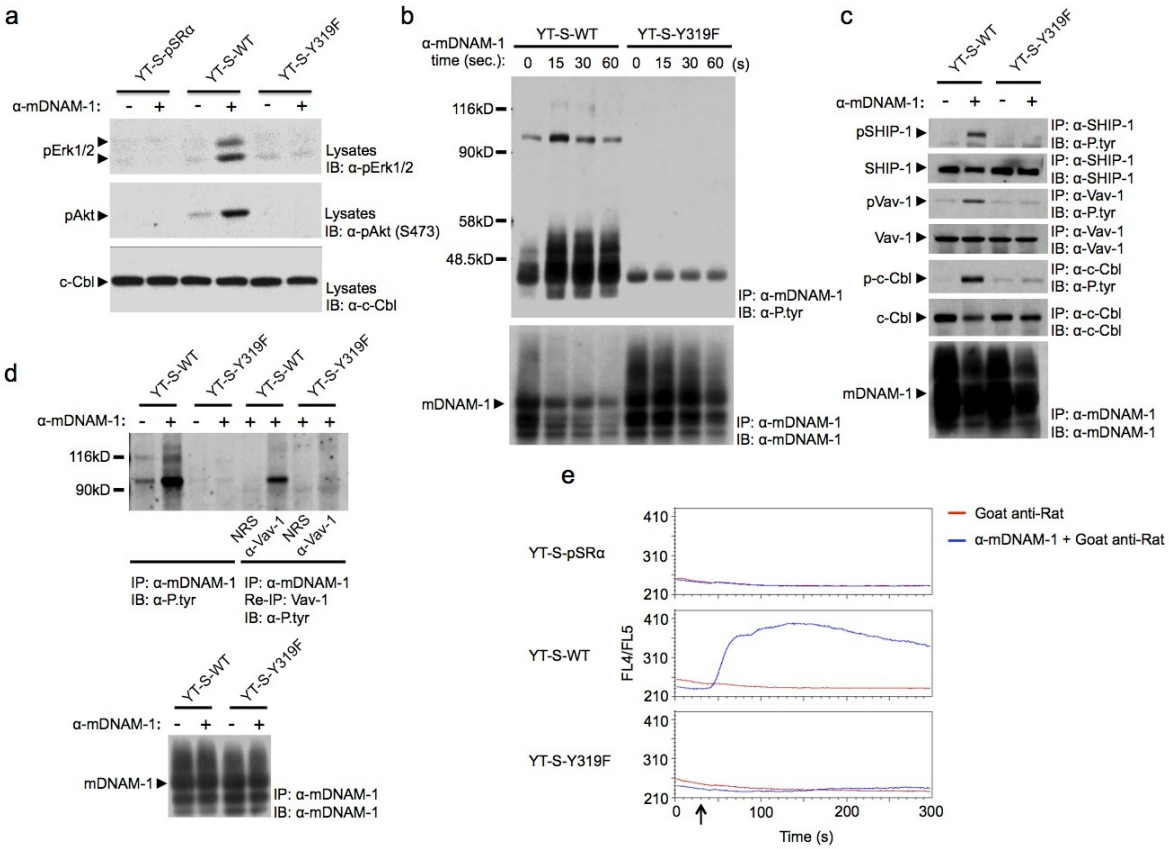

Figure 2.5 Y319 is critical for DNAM-1-induced signals

(a) Immunoblot analysis of phosphorylation of Erk and Akt in total cell lysates of YT-S cells stimulated with 480.2, followed by goat anti-rat (2 min at 37°C), or treated by goat anti-rat alone (unstimulated; -). Blot of c-Cbl as loading control. (b) Immunoblot analysis of phosphorylation of mDNAM-1 after stimulation. YT-S cells expressing WT or Y319F mDNAM-1 were treated with 480.2, followed by goat anti-rat at 37°C for indicated time. mDNAM-1 was immunoprecipitated (IP) from total cell lysates by anti-mDNAM-1 antibodies and blotted for total phosphotyrosine with 4G10. (c) Immunoblot analysis of tyrosine phosphorylation of SHIP-1, Vav-1 and c-Cbl after mDNAM-1 engagement. YT-S cells were treated as described in (a) and immunoprecipitated (IP) with anti-SHIP-1, anti-Vav-1 or anti-c-Cbl. (d)

