Innate immune recognition by stimulatory immunoreceptors
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The specificity of cells of the innate immune system is determined in part by various stimulatory receptors that function in different forms of immune recognition. NKG2D, a stimulatory receptor expressed by natural killer (NK) cells, macrophages and certain T cell subsets, recognizes various families of ‘induced-self’ ligands. The ligands are distantly related to class I MHC molecules and are induced in ‘distressed’ cells as markers of abnormal self. Another form of innate immune recognition is exemplified by the Ly49H receptor, which is expressed by a subset of NK cells. The Ly49H receptor directly recognizes a virus-encoded protein expressed by cells infected with mouse cytomegalovirus (MCMV) and the Ly49h gene is identical to the Cmv1β gene, which confers resistance to MCMV infections. Yet another group of receptors (the triggering receptors expressed by myeloid cells, or TREMs), which are exclusively expressed by myeloid cells, have been shown to amplify cytokine responses to bacterial products and have also been implicated in the pathogenesis of septic shock.

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Abbreviations
BCR B cell receptor
GM-CSF granulocyte-monocyte colony-stimulating factor
IFN interferon
ITAM immunoreceptor tyrosine-based activation motif
ITIM immunoreceptor tyrosine-based inhibition motif
KARAP killer cell-activating receptor-associated protein
MCMV mouse cytomegalovirus
MHC major histocompatibility complex
MICA/B MHC class I chain-related proteins A and B
Rae1 retinoic acid early inducible 1 protein
TCR T cell receptor
TNF tumor necrosis factor
TREM triggering receptor expressed by myeloid cells
ULBP UL16-binding protein

Introduction
Stimulatory immunoreceptors occupy center stage in the recognition of foreign antigens or pathogens by the immune system. Archetypal examples of stimulatory immunoreceptors are the B cell receptor (BCR) and T cell receptor (TCR), which are encoded by rearranging genes and are the major structures employed by B and T cells to discriminate between self and nonself molecules. Stimulatory immunoreceptors are multisubunit structures composed of ligand-binding subunits (e.g., the TCR α and β chains and the BCR heavy and light chains) and associated transmembrane signaling adaptor protein(s). The cytoplasmic domains of adaptor proteins contain an immunoreceptor tyrosine-based activation motif (ITAM) with the consensus sequence YxxL/IXxYxxL/I (with x representing any amino acid, the other residues are represented by amino acid one-letter code). The TCR associates with several adaptor proteins including the TCRζ chain (also called CD3ζ), and the BCR associates with the Igα and Igβ adaptor proteins. Crosslinking of stimulatory immunoreceptors leads to activation of the corresponding cells and initiates immune responses against pathogens and tumors.

The cells of the innate immune system do not express highly diversified receptors encoded by rearranging genes. Instead, they express various other stimulatory immunoreceptors that also associate with ITAM-containing signaling adaptor proteins, including CD3ζ, FcRγ and killer cell-activating receptor-associated protein (KARAP, also called DAP12). The biological roles of many of these receptors are not well understood, primarily because many of the ligands have not been identified and genetic loss-of-function studies have not yet been carried out in most cases.

This review focuses on three stimulatory receptor systems that fulfill very distinct functions in the innate immune response: NKG2D, Ly49H and the triggering receptors expressed by myeloid cells (TREMs). These three receptor systems illustrate distinct paradigms of how innate immune recognition via stimulatory receptors is achieved.

NKG2D is a stimulatory receptor expressed by lymphoid and myeloid cells that recognizes various cell surface ligands that are distantly related to class I MHC molecules and upregulated in infected, transformed or stressed cells [1]. Ly49H is expressed by a subset of NK cells and directly recognizes a viral gene product from mouse cytomegalovirus (MCMV), suggesting that this receptor may be involved in the immune response to viruses [2**–6**]. The TREMs are a small family of stimulatory receptors exclusively expressed by myeloid cells. The ligands for these receptors are unknown. However, the
blockade of TREM-1 in mice prevents various types of sepsis syndromes, suggesting that this receptor is involved in enhancing innate immune responses to several types of pathogens [7**]. All of these immunoreceptors use the KARAP/DAP12 molecule as a signaling adaptor protein ([6**,7**], see also Update).

