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The balancing act: Inhibitory Ly49 regulate NKG2D-mediated NK cell functions

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Abstract

NK cells use NKG2D receptor to recognize '*induced-self*'. In apparent violation of the '*missing-self*' hypothesis, NK cells stimulated through NKG2D can lyse target cells despite normal expression levels of MHC class I molecules. Although, '*overriding*' of the inhibitory by the activating signals had been postulated the precise role of inhibitory Ly49 receptors on NKG2D-mediated activation has only started emerging. We propose that NKG2D-mediated activation is a function of '*altering the balance*' in the signaling strength between the activating NKG2D and inhibiting Ly49 receptors. Balance in the signaling strength depends on the expression levels of activating ligands on the target cells. Qualitative and quantitative variations of MHC class I molecules expressed on the target cells also plays a major role in determining this '*altered-balance*'. Consequently, the nature of Ly49 receptors expressed on specific NK subsets determines the level of NKG2D-mediated NK cell activation. These observations provide a firm basis of '*altered-balance*' in NK signaling and describe an active interplay between inhibitory Ly49 and activating NKG2D receptors.

Keywords

Natural killer cells; NKG2D; H60; Ly49; Altered-balance

1. Introduction

Natural killer (NK) cells are an important and necessary effector population of the innate immunity [1]. NK cells are derived from the bone marrow and play vital roles in the clearance of virally infected or malignant tumor cells [2]. NK cells also have the ability to regulate the adoptive immunity through the generation of a variety of cytokines and chemokines [3–5] NK cells have also been shown to play key roles in bone marrow transplant rejections [6–10], maternal–fetal interactions, viral/bacterial infections [11,12], autoimmune and inflammatory diseases [13]. For the purpose of functional analyses, NK cells have long been studied as a classical example of an effector lymphocyte capable of utilizing both activating and inhibitory receptors. This review specifically discusses the effect of inhibitory Ly49 on the activating NKG2D receptor. I propose that the signalling balance between these two types of receptors forms the molecular cues for the NK cells to mediate select effector function(s).

Each NK cell is known to express one or more inhibitory receptors, which interact with a specific MHC class I molecules on the target cells [14]. Interaction of MHC class I with the Ly49 receptors prevent the activation of NK cells and thereby the lysis of the target cell. Thus, NK cells utilize the Ly49 receptors to differentiate '*self*' from '*missing-self*' (Fig. 1). The

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'*missing-self*' hypothesis proposes that NK cells lyse target cells that have lost expression of MHC class I molecules, i.e., that are missing self [15]. Thus, in an unchallenged steady-state, murine NK cells discriminate '*self*' cells from those that are '*missing-self*' through an array of Ly49 receptors, which inhibit lysis upon recognition of MHC class I molecules on target cells [16–18]. Loss of MHC class I molecules on target cells relieves the NK cell of Ly49-mediated inhibition, thus allowing the NK cells to mediate cytotoxicity [19]. Inhibition mediated through Ly49 receptors are thought to globally affect NK cell functions. However, the possibility that inhibitory Ly49 function as regulatory receptors, dictating specific effector functions have not been explored.

Our recent analyses indicate that the NKG2D receptor is also subject to the regulation of inhibitory Ly49 receptors [20]. In particular, the levels of H60 and the nature of MHC class I molecules on the target cells directly influenced the magnitude of NKG2D-mediated cytotoxicity. Successful engagement of specific inhibitory Ly49 receptor to their cognate MHC class I molecules determined the extent of inhibition on NKG2D-mediated activation. Furthermore, the level of inflammatory cytokines such as IFN-γ and GM-CSF generated by NK cells through NKG2D-mediated activation is also regulated by the Ly49 receptor. Independent studies by Carbone et al., demonstrates that the ability of the human NK cells to recognize and clear multiple myeloma cells through NKG2D receptor depends not only on the expression of activating ligands such as MIC-A but also level of MHC class I expression [21].

Thus, it appears that the exclusive '*missing-self*' hypothesis proposed by Karre et al. [22] almost thirty years ago holds also true in the context of '*induced-self*'. In this review, I discuss the role of specific interplay between inhibitory Ly49 and activating NKG2D receptors and its role in the regulation of NK cell-mediated effector functions. Studies directed towards unraveling the balance between inhibitory and activating receptors will provide us a critical understanding in the formulation of NK-based cellular immunotherapies directed to hematological malignancies.

