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Regulation of immune cell function and differentiation by the NKG2D receptor

Biljana Zafirova · Felix M. Wensveen ·
Maja Gulin · Bojan Polić

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Abstract NKG2D is one of the most intensively studied immune receptors of the past decade. Its unique binding and signaling properties, expression pattern, and functions have been attracting much interest within the field due to its potent antiviral and anti-tumor properties. As an activating receptor, NKG2D is expressed on cells of the innate and adaptive immune system. It recognizes stress-induced MHC class I-like ligands and acts as a molecular sensor for cells jeopardized by viral infections or DNA damage. Although the activating functions of NKG2D have been well documented, recent analysis of NKG2D-deficient mice suggests that this receptor may have a regulatory role during NK cell development. In this review, we will revisit known aspects of NKG2D functions and present new insights in the proposed influence of this molecule on hematopoietic differentiation.

Keywords NKG2D · NKG2D ligands · NK cell development · Viruses · Tumors · Autoimmunity

The NKG2D receptor and its ligands

NKG2D is a potent activating receptor expressed on virtually all NK cells [1]. It is also expressed on most NKT cells and subpopulations of $\gamma\delta$ T cells [2]. All human CD8⁺ $\alpha\beta$ T cells express NKG2D, whereas NKG2D expression on CD4⁺ $\alpha\beta$ T cells has primarily been reported in some pathological conditions [3–6]. Mice express NKG2D only on activated and memory type $\alpha\beta$ CD8⁺ T cells. Thus,

NKG2D is expressed on effector cells of both innate and adaptive immune responses and is implicated in the surveillance of viral infections and cancers as well as in some autoimmune processes and transplantation reactions [7–15].

NKG2D is a C-type lectin-like type receptor and belongs to the NK group 2 (NKG2) of receptors as member D. It has also been classified as killer cell lectin-like receptor of the subfamily K, member 1 (KLRK1) [16]. NKG2D (KLRK1) is encoded by the *Nkg2d* (*Klrk1*) gene that is located within the NK gene complex (NKC) situated on chromosome 6 in mice and on chromosome 12 in humans [17, 18]. NKG2D is atypical in comparison with other NK receptors. Although NKG2D belongs to the NKG2 family, it does not share most of their properties. In contrast to other members of the family (NKG2A/B, NKG2C, and NKG2E), which form heterodimers with CD94 and recognize MHC class Ib molecules (HLA-E in humans and Qa1 in mouse), NKG2D is a homodimer and recognizes a number of stress induced MHC class I-like ligands [2, 19]. There is no inhibitory counterpart known for NKG2D and NKG2D is capable of overriding signals provided by inhibitory receptors on NK cells engaging MHC class Ia and Ib molecules. Thus, NKG2D plays a role as a molecular sensor detecting “induced self” on cells in danger, which is mostly triggered by viral infections and by factors causing DNA damage and tumor transformation [19, 20].

The NKG2D receptor consists of two disulfide-linked type II transmembrane proteins with positively charged amino acid residues in their transmembrane domains and very short intracellular tails that do not have any signaling properties [21]. Signal transduction operates through two adaptor proteins, DAP10 and DAP12, which associate with the receptor as homodimers [22, 23]. The NKG2D

B. Zafirova · F. M. Wensveen · M. Gulin · B. Polić (✉)
Department of Histology and Embryology, Faculty of Medicine,
University of Rijeka, B. Branchetta 20, 51000 Rijeka, Croatia
e-mail: bojanp@medri.hr

signaling complex appears as a hexameric structure since each NKG2D homodimer binds two adaptor homodimers [24]. In mice, there are two NKG2D isoforms that differ in length of their intracellular sequence by 13 amino acid residues and have different association properties for the two adaptor proteins. The short isoform of NKG2D (NKG2D-S) associates with both adaptor proteins, while the long one (NKG2D-L) only binds to DAP10 [22, 23, 25]. Humans possess only the NKG2D-L isoform.

DAP10 has a YINM motif in its cytoplasmic tail which, upon phosphorylation, recruits the p85 subunit of phosphoinositide-3-kinase (PI3K) and Grb2–Vav1. This signal stimulates survival and cytotoxicity of NK cells and provides co-stimulation to activated T cells [26–28]. DAP12 possesses an immunoreceptor tyrosine-based activation motif (ITAM) which, after phosphorylation, recruits Src family kinases ZAP70 and Syk, responsible for cytokine release and enhancement of cytotoxicity in NK cells [29]. Even though there are some reports that DAP12 can also be expressed by T cells [30], deficiency of DAP10 results in complete loss of NKG2D signaling in T cells and therefore appears to be the most important adaptor for NKG2D signaling in these cells [13, 22, 23].