**Stimulatory receptors expressed by NK cells**

The activation of NK cells is regulated by a balance of signals received from inhibitory and stimulatory receptors. NK cells are known to be activated by many tumor cells and virus-infected cells. How NK cells selectively recognize these cells is largely unknown. NK cells express various families of inhibitory receptors, all of which interact with class I MHC molecules. These receptors prevent NK cells from attacking normal self cells, while allowing them to attack cells that downregulate class I MHC molecules, as often occurs in tumor cells and virus-infected cells [8]. This form of interaction is called ‘missing self’ recognition (Figure 1b; [9]).

Similar to other lymphocytes, NK cells must be triggered to become activated. A variety of stimulatory receptors expressed by NK cells have been identified over the years [1,10], but the specificity and function of most of these receptors has been difficult to determine. Recently, ligands for some of the stimulatory receptors (e.g. NKG2D, Ly49H) have been identified, which has aided in developing a nascent understanding of their functions.

**The NKG2D receptor recognizes ‘induced self’ ligands as markers of abnormal self**

**NKG2D receptor and its expression**

NKG2D is expressed by various lymphoid and myeloid cells. In mice, NKG2D is expressed by all NK cells, by all epidermal γδ T cells, and by subsets of NKT cells and splenic γδ T cells (but not by intestinal epithelial γδ T cells) [11,12**]. Additionally, NKG2D is expressed by essentially all CD8+ T cells after T cell receptor triggering and by macrophages after stimulation with LPS, IFN-γ or IFN-αβ [11,12**]. In humans a slightly different expression pattern is observed; in addition to NK cells, all CD8+ T cells and all intraepithelial lymphocyte (IEL) γδ T cells constitutively express NKG2D [13–15].

**Ligands for the NKG2D receptor**

NKG2D recognizes several families of cellular ligands, all of which are distantly related to MHC class I molecules in sequence and are upregulated on tumor cells, virally infected cells and ‘stressed’ cells (Figure 1c; [16]). The first ligands to be identified were a family of non-classical class I MHC molecules, the MHC class I chain-related proteins A and B (MICA/B; [13]). These molecules were initially identified as ligands for a subset of human γδ T cells [17,18**]. Interestingly, MICA/B are not expressed by most normal tissues but are upregulated in many epithelial tumor cells [19], in cells infected with human cytomegalovirus [20**], in bacterially infected cells [21] and in ‘stressed’ cells [22]. A low level of MICA/B expression is maintained on the epithelial cells lining the gastrointestinal surfaces, which may be due to interactions of these cells with various environmental ‘stressors’ [22,23]. No MICA/B homologs have been identified in mice.

We, and others, recently cloned two novel families of ligands for the mouse NKG2D receptor, Rae1 and H60 [11,24]. The retinoic acid early inducible 1 proteins (Rae1s) are encoded by a family of five very closely related genes (Rae1a–e) [25,26,27*,28]. H60 was initially identified as a dominant minor histocompatibility antigen in the response of C57BL/6 mice against BALB.C cells [29]. H60 and Rae1 proteins are distantly related to class I MHC molecules in sequence, although they lack an α2-like domain. Furthermore, unlike the MHC-encoded MICA/B genes, the H60 and Rae1 genes are all co-localized away from the MHC on mouse chromosome 10 [25,29]. Most interestingly, the Rae1 proteins are not expressed by most normal cells, but are upregulated by many tumor cells of diverse origin [11,27*,30**]. H60 is expressed by some tumor cells from BALB/c mice but is also expressed at low levels by activated lymphoblasts and at high levels by thymocytes from BALB/c mice [1,11,29].

Interestingly, a region of human chromosome 6 (q24.2–q25.3), which is syntenic to the murine Rae1/H60 locus contains a related gene family that encodes NKG2D ligands. This family of proteins, variously called UL16 binding proteins (ULBPs; [31**]), ALCAN [32] or human RAE1 proteins [33], is encoded by 10 related genes, six of which encode potentially functional glycoproteins and four of which are pseudogenes [33]. The ULBPs were initially identified based on the ability of some members (ULBP1 and 2) to interact with UL16, a protein encoded by human cytomegalovirus (HCMV). It has been proposed that UL16 helps the virus evade the NK cell response by binding to and inactivating ULBPs in infected cells [31**].