2. 'Self' versus 'missing-self' versus 'induced-self'

Over a period of time, distinct mechanisms have been proposed to explain NK cell activations, which appear to fall into multiple categories. First, NK cell activations occur due to the absence of inhibition. The '*missing-self*' hypothesis proposes that NK cells lyse target cells that have lost the expression of MHC class I molecules (Fig. 1) [23–25]. Loss of MHC class I molecules on target cells relieves the NK cell of Ly49-mediated inhibition, thus allowing cytotoxicity [26].

The second immunological situation is the activation of NK cells due to absence of inhibition and presence of stimulatory receptors. A set of widely expressed activation receptors, NKp30, NKp44, NKp46, and 2B4, have been implicated in their ability to mediate NK cell stimulation which are otherwise '*masked*' under the strong negative signaling from inhibitory receptor-MHC class I molecules interactions. This phenomenon of MHC class I obstruction to NK cell activation is referred as '*target interference*' [27–30].

The third immunological concept that defines NK cell activation is the '*induced-self*', where overriding of inhibitory receptors occur by the signals mediated through activating receptors. Under challenged conditions, target cells express a family of stress-inducible ligands, including ULBP [31,32], MICA/B in humans [33], and H60 [34,35], Rae-1 [36], and Mult-1 [37,38] in mice, which are recognized by the activating receptor, NKG2D [34,39–42]. Although, these findings demonstrated a non-requirement for the down-regulation MHC class I, the interplay between NKG2D-mediated activation and Ly49 receptor-mediated inhibition has only started emerging. Below we have a detailed description of these phenomena.

2.1. 'Self' versus 'missing-self'

'*Missing self*' hypothesis was put forward by Karre and coworkers which formed the basis for how NK cell functions are regulated [43]. Expression levels of MHC class I on target cells defined '*self*'. In contrary, significant reduction in the expression of MHC class I on tumor or virally infected cells lead to activation of NK cells [44–46]. In this context, it is important to point that several families of inhibitory receptors have been defined to function on NK cells. They belong to immunoglobulin super-family such as KIR [47] molecules (human) or, C-type lectin [48–55] super-family (murine). Majority of the C-type lectins belong to Ly49 sub-family that recognize classical MHC class I molecules. There are 21 defined murine Ly49 genes out of which 18 of them are known to be transcribed [56–62]. The second set of lectin members are the CD94/NKG2 (A&B) that recognize non-classical MHC (HLA-E in human and Qa-1 in mouse) [63]. The last set of lectin receptors are collectively called as NK1.1 complex which are also known as NKR-P1 and interestingly their inhibitory ligands are encoded by the *Clr* gene family [64,65]. These C-type lectin-like molecules or structurally distinct killer immunoglobulins like inhibitory receptors (KIR) function through a well-defined cytoplasmic consensus amino acid sequence defined as immunoreceptor tyrosine-based inhibitory motif (ITIM) [66–71]. The prototype six amino acid ITIM sequence is (Ile/Val/Leu/Ser)-X-Tyr-X-X, where X denotes any amino acid [72].

2.2. 'Target interference'

A wide array of NK-activating receptors have been characterized in humans, including KIR2DS, KIR3DS, NKp36, NKp44, NKp46 and p75/AIRM, collectively called 'Natural Killer Cell Receptors' (NCRs) [73–75]. Recent studies have also implicated some of the defined T cell co-stimulatory molecules such as CD2, CD28, 2B4 and LFA-1 also as activating receptors for NK cells [76–78]. When normal levels of MHC class I are expressed on the target cells, these receptors fail to activate NK cells due to the strong negative signals received by the NK cells through their inhibitory receptors. This phenomenon is defined as '*target interference*'.

2.3. 'Self' versus 'Induced-self'

Recently, a novel paradigm defined as '*induced-self*' has been introduced to explain NK cell activation mediated through the NKG2D receptor. Eloquent studies by Raulet and Lanier laboratories have demonstrated that interaction of NKG2D with its cognate ligands can override the negative signals provided by inhibitory receptors [34,35]. These findings while promoting a novel hypothesis '*induced-self*' [34,35] mediated by NKG2D receptor also changed the long-held '*self*' versus '*missing-self*'-NK cell paradigm [79–81]. Their studies equivocally argued that activation through NKG2D can lead to target cell lysis despite their normal expression levels of MHC class I molecules [82,83].