Immunoblot analysis of the phosphorylated protein recruited to mDNAM-1 after stimulation. YT-S cells were stimulated for 15s with 480.2 followed by goat anti-rat as described in (b), immunoprecipitated (IP) with anti-mDNAM-1, re-immunoprecipitated (re-IP) with anti-Vav-1 or normal rabbit serum (NRS) as control. Data are representative of three (a, b, d), two (c) or one (e) independent experiments. (e) Flow cytometry analysis of changes in intracellular calcium over time in YT-S cells treated as described in (a), presented as the FL4/FL5 ratio. Goat anti-rat was added at 30s as indicated by the arrow.

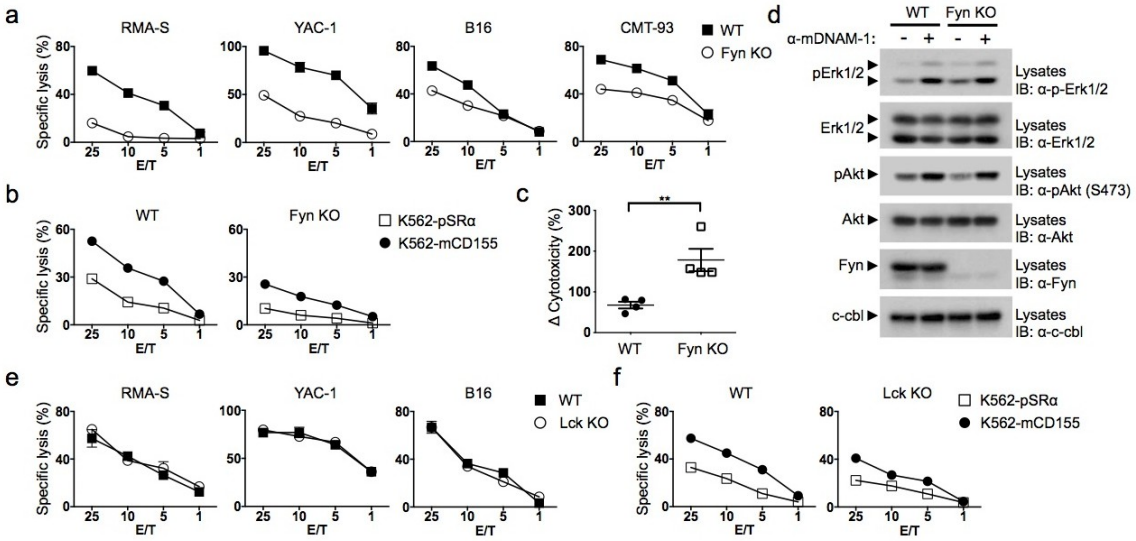

Figure 2.6 Deficiency of Fyn or Lck does not abolish DNAM-1 function

(a and b) ^{51}Cr-release assay of the ability of IL-2-activated NK cells from WT or Fyn KO mice to kill RMA-S, YAC-1, B16, CMT-93 (a) and K562 expressing or not mCD155 (b). (c) The difference in cytotoxicity (Δ cytotoxicity) of IL-2-activated NK cells toward K562 expressing mCD155 versus K562 lacking mCD155 was calculated at the E/T ratio 25. Each dot represents one mouse. (d) Immunoblot analysis of phosphorylation of Erk and Akt in total cell lysates of IL2-expanded NK cells stimulated with 480.2, followed by goat anti-rat, or treated with goat anti-rat alone (unstimulated; -). (e and f) ^{51}Cr-release assay of the ability of IL-2-activated NK cells from WT or Lck KO mice to kill RMA-S, YAC-1, B16, (e) and K562 expressing or not mCD155 (f). Data are representative of four (a, b) or two (d, e, f) independent experiments.