**NKG2D function**

The expression of NKG2D ligands on target cells potently induces NK cell cytotoxicity (Figure 1c; [11,12**,13,30**]). Depending on the levels of NKG2D ligands, the stimulatory signal can override coexisting inhibitory signals provided by the same target cell [11,13,24,30**]. However, the stimulatory signal provided by NKG2D is not entirely refractory to inhibitory signals [14].

Crosslinking of the NKG2D receptor on NK cells triggers several effector mechanisms from NK cells (e.g. mobilization of intracellular Ca2+ [12**], production of cytokines
including IFN-γ [11,12**,30**,34,35], GM-CSF [31**,35], TNF-α [34] and TNF-β (LT-α) [31**], and production of chemokines such as macrophage inflammatory protein (MIP-1β) [34,35] and I-309 [31**]. Interestingly, triggering of the NKG2D receptor alone is sufficient to stimulate NK cell activation, contradicting the notion that NKG2D is a co-stimulatory receptor in NK cells [12**]. The cross-linking of NKG2D expressed by activated macrophages also led to the induction of effector mechanisms (e.g. production of nitric oxide and TNF-α [11,12**]). By contrast, activated CD8+ T cells that express NKG2D cannot be directly stimulated via NKG2D [12**,20**]. In CD8+ T cells, including CD8α CD8+ T cells, however, NKG2D provides a co-stimulatory signal that synergizes with T cell receptor signals [12**,15,20**,30**]. Hence, interactions of NKG2D with induced ligands provide functions appropriate to the cells in which it is expressed: direct activation of innate immune cells and enhancement of the response of antigen-specific CD8+ T cells.

NKG2D in anti-tumor immune responses

Many human tumors of epithelial origin express MICA/B [19], and most mouse tumor cell lines of diverse origin also express ligands for NKG2D [11,30**]. Blockade of the NKG2D receptor–ligand interaction resulted in reduced NK killing of all NKG2D ligand-positive tumor cell lines tested, suggesting that NKG2D plays a significant role in natural cytotoxicity to tumor cells [12**].

Ectopic expression of the NKG2D ligands Rae1 or H60 in rare tumor cell lines that do not naturally express ligands (the RMA lymphoma and its MHC class I low variant RMA-S, the EL4 thymoma and the B16-BL6 melanoma) resulted in uniform rejection of the tumor cells by syngeneic mice [30**,36**,37*]. The rejection was mediated by NK cells in the case of the B16-BL6 melanoma, EL4 thymoma and MHC class I- RMA-S lymphoma [30**,37*], or cooperatively by CD8+ T cells and NK cells in the case of the MHC class I- RMA lymphoma [30**,37*]. Interestingly, rejection of NKG2D ligand-expressing RNA or RMA-S tumor cells required functional perforin but not IFN-γ, suggesting that pore-forming cytotoxic granules, but not IFN-γ, are used by NK cells to CD8+ T cells, are the main effector mechanism for tumor rejection [37*]. Strikingly, mice that had rejected tumor cells expressing ligands for NKG2D were immune when subsequently rechallenged with the parental tumor cell lines that lacked NKG2D ligands. This efficient vaccination effect was dependent on tumor-specific CD8+ T cells [30**]. Also, vaccination of mice with irradiated ligand-positive tumor cells, but not with the ligand-negative parental cells, led to potent priming of tumor-specific CTLs in an NK cell-independent manner [30**]. These data suggest that tumor cell expression of NKG2D ligands can enhance adaptive immune responses specific for tumor cells.

In summary, the data suggest that the NKG2D immunoreceptor is an important ‘sentinel’ used by various lymphoid and myeloid cells to detect cells that have upregulated induced-self ligands as a result of various cellular insults (Figure 1c).

Direct recognition of viral proteins by NK cells

Ly49H is another stimulatory receptor expressed by NK cells. Ly49H is a C-type lectin-like receptor that is a member of the Ly49 family of MHC class I-specific receptors [38]. In contrast to the majority of the Ly49 molecules, Ly49H does not contain an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain. Instead, Ly49H associates with the signaling adaptor protein KARAP/DAP12, which contains an ITAM and couples the Ly49H receptor to the syk/ZAP70 signaling pathway [39]. Crosslinking of Ly49H induces cytotoxicity against target cells and the production of cytokines (GM-CSF, IFN-γ; [40]). Similar to other Ly49 molecules, Ly49H is expressed in a variegated fashion by approximately 50% of NK cells [40,41]. Unlike most other Ly49 family members, however, Ly49H does not bind to any known class I MHC molecule.