Although, the above phenomena have been proved in different systems, recent studies are pointing to a unified molecular mechanism as the common basis for NK cell activation. Based on others and our studies, we predict that NK cell activation is purely a function of balance between the activating and inhibitory stimuli. One exciting possibility is that this balance in the signaling strength could very well dictate the nature of NK-mediated effector functions *in vivo*.

3. Role of activating ligand density and NKG2D-mediated NK cell activation

Multiple 'stress-inducible' activating ligands belonging to a non-classical MHC class I family (H60, Rae1α-ε and Mult-1) have been defined to interact with NKG2D receptor on murine NK cells. The requirements for their optimal *in vivo* expression have yet to be determined. Specific cell types which have the ability to express these activating ligands are not well defined. Our preliminary studies demonstrate that some of these ligands can be expressed in T, B,

macrophage and dendritic cells (Haiyan Chu and Malarkannan, unpublished observations). In view of the fact that the activating ligands are inducible, it is expected that depending on the type and potency of the stimuli, the expression levels of these ligands may vary. Along these lines, it is arguable that a normal but activated B or dendritic cell may express H60 or Rae1 during an immune response. Further, these cells may not necessarily down-regulate the expression levels of MHC class I molecules. Under these circumstances, it may not be obligatory for the NK cells to mediate cytotoxicity against these activated yet normal cells. Therefore, it is highly possible that the balance between the activating and the inhibitory signals may form the cues for the NK cells to perform chosen effector functions.

One of the ways to determine the level of negative inhibition from Ly49 receptors on NKG2D-mediated activation is varying the levels of activating ligands such as H60. To mimic different intensity of stress, we generated and used stable cell lines expressing varied levels of H60 [20]. As expected, the percentages of NK-mediated cytotoxicity were dependent of the level of H60 expression (Fig. 2). Interestingly, blocking the interaction of Ly49C to H2-K^b on a model tumor cell with moderate levels of H60 (similar to lipopolysaccharide stimulated splenocytes of BALB origin) augmented the level of NK-mediated cytotoxicity. This provided the first clue that the activation through NKG2D receptor was under the dynamic control of Ly49 receptors. Blocking Ly49C/I receptors with specific monoclonal antibodies or using purified, homogeneous $Ly49C^+/I^+$ or $Ly49C^-/I^-$ NK subsets demonstrated that the expression levels of activating ligand (H60) on target cells play a major role in balancing the effect of inhibitory receptors (Fig. 2). Thus, at an optimal level of H60 expression, activation through NKG2D receptor is highly susceptible to Ly49C/I receptor-mediated inhibition. In contrast, when target cells made to express significantly higher levels of H60, blocking the interaction between Ly49C and H2-K^b had no effect. There were two key functional outcomes from these observations. They are: (a) NK cell activation by the physiological levels of H60 (such that of splenocytes) is subjected to strong negative regulation of inhibitory receptors, and (b), however, an over expression of H60 (pathological) can generate a stronger positive signal leading to a possible over-riding of inhibition by NKG2D-mediated activation.

4. Nature of Ly49 receptors affects the strength of NKG2D-mediated activation

It is also important to discuss the inherent limitations with the tumor model discussed in the earlier section that was used by us and others. To our knowledge, expression of H60 protein is restricted to the BALB and 129J backgrounds and the transcription of its gene is undetectable in either C57BL/6 or B10 strains. The other major difference between BALB and C57BL/6 are their MHC complexes. Ly49 receptor acquisition and expression during the terminal maturation of NK cells are known to be strongly '*calibrated*' by the '*self*' MHC molecules. Recent studies provide further proof for an active process defined as '*licensing*' that is mediated by '*self*' MHC class I molecules [84,85]. This process governs both the functional maturation of NK cell subsets and prevention of autoimmune reactivity. However, the molecular mechanism by which a specific MHC complex impacts upon the terminal maturation of NK cells and thereby the strength of NKG2D-mediated activation is currently not well understood.