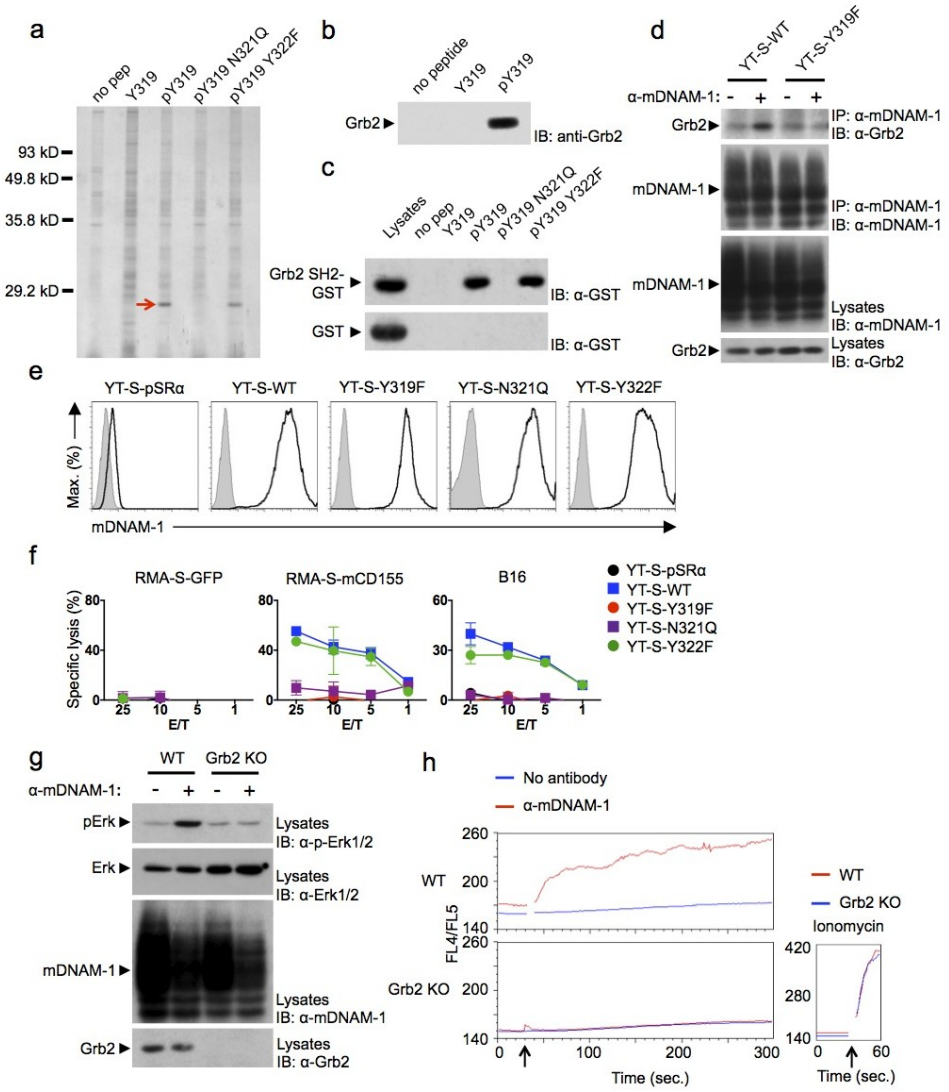

Figure 2.7 Signal transduction of DNAM-1 requires Grb2

(a) Silver staining analysis of the proteins pulled down by mDNAM-1 peptides from YT-S cell lysates. No pep, no peptide was added. Y319, mDNAM-1 peptide with Y319 unphosphorylated. pY319, mDNAM-1 peptide with Y319 phosphorylated. pY319 N321Q, asparagine 321 is mutated to glutamine in pY319 peptide. pY319 Y322F, tyrosine 322 is mutated to phenylalanine in pY319 peptide. The red arrow indicates the protein that can only be pulled down by the pY319 and Y322 mutant peptides but not the N321 mutant peptide. (b) Immunoblot analysis of the protein indicated in (a) with antibody to Grb2. (c) Immunoblot analysis of the ability of