NKG2D is able to bind a number of MHC class I-like ligands because of its unique protein structure [31–33]. In mice, it binds to the family of retinoic acid early inducible proteins (Rae1) [34, 35]. There are five members of these GPI-anchored proteins (Rae1- α , - β , - γ , - δ , and - ϵ) and their expression differs between mouse strains [19]. NKG2D also binds to the closely related group of histocompatibility antigen 60 (H60) glycoproteins, which consists of three members (H60a–c). H60a and b are transmembrane proteins, while H60c is GPI-anchored [36–38]. Whereas H60a is widely expressed, H60b and c proteins display highly tissue- (but not mouse strain)-specific expression patterns [19]. Murine UL-16-binding protein-like transcript 1 (MULT1), which binds NKG2D with the highest affinity of all ligands, is so far the only transmembrane glycoprotein that belongs to the third group of murine NKG2D-binding proteins [39, 40].

In humans, NKG2D binds to the MHC class-I related proteins MICA and MICB (MHC class I chain-related protein A and B) [1]. NKG2D also recognizes surface glycoproteins that bind human cytomegalovirus (HCMV) UL-16 protein (ULBPs) [12, 41–43]. There are six members of the ULBP family of proteins, which are closely related to the Rae1 molecules in mice. ULBP1, -2, and -3 and -6 are GPI-anchored, while RAET1E and -G (also known as ULBP4 and ULBP5) are transmembrane proteins. NKG2D ligands are mostly induced by cellular stress (“induced self”), although some of them are expressed at low levels in different tissues [37–39, 44]. However, expression of the ligands is very tightly controlled at

transcriptional or/and posttranscriptional levels, since unbalanced expression may trigger activation of the immune system and autoimmune responses [45].

NKG2D in the control of viral infections

Viruses, as intracellular pathogens, are usually efficiently controlled by NK and CD8⁺ T cells. NK cells limit viral replication and viral load in different tissues early upon infection, while CD8⁺ $\alpha\beta$ T cells are responsible for the final viral clearance and, in some cases, for the establishment of viral latency. NKG2D is expressed on both of these cell types and plays an important role in the control of viral infections. Different viruses are able to induce expression of NKG2D ligands on infected cells [46–49]. They cause strong activation of NK cells as well as enhancement of antiviral effector functions of activated CD8⁺ $\alpha\beta$ T cells, which can be prevented by NKG2D-specific mAbs [1, 13, 46, 48, 50]. Thus, NKG2D-mediated control represents a powerful and efficient mechanism to cope with viral infections.

However, the NKG2D-mediated immunosurveillance may exert considerable selective pressure on viruses in the course of the co-evolution with their hosts. Therefore, it is not surprising that some of them have developed mechanisms to evade NKG2D-mediated activation of the immune system [14].

Cytomegaloviruses (CMVs) have particularly well-developed mechanisms of interference with NKG2D-mediated immune cell activation. The HCMV-encoded membrane glycoprotein UL16 binds the NKG2D ligands ULBP1, ULBP2, and MICB [12, 51, 52], thus preventing their expression on the cell surface. HCMV gene product UL142 retains ULBP-3 in the *cis*-Golgi complex and prevents its transport to the membrane [53]. UL142 also retains newly synthesized full-length MICA (allele 001) in the *cis*-Golgi [54, 55]. Interestingly, the MICA allele 008, which lacks the cytoplasmic domain, shows resistance to UL142 binding. The fact that the truncated form of MICA is the most common allele in several human populations suggests a profound advantage of the hosts' ability to control virus infection through NKG2D-dependent activation of NK cells [56].

Although MICA and MICB genes are transcribed in all cells [57], their translation in healthy cells is repressed by endogenous cellular miRNAs [58]. Viruses exploit these structures to prevent NKG2D ligand upregulation. For example, the HCMV-derived miRNA, miR-UL112, protects infected cells from NKG2D-mediated lysis by acting as a sponge for MICB mRNA [59].

Mouse cytomegalovirus (MCMV) has also been shown to extensively downregulate NKG2D ligands. Four viral

proteins are involved in this interference. MCMV protein m145 downmodulates cell surface expression of MULT-1 [60], m152 interferes with the expression of Rae1 and H60 proteins [61, 62], while m155 is involved in downmodulation of H60 [63]. In addition, m138/fcr-1 plays an important role in downmodulation of MULT-1, Rae1 ϵ and H60 [64, 65].