Antibody-mediated blocking experiments described above were mostly directed to Ly49C NK subset. Ly49C subset constitutes approximately 20–30% of the total NK population in both C57BL/6 and BALB strains. NKG2D-mediated activation of Ly49C+ subsets were under significantly stronger inhibition when the target cells expressed only a 'physiologically' comparable, moderate level of H60. This particular system dealt with a single Ly49 receptor and its interaction to its MHC ligand. However, tumor cells, particularly of human origin, compared to that of inbred mice may express more than two MHC class I molecules. Thus, tumor cells expressing multiple MHC may have the ability to mediate additional inhibitions

through more KIR/Ly49 receptors. Therefore, to further offset the balance between activation and inhibition, additional MHC class I molecule, H2-D^d, were introduced into the model tumor cells (Fig. 2). Compared to Ly49C/I-mediated inhibition, expression of H2-D^d that serves as a ligand for inhibitory Ly49A/G receptors, on target cells, significantly reduced NKG2D-mediated NK cell cytotoxicity [20].

We conclude that the operation of '*missing-self*' hypothesis in NKG2D-mediated '*induced-self*' is convincingly evident due to the following reasons. In the case of H-2^{d+} strain-derived Ly49A/G+ NK cell subsets (BALB/c and B10.D2), the cytotoxicity through NKG2D receptor is significantly abolished when the target cells express normal levels of H2-D^d, thereby, establishing '*self*'. Even an augmented expression of H60 could not over ride the inhibition mediated through the Ly49A/G receptors. Therefore, 'down-regulation' of self-MHC is necessitated to establish '*missing-self*' for the Ly49A/G+ NK cells to mediate the efficient lysis. Recognition of '*self*' is also evident in NK cells from H-2^{b+} strain (C57BL/6), but the extent of inhibition is strongly subjected to the level of activating ligand due to the insufficient inhibition mediated by Ly49C/I receptors. Molecular basis for the affinity variations between distinct Ly49 receptors to their respective MHC class I molecules that dictate the strength of inhibition is started emerging [86]. These results also demonstrate specific NK subsets would tender select effector functions depending on the balance between positive and negative signals for a given target cell. Collectively, a successful immune surveillance by NK cells is determined not only by the interaction of NKG2D to the activating ligand H60 but also dependent on the scanning for specific MHC class I molecules on the target cells by the inhibitory Ly49 receptors. Thus, the quantitative and qualitative strength of Ly49-mediated inhibitory signals help determining the level of NKG2D-mediated effector functions.

Does the balance between signal strengths regulate distinct effector functions mediated by NK cells? Can the interplay between the activating (NKG2D) and the inhibitory (Ly49) signals form the environmental cues determining specific NK-mediated effector functions? In a more physiological context, what is the functional outcome of an NK cell recognizing an activated B cell expressing H60 and normal levels of MHC class I? Interaction of inhibitory Ly49 receptors to MHC class I molecules results in the recruitment of SH2 domain-containing phosphatases to immuno tyrosine-based inhibitory motifs [87,88]. Activated phosphatases are known to dephosphorylate membrane proximal signaling substrates [89]. However, the identities of many of these substrates are yet to be determined. Many of the membrane proximal signaling events occurring downstream of NKG2D receptor have been defined. However, only minimal information is currently available on the distant signaling events downstream of NKG2D receptor. Although, signaling requirements for cytotoxicity and cytokine generations through NKG2D receptor are understandably different, future studies are needed to distinguish 'global' versus 'selective' effects of the inhibitory signals. Together, we postulate that the '*altered-balance*' will determine the functional outcome of NK cell activations in terms of cytotoxicity, cytokine or chemokine productions and other non-cytotoxic functions.

5. Concluding remarks

The magnitude of activation through NKG2D is a function of '*altered-balance*' in the signaling strength between activating NKG2D and inhibitory Ly49 receptors. The magnitude of NKG2D-mediated cytotoxicity depends on the quantities of H60, MHC class I and the nature of MHC class I expressed on the target cells. Based on these observations, we put forward an integrative hypothesis in which a balance in the signaling strength may govern distinct effector functions of NK cells. To better understand '*altered-balance*', a detailed future study involving the intracellular signaling molecules is needed in delineating the specific activation pathway (s) that are targeted by the Ly49-recruited phosphatases. Also, similar to that of the murine system described here, a parallel analysis is required for human NK cells. This can lead to

successful formulation of cellular immunotherapy that will be based on the adoptive transfer of HLA-mismatched NK cells for leukemia ablation.