mDNAM-1 peptides to pull down Grb2 SH2 domain-GST fusion protein. (d) Immunoblot analysis of binding of Grb2 to mDNAM-1 in YT-S cells. YT-S cells were stimulated with 480.2, followed by goat anti-rat (15s at 37°C), or treated with goat anti-rat alone (unstimulated; -). Cell lysates were immunoprecipitated (IP) with anti-mDNAM-1 and blotted with anti-Grb2. (e) Flow cytometry analysis of expression of mDNAM-1 on YT-S cells transfected with WT, Y319F, N321Q, or Y322F mDNAM-1 or transfected with blank vector (YT-S-pSRα). Open histograms represent staining with anti-mDNAM-1, whereas filled histograms showed isotype staining. (f) ^{51}Cr-release assay of the capacity of YT-S derivatives to kill B16 and RMA-S expressing or not mCD155. (g) Immunoblot analysis of phosphorylation of Erk in total cell lysates of WT or Grb2 KO DT40 cells expressing mDNAM-1. DT40 cells were stimulated with 480.2, followed by goat anti-rat (2min at 37°C), or treated with goat anti-rat alone (unstimulated; -). (h) Flow cytometry analysis of changes in intracellular calcium over time in DT40 cells treated as described in (g), presented as the FL4/FL5 ratio. Goat anti-rat was added at 30s as indicated by the arrow. Cell responsiveness was verified by treatment with ionomycin. Data are representative of three (a, b, d, e, f, g, h) or two (c) independent experiments.

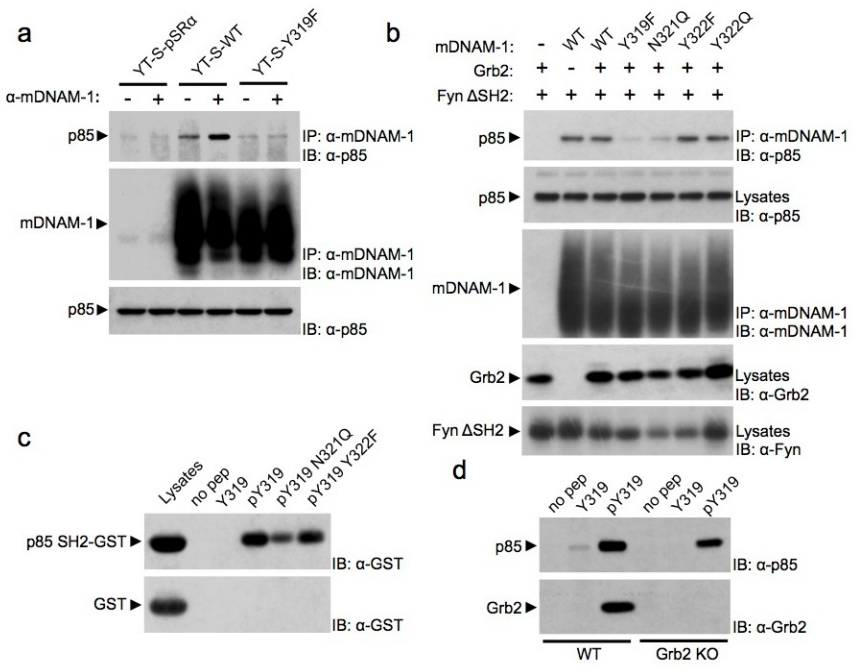

Figure 2.8 p85 binds to DNAM-1 in Grb2-dependent and Grb2-independent manner.

(a) Immunoblot analysis of binding of p85 to mDNAM-1 in YT-S cells after stimulation. YT-S cells were stimulated with 480.2 and goat anti-rat (15s at 37°C), or treated with goat anti-rat alone (unstimulated; -). Cell lysates were immunoprecipitated with anti-mDNAM-1 and blotted with anti-p85. (b) Immunoblot analysis of binding of p85 to mDNAM-1 in COS-1 cells. COS-1 cells were transfected with Grb2, Fyn without SH2 domain (Fyn ΔSH2) and mDNAM-1 harboring different mutations. Cell lysates were immunoprecipitated with anti-mDNAM-1 and blotted with anti-p85. (c) Immunoblot analysis of the ability of mDNAM-1 peptides to pull down p85 SH2 domain-GST fusion protein. (d) Immunoblot analysis of the capacity of mDNAM-1 peptides to precipitate p85 and Grb2 from cell lysates of WT or Grb2 KO DT40 cells. Data are representative of three (a), two (c), or one (b, d) independent experiments.