Kaposi's sarcoma-associated herpesvirus (KSHV) immune evasion gene K5 reduces cell surface expression of MICA and MICB as well as of the NKp80 ligand activation-induced C-type lectin (AICL) via ubiquitination of lysine residues in cytoplasmic tails of the ligands [66].

Epstein–Barr virus (EBV) can cause B cell lymphomas in immunosuppressed patients. EBV-transformed B cell lines express a high level of MHC class I molecules rendering them NK cell-resistant. However, reactivating latent EBV in transformed B cells increases susceptibility to NKG2D- and DNAM1-dependent NK cell lysis [67].

Ectromelia virus (ECTV) belongs to the orthopoxvirus genus, which includes variola virus (VARV), the causative agent of smallpox, and vaccinia virus (VV). Depletion of NK cells in mousepox-resistant mouse strains (C57BL/6) results in massive viremia and death [68]. ECTV induces expression of NKG2D ligands on infected cells. Neutralization of NKG2D recognition by antibodies causes increased viral titers and mortality [46]. Zoonotic orthopoxviruses (monkeypox and cowpox viruses) encode for a soluble antagonist of NKG2D [69]: orthopoxvirus MHC class I-like protein (OMCP). OMCP, which is conserved among cowpox and monkeypox viruses, is secreted by infected cells and blocks recognition of NKG2D ligands [69]. Binding of OMCP causes internalization of NKG2D, thus lowering the amount of available NKG2D receptor and inhibiting NKG2D-dependent killing by NK cells [69].

Human immunodeficiency virus (HIV)-encoded protein Nef (negative factor) has been shown to downregulate expression of the NKG2D ligands MICA, ULBP1, and ULBP2 on Jurkat and primary CD4⁺ T cells in vitro [70], in addition to HLA-A and -B [71]. In contrast to Nef, another HIV-encoded protein Vpr (viral protein R) has recently been shown to upregulate expression of NKG2D ligands ULBP1, -2, -3, but not MICA or MICB, both in vitro and in vivo [72]. Vpr causes upregulation of the ligands through activation of DNA damage/stress-sensing ATR kinase [48]. It seems that Vpr not only contributes to HIV-1-induced CD4⁺ T-lymphocyte depletion but also takes part in HIV-1-induced NK cell dysfunction [72].

In conclusion, NKG2D-mediated control appears to be an important and powerful mechanism in the immunosurveillance of viral infections. A number of viral evasion mechanisms targeting the NKG2D pathway just emphasize its importance and this fact should be seriously considered in the development of future vaccines.

NKG2D in the control of cancer

The potential role of NKG2D in cytotoxic anti-tumor responses was quickly recognized and discussed as a revival of the tumor surveillance model [73]. Early experiments showed that overexpression of NKG2D ligands in cancer cells caused tumor rejection after transplantation in mice [74, 75]. In humans, it was found that expression of NKG2D ligands highly correlated with the amount of T cell infiltrates in solid tumors [76]. Paradoxically, some tumors were shown to actively upregulate NKG2D ligands, making them prone to recognition by NK cells and cytotoxic T cells [76]. Various explanations have been proposed for this observation. As described in the previous section, viral infection leads to upregulation of NKG2D ligands. Some virally induced tumors therefore show enhanced expression of these proteins in vivo [77]. However, also tumors without viral origin can have high levels of NKG2D ligands, suggesting a different cause. One hypothesis is that induction of NKG2D ligands is the result of the oncogenic process itself. NKG2D ligands are stress-response genes and are upregulated by stimuli such as DNA damage [20, 78], heat-shock [44, 79], and shifts in hormone levels [80]. Many, if not all of these process readily occur in cancer cells. Oncogenesis is usually associated with genomic instability [81], heat-shock proteins are often hijacked by tumor cells to promote their survival [82] and many tumors, such as breast, endometrial and ovarian cancers use estrogens to promote their growth [83]. In addition, rapidly proliferating cells have been shown to induce NKG2D ligands [84]. Upregulation of these molecules in cancer cells might therefore be a bystander effect of the oncogenic process. Preventing lysis by NKG2D-expressing NK and CD8 T cells must therefore be a feature that a cancer cell acquires in order to sustain its growth.