Recently, two groups reported evidence that a gene previously shown to confer resistance to MCMV infection, Cmv1R, is identical to the Ly49h gene on mouse chromosome 6 [2**,3**]. Furthermore, the blockade of Ly49H in C57BL/6 mice, which express Cmv1R, rendered these mice susceptible to infection with MCMV [2**,4**]. More recently, two groups reported that Ly49H directly binds to the product of the MCMV m157 gene, a protein that is expressed on the surface of infected cells [5**,6**]. Although the sequence of m157 is not notably homologous to MHC proteins, structural prediction programs suggest that the protein is similar to class I MHC molecules in structure, with similarities to the H-2M3, MICA and CD1d nonclassical MHC class I molecules [5**,6**]. Target cells transfected with m157 triggered cytotoxicity and the production of IFN-γ and activation-induced T cell derived and chemokine-related cytokine (ATAC)/lymphotactin by NK cells from C57BL/6 mice [5**,6**]. A possible beneficial function of m157 expression for MCMV was suggested by the finding that, in addition to binding Ly49H in C57BL/6 mice, m157 binds the inhibitory MHC-specific Ly49I receptor in another mouse strain, 129/J [5**]. Therefore, m157 may help the virus inhibit and therefore evade NK cells in some mouse strains (Figure 1b). The reactivity of Ly49H with m157 may have evolved in the host as an immune countermeasure (Figure 1a). These notions would be supported if it could be shown that recombinant viruses lacking the m157 gene are more pathogenic in resistant strains such as C57BL/6 and less pathogenic in sensitive strains such as 129/J.
**Stimulatory receptors expressed by myeloid cells**

Several stimulatory receptors have been identified over the years that are expressed by myeloid cells and signal through ITAM-containing adaptor proteins, usually KARAP/DAP12 [42]. As discussed above, NKG2D is one receptor of this type [11,12**; see also Update]. The specificities and biological functions of most of the other receptors in this category are unknown. Recently, the TREM family of receptors has attracted considerable attention based on evidence that one family member (TREM-1) participates in the pathogenesis of septic shock in mice infected with bacteria [7**].

**The TREM family**

Five Trem genes have been identified to date, four of which (Trem 1–4) encode potentially functional type I transmembrane glycoproteins [43]. The Trem genes are clustered on mouse chromosome 17/human chromosome 6. TREMs display a single extracellular Ig-like domain. For signaling, all known TREMs associate with the KARAP/DAP12 signaling adaptor protein [43–46]. Interestingly, the TREMs are distant relatives to a stimulatory receptor expressed by human NK cells, Nkp44 [47,48]. Nkp44 also associates with KARAP/DAP12 and maps to the same region on human chromosome 6 [44].

The TREMs are expressed by different subsets of myeloid cells. Whereas Trem-2 is exclusively expressed by immature monocyte-derived dendritic cells [45], TREM-1 is expressed by neutrophils and a subset of CD14high monocytes. Significantly, the stimulation of TREM-1-expressing cells with bacteria (both Gram positive and Gram negative) and fungi, or their cell-wall products, results in marked upregulation of TREM-1 on monocytes and neutrophils [7**.44]. In addition, TREM-1 is also specifically upregulated in situ on neutrophils and monocytes from peritoneal lavages of patients with bacterial peritonitis [7**]. Triggering of TREM-1 on neutrophils leads to the release of IL-8 and myeloperoxidase [44]. Monocytes produce IL-1β, TNF-α and monocyte chemoattractant protein (MCP)-1 after stimulation of TREM-1 [7**.44]. TREM-1 stimulation also upregulates a variety of adhesion molecules (ICAM-1, CD11b, CD49e, CD29) and co-stimulatory receptors (CD40, CD86/B7.2) expressed by monocytes and/or neutrophils [7**].