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Abbreviations

NK
: natural killer cells

KIR
: killer immunoglobulin-like receptor

IFN-γ
: interferon gamma

GM-CSF
: granulocyte-macrophage colony stimulating factor

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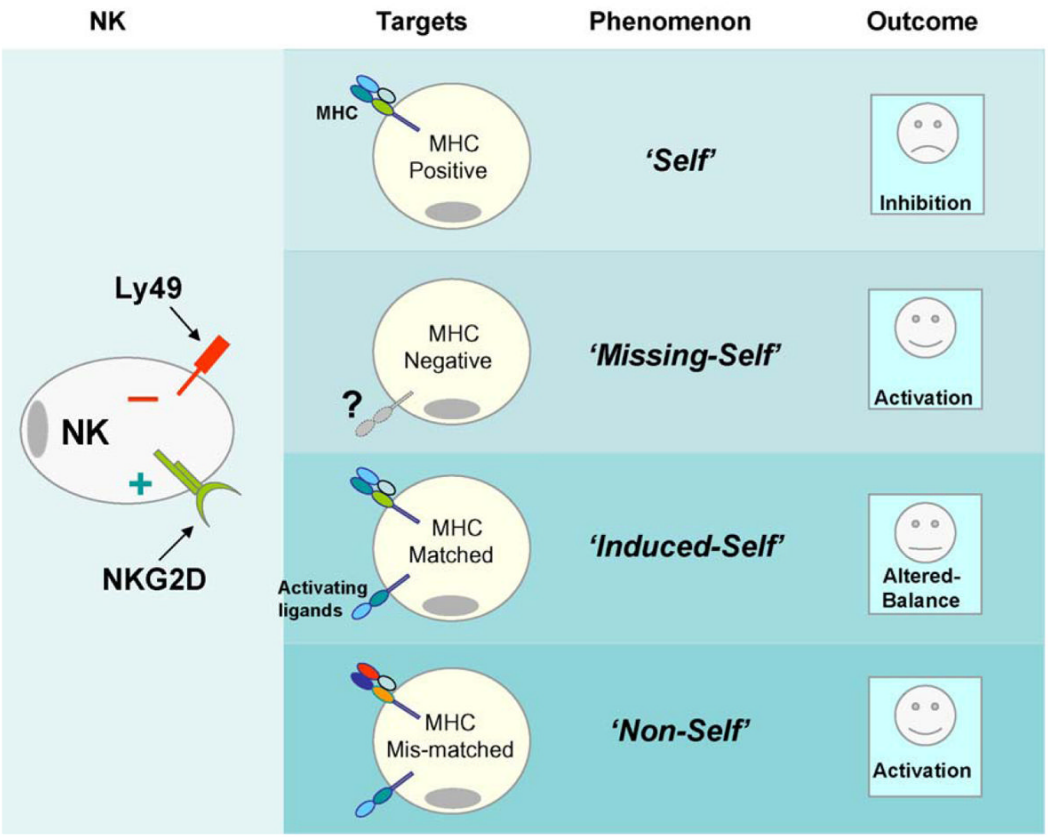

Fig. 1.

Different mechanisms through which NK cells can be activated. Normal cells express optimal levels of MHC class I. Interaction of MHC class I to Ly49 receptors leads to the induction of negative signals through recruitment and activation of phosphatases. These negative signals are known to actively down-regulate the effector functions of NK cells. Each NK cell is known to express one or more inhibitory Ly49 receptors, which interact with a specific MHC class I molecules on the target cells. Thus, expression of optimal levels of MHC class I defines 'self' recognition by NK cells. However, a reduction in the MHC class I due to viral infection or tumor transformation leads to the activation of NK cells. Thus, NK cells utilize inhibitory Ly49 receptors to differentiate '*self*' from '*missing-self*'. On the other hand, target cells can also express inducible ligands such as H60, which are recognized by activating NKG2D receptor. This phenomenon is defined as '*induced-self*' and concomitant activation through NKG2D and inhibition through Ly49 receptor results in '*altered-balance*'. Strength of NKG2D-mediated stimulation depends on the balance between activating and inhibitory signals. Another clinically relevant phenomenon is the '*non-self*', where target and NK cells are derived from different individuals. Since each subset of NK are defined by the type of Ly49 or KIR receptor they express, it is possible to formulate NK-based cellular immunotherapy by miss-matching MHC and inhibitory receptor combinations to prevent potential inhibition and thereby successful activation of NK cells.