Multiple strategies are used by cancer cells in order to prevent NKG2D mediated killing. The first and most obvious strategy is downmodulation of NKG2D ligands. Despite significant amounts of DNA damage or expression of heat-shock proteins, many tumors have been shown to actively inhibit NKG2D ligand expression [9, 85–87]. An important mechanism for NKG2D ligand downregulation appears to be the production of immunomodulatory cytokines such as TGF β , which can be directly excreted by tumor cells themselves, or by regulatory immune cells that are expanded during tumor progression [88–91]. In addition to this system, tumors decrease surface NKG2D ligand expression via shedding of the extracellular domain by metalloproteases or with the assistance of the disulphide-isomerase ERp5 [92, 93]. Thirdly, expression of NKG2D ligands is actively regulated by microRNAs, and tumor cells also have been shown to manipulate this system via

miRNA overexpression [58]. Experimental evidence for the *in vivo* relevance of NKG2D ligand downmodulation in the formation of tumors was recently shown in mice deficient for NKG2D. In animal models for spontaneous tumor formation, absence of NKG2D resulted in enhanced formation of aggressive tumors. In addition, these tumors were shown to have increased expression of Rae1 molecules on their surface [9].

Some tumors exploit an opposite strategy to manipulate NKG2D mediated signaling and instead induce high levels of its ligands [94]. This concept remained relatively ill-understood until it was mimicked in mice overexpressing Rae1 and MICA molecules [15, 95, 96]. In these animals, surface expression of NKG2D was greatly reduced, both on NK cells and on CD8 T cells, resulting in impaired anti-tumor responses by these immune cells [15, 95]. Interestingly, the impact of NKG2D ligand overexpression on CD8 T cell responses against pathogens appears to depend highly on the model used. Infection of transgenic mice with *L. monocytogenes* resulted in reduced anti-bacterial CD8 T cell numbers [15]. Antiviral responses upon mCMV infection, on the other hand, did not functionally impair CD8 T cell responses [96]. One explanation for these observations may be the differential co-stimulation of CD8 T cells upon infection with different pathogens. Unlike CD28 triggering, NKG2D co-stimulation is not required for T cell function [97]. Rather, NKG2D engagement appears to enhance the cytotoxic capacity of these cells. It will therefore depend on the pathogen or kind of tumor encountered to what extent NKG2D signaling is required for T cell-mediated cytotoxicity.

For NK cells, NKG2D is a directly activating receptor and NK cell function was impaired in MICA- and Rae1 ϵ -transgenic animals. Not surprisingly, both *in vivo* and *in vitro* killing of tumor cells expressing NKG2D ligands by NK cells of these mice was reduced. Oppenheim and coworkers also suggested that constitutive engagement of NKG2D impaired NK cell function beyond its downmodulation and subsequent inability to engage its ligands on tumor cells [95]. However, other studies that directly addressed this issue indicate that this is not the case [15, 96]. Interestingly, NKG2D downmodulation via hyperstimulation appears to have different effects than inhibition of NKG2D signaling by omitting the molecules involved in transducing NKG2D-signaling. Downmodulation of NKG2D via antibody treatment or in Rae1 transgenic animals resulted in increased tumor cell growth in a model of chemically induced cancer formation [95, 98]. However, the same model showed no differences when NKG2D-deficient animals were compared with wild type controls [9]. The explanation for these differences is currently lacking, but it appears likely that NKG2D hyperligation

induces compensatory and/or regulatory mechanisms which are absent in NKG2D-deficient animals.

In support of this notion is the observation that NKG2D stimulation promotes the specific outgrowth of regulatory T cell subsets in humans. NKG2D ligation enhances proliferation of a characteristic regulatory NKG2D⁺CD4⁺ T cell pool that is rare under normal conditions [6, 99]. This cell subset produces IL-10 and TGF β , thus inhibiting immune responses in a paracrine fashion [99]. In addition, these cells express high levels of Fas ligand (FASL), which induces apoptosis in neighboring activated T cells, whereas these regulatory cells themselves appear refractory to this FASL [6]. Apart from directly presenting NKG2D ligands *in cis*, human tumor cells can manipulate the immune system by releasing a soluble form of NKG2D ligands. With the help of metalloproteases or of the cell surface-bound protein ERp5, the NKG2D ligands MICA, MICB, and ULBP2 are cleaved of the membrane and are capable of affecting NKG2D expressing cells *in trans* [100–102]. In addition, ULBP4 can be expressed in soluble form via alternative splicing [103]. Apart from producing soluble proteins, tumor cells produce exosomes with high levels of NKG2D ligands [104, 105]. Both soluble ligands and ligands expressed on exosomes have been shown to downmodulate NKG2D on cytotoxic cells and thus impair their anti-tumor activity.

In summary, it appears that tumor cells in general adapt methods to inhibit NKG2D signaling. They do this either via downmodulation of NKG2D ligands or via hyperexpression of NKG2D ligands on their surface or in soluble form. In addition, however, there are also tumors that do not seem to modify the NKG2D signaling pathway at all, yet still escape from destruction. These tumors also arise in experimental models [9], indicating that there is a ‘third’ method to avoid NKG2D-mediated killing. The molecular mechanism behind this phenomenon has yet to be revealed.