Importantly, TREM-1 synergizes with LPS in the induction of cytokine and chemokine responses. Notably, TREM-1 engagement enhances the release of TNF-α and IL-1β, pro-inflammatory cytokines that mediate septic shock. Strikingly, blocking experiments performed with TREM-1-Ig fusion proteins prevented septic shock and inflammatory responses in three different experimental models of septic shock. These results indicate that TREM-1 recognition participates in the pathogenesis of septic shock [7**]. The results also suggest that LPS and TREM-1 act cooperatively, but how the respective signaling pathways interact is not yet well understood. Furthermore, because TREM ligands have not yet been identified, it is not known whether TREMs recognize components of the pathogen or ‘induced-self ligands’.

**Perspectives**

Stimulatory receptors on lymphoid and myeloid cells are multisubunit receptor complexes composed of extracellular ligand-binding subunits that associate with various ITAM-containing signaling molecules. Some of these receptors are primary immune recognition receptors (e.g. BCR, TCR, Ly49H), whereas others may act as amplifiers of a response (TREM’s may fall into this category). NKG2D does both, functioning as a primary stimulatory receptor on NK cells and macrophages and as a co-stimulatory receptor on CD8+ T cells. Despite their similarity in subunit structure, stimulatory receptors play different roles in the immune system, which in some cases illustrates distinct strategies of immunity.

The first and most familiar strategy of immune recognition is the direct recognition of foreign molecular structures (Figure 1a). The adaptive immune system, by virtue of its great diversity of specificities, can respond to virtually any foreign molecular structure. Components of the innate immune system, by contrast, are less diverse and appear to have evolved to recognize a much smaller universe of foreign structures, albeit ones that are characteristic of specific classes of potential pathogens. This concept is well illustrated by the Toll-like receptors (TLRs), which participate in the recognition of various common components of microbes and viruses. The recognition of viral components by Ly49H, and possibly other NK receptors, such as Nkp46 and Nkp44, is apparently yet another example of this type of recognition (Figure 1a; [5**.6**.49]).

A second general strategy of immunity is the concept of ‘missing-self’ recognition, in which immune cells express inhibitory receptors specific for markers of normal self cells (Figure 1b; [9]). When expression of the normal self markers is prevented in a cell, inhibition is disrupted and the cell is attacked. This type of recognition operates in the complement system, but perhaps the best characterized example is the recognition of MHC class I molecules by inhibitory NK receptors such as Ly49s, KIRs and CD94/NKG2A. MHC downregulation, as occurs frequently during viral infection and transformation, can lead to susceptibility of an infected cell to NK cell attack. This type of system is vulnerable to evasion by viruses, which may produce proteins that engage inhibitory receptors and therefore prevent NK responses. It appears likely that this has occurred in the case of MCMV m157, which binds to at least one of the inhibitory receptors expressed by mouse NK cells (Figure 1b). As a possible countermeasure, mice have evolved stimulatory Ly49
isoforms (e.g. Ly49H), which bind the same viral ligand (Figure 1a).

The recognition of ‘induced-self ligands’ by the NKG2D receptor is a paradigm for a third strategy of immune recognition (Figure 1c; [1]). NKG2D ligands are all distant related to class I MHC molecules and are not expressed by most normal cells [1]. Upregulation of these ligands in response to various cellular insults (transformation [11], viral infection [20**, bacterial infection [21] and several other forms of cellular abuse [22]) results in recognition of these cells by the immune system (Figure 1c; [1,50]). Interestingly, this type of recognition system shifts the burden of discriminating ‘normal’ and ‘abnormal’ from the immune system itself to the potential target cell, which must ‘decide’ whether to upregulate the ligands. Strikingly, as has also been revealed in studies of foreign pattern recognition by TLRs, induced self recognition (via NKG2D) activates not only the innate immune system, but also provides key signals in the activation of adaptive immune responses [30**,51**].