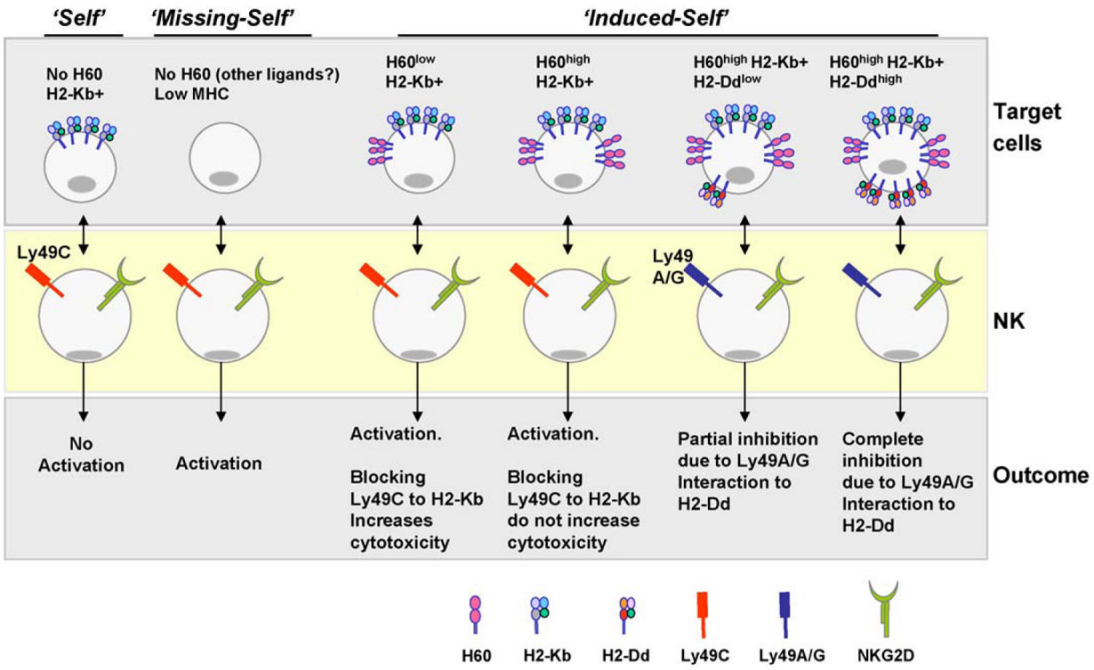

Fig. 2.

Activation through NKG2D receptor is subjected to the negative inhibition of Ly49 receptors. Cells expressing optimal levels of MHC class I ('*Self*') and no activating ligands such as H60 were not lysed by the NK cells. We assume that in these combinations the NK cells are under active inhibition and any stimulatory ligands present on the target cells are unable to overcome the inhibition. Pathological conditions may lead to the down-regulation of MHC class I leading to the '*missing-self*' recognition by the NK cells. Absence of inhibition leads to the activation. However, the activating ligand(s) involved in these specific combinations have not been defined in murine target cells. Target cells under stress express self ligands which has been defined as '*induced-self*'. Our studies demonstrate that the '*induced-self*' is a component of '*self*' and '*missing-self*' recognition. Optimal expression of H60 along with normal expression of MHC on the target cells leads to the activation of NK cells. However, blocking the interaction of Ly49C to H2-K^b augments the ability of NK cells to mediate cytotoxicity. This indicates active inhibitory signals along with the NKG2D-mediated activating signals occurring concomitantly. Increasing the levels of H60 on the target cells resulted in a higher level of NKG2D-mediated cytotoxicity and blocking Ly49C to H2-K^b did not alter the outcomes. Interestingly, introduction of additional MHC class I molecule, H2-D^d, on target cells could significantly affect the activation mediated through NKG2D receptor. Thus, the functional outcome of NK cell activation depends on the balance between the activatory and inhibitory signals.