Due to its prominent role in tumor cell biology, the NKG2D signaling pathway has been under extensive investigation in the cancer field, both as a diagnostic tool and as a therapeutic target. MICA has been shown to be one of the most polymorphic genes within the group of MHC class I-related molecules [106] and several alleles, supposedly of reduced NKG2D affinity, have been associated with cancer [107–109]. Also, expression of NKG2D ligands, both the soluble and the membrane-bound form, has been shown to be a reliable marker for disease progression in a variety of malignancies [85, 94, 110], illustrating its value as a clinical marker.

Induction of NKG2D ligand expression on tumors appears to be a promising therapeutic strategy in cancer. Expression of the NKG2D ligand MULT1 is low under

homeostatic conditions, due to its continuous ubiquitination and subsequent targeting to the proteasome. In stressed cells, this ubiquitination process is inhibited, leading to rapid upregulation of surface-bound MULT1 [45, 111]. In tumor cells, NKG2D ligand expression can therefore be specifically induced with proteasome inhibitors, making tumors sensitive to NKG2D-mediated killing [112]. In addition, HDAC inhibitors appear to stimulate NKG2D ligand expression on tumor cells and their immunogenicity for allogeneic NK cells [113].

Finally, the NKG2D receptor system has been used as a target for anti-cancer therapy. Therapeutic MICA-specific antibodies effectively opsonized cancer cells and induced DC-mediated cross-presentation of tumor antigens [114]. Bifunctional proteins consisting of a tumor-antigen directed antibody fused to NKG2D ligands effectively 'coated' tumor cells with activating ligands and increased their killing [115, 116].

In summary, the two sides of NKG2D in tumor biology make this protein a key molecule of interest for future oncological research. On one hand, the NKG2D receptor system may be exploited in anti-tumor strategies, and on the other hand, over-stimulation of NKG2D should be prevented in order to potentiate tumor surveillance.

NKG2D in autoimmunity

As previously mentioned, inappropriate or deregulated expression of NKG2D ligands on healthy cells can break the delicate balance between immune activation and tolerance, and trigger autoimmune response [117]. Several autoimmune diseases have therefore been associated with NKG2D signaling.

Insulin-dependent diabetes mellitus/type 1 diabetes (T1D) is a chronic autoimmune disorder in which insulin-producing Langerhans islets are destroyed by autoreactive immune cells. Genetic linkage studies have shown that some MICA alleles are positively associated with human T1D, but the functional relevance of this polymorphism is far from clear [118, 119]. The nonobese diabetic (NOD) mouse is widely studied as a model of human T1D [120]. Prediabetic NOD mice express Rae1 ligands on their islet cells [121]. The development of disease can be completely prevented by treatment with NKG2D-blocking mAbs, which reduce expansion and function of autoreactive CD8⁺ T cells [10].

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disorder in which immune cells, especially T cells, cause inflammation and destruction of the joints [122]. RA patients have high levels of IL-15 and TNF- α in the sera and inflamed joints [5], which induce expression of NKG2D on CD4⁺CD28⁻ subset of T cells [5]. Since

MICA and MICB molecules are also dramatically upregulated in RA synoviocytes, they activate the T cells in an NKG2D-dependent manner [5].

Celiac disease is an autoimmune disorder of the small intestine that occurs in genetically susceptible individuals as a reaction to wheat gliadin protein [123]. One of the diagnostic hallmarks of celiac disease is a massive infiltration of intraepithelial NKG2D⁺ CD8⁺ $\gamma\delta$ T lymphocytes (IELs) in the gut [124]. MIC proteins, normally found intracellularly in enterocytes, become strongly expressed on the surface of epithelial cells in patients with active disease. There is evidence that this process is IL-15-dependent [125, 126].

Crohn's disease (CD) belongs to the group of inflammatory bowel diseases. Significantly increased levels of MIC expression were found on intestinal epithelial cells. It has been shown that the number of intestinal epithelial CD4⁺ T cells expressing NKG2D highly correlates with the amount of intestinal inflammation and therefore are thought to play an important role in disease progression [4].

NKG2D in NK cell development

NKG2D is expressed very early in the development of NK cells, already at the stage of NK precursors (NKPs), the earliest NK committed cells [127]. About 10% of CD122⁺ NK1.1⁻ NKPs (stage I) exhibit this receptor on the cell surface, whereas all