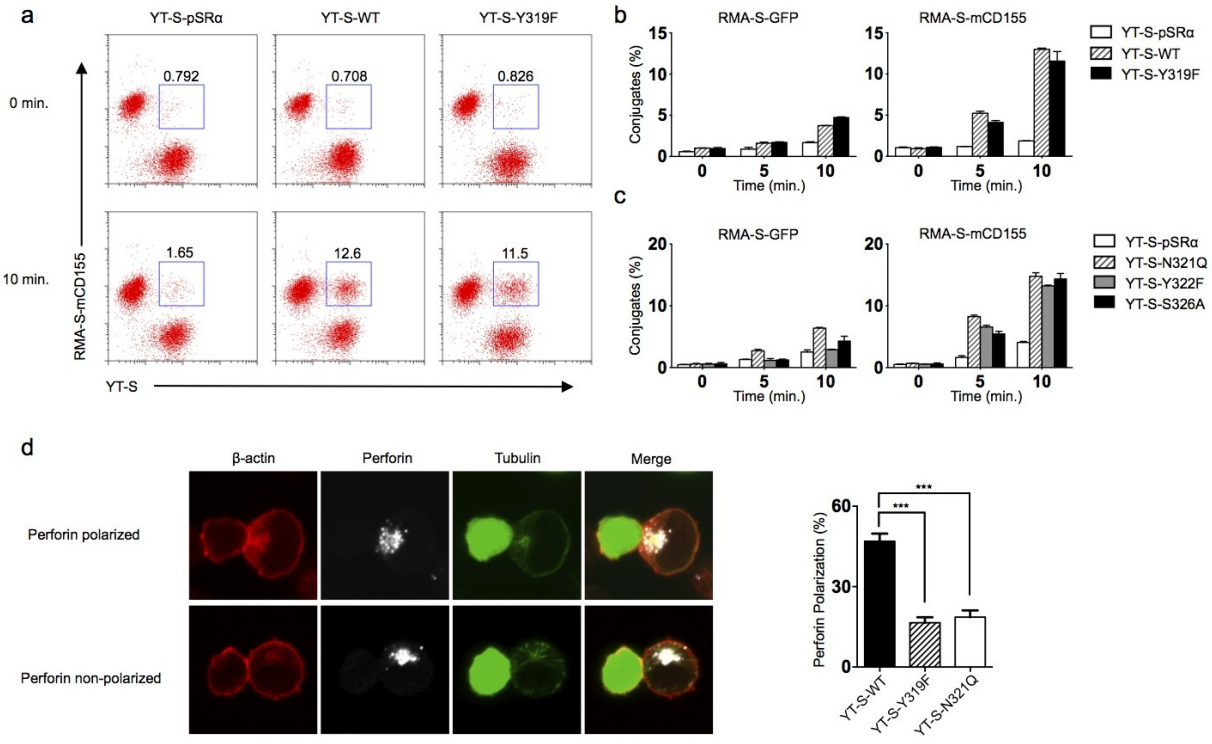

Figure 2.9 Residues Y319 and N321 of DNAM-1 are critical for perforin polarization but not conjugate formation.

(a, b and c) Flow cytometry analysis of conjugate formation between YT-S derivatives and RMA-S expressing or not mCD155. YT-S cells were labeled by Brilliant Violet 421-conjugated anti-hNKp46, whereas RMA-S cells were labeled by Alexa 647-conjugated anti-mSLAM. YT-S and RMA-S Cells were mixed together and incubated at 37°C for 0, 5, and 10 min before the reaction was terminated by adding fixation buffer. (a) Representative experiments and time points are shown. YT-S-RMA-S conjugates are identified as $NKp46^{+}SLAM^{+}$ and boxed. The percentages of conjugates formed are shown at the top. (b and c) Graphic representations with standard deviations of triplicate values. (d) Confocal microscopy analysis of perforin polarization in YT-S cells in response to RMA-S expressing mCD155. YT-S cells expressing WT, Y319F or N321Q mDNAM-1 were incubated

with RMA-S-mCD155 for 30 min at 37°C. Cell mixtures were loaded to poly-l-lysine-coated slides, fixed and stained with anti-β-actin, anti-perforin, and anti-tubulin. Left: Representative images of conjugates with perforins in YT-S cells polarized or not to the edge of synapses with RMA-S. Right: The percentages of conjugates with perforins in YT-S cells polarized to the synapses. Data are representative of seven (a, b) or one (c) independent experiments. (d) More than 50 conjugates were pictured for each condition, and data are pooled from two independent experiments.

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