**Update**

Recent studies have shed light on how the NKG2D receptor provides different signals in different cell types. Two NKG2D splice variants associate with different signaling adaptor proteins, resulting in qualitatively different signals [52**]. In CD8+ T cells, NKG2D associates solely with the signaling molecule DAP10, which provides a primarily co-stimulatory signal. In NK cells and macrophages, by contrast, NKG2D associates with both DAP10 and the ITAM-containing KARAP/DAP12 signaling molecule, which together provide both co-stimulatory and direct stimulatory signals in these cells. A recent analysis of mice genetically deficient for DAP10 arrived at a similar conclusion [53**]. In these mice, CD8+ T cells do not express cell surface NKG2D, and NKG2D function is abolished in these cells. In contrast, activated NK cells show normal surface expression of NKG2D and the function of NKG2D is only moderately impaired in these cells. Most strikingly, tumor cells expressing

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Figure 1

(a) Normal self cell | Infected cell displaying microbial ‘non-self’
Ly49H | m157
KARAP/DAP12

(b) Normal self cell | Infected cell downregulates ‘normal self’ marker | Infected cell displaying ‘stolen identity’
Ly49s, KIRs, CD94/NKG2A | MHC class I
Activated

(c) Normal self cell | ‘Stressed’ cell upregulates ‘induced self’ ligands as markers of abnormal self
NKG2D ligand (MICA/B, Raes1s, H60, ULBPs)
Unactivated | Activated
NK cell

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Innate immune recognition by NK cells. The figure illustrates three different modes of innate immune recognition, as mediated by NK cells in this example. (a) Direct recognition of microbial ‘nonself’. MCMV-infected target cell expresses the viral protein m157. Ly49H binds to m157, resulting in activation of the NK cell by KARAP/DAP12-mediated signaling events. (b) ‘Missing-self’ recognition. Inhibitory receptors (Ly49s, KIRs, CD94/NKG2A) expressed by NK cells interact with self class I MHC molecules on normal self cells. The ITIM motif in the cytoplasmic domain of these inhibitory receptors relays an inhibitory signal to the NK cell. Infection or transformation is often accompanied by downregulation of self class I MHC, unleashing the NK cell. Some viruses evade NK immunity by expressing molecules that mimic surface MHC class I, which can inhibit NK cells despite the downregulation of endogenous class I MHC molecules. (c) Recognition of ‘induced self’ ligands as markers of abnormal self. Stressed cells (e.g. infected, transformed) upregulate ‘induced self’ ligands for the NKG2D receptor, leading to NK cell activation via KARAP/DAP12 and/or DAP10 signaling events.
NKG2D ligands were rejected to the same extent in DAP10 deficient mice as in wild-type controls. Taken together, these data provide strong evidence that the NKG2D receptor fulfills distinct functions in the innate and adaptive immune system. In NK cells and macrophages NKG2D acts as a direct stimulatory receptor by associating with both ITAM-containing stimulatory adaptor proteins (KARAP/DAP12) and a co-stimulatory adaptor protein (DAP10). In the adaptive immune system NKG2D has a co-stimulatory function, amplifying antigen-specific signals provided by the TCR, by virtue of its sole association with a co-stimulatory adaptor protein (DAP10).

Another recently reported finding is that tumor cells expressing the MICA/B ligands for the human NKG2D receptor can release a soluble form of these proteins that are detectable in the serum of patients with MICA/B-positive tumors [54**]. Binding of soluble MICA/B to NKG2D induces downregulation of cell surface NKG2D on human CD8+ T cells as a result of ligand-induced endocytosis and probably lysosomal degradation. Malignoma antigen-specific CD8+ T cell clones that have down-regulated NKG2D after incubation with soluble MICA/B proteins can no longer be costimulated by NKG2D ligand-positive tumor cell lines. This paper provides the first evidence for an immune evasion mechanism employed by tumor cells to avoid recognition via NKG2D.

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References and recommended reading
Papers of particular interest, published within the annual period of review, have been highlighted as:
• of special interest
** of outstanding interest
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This paper provides biochemical, molecular and genetic evidence that NKG2D associates with both KARAP/DAP12 and DAP10 in NK cells and macrophages and solely with DAP10 in CD8\(^+\) T cells. Accordingly, NK cells and macrophages from KARAP/DAP12 loss-of-function mice were severely impaired in NKG2D-mediated functions, whereas NKG2D co-stimulatory function was unimpaired in CD8\(^+\) T cells. Ectopic expression of KARAP/DAP12 in CD8\(^+\) T cells conveyed direct stimulatory functions to the NKG2D receptor in these cells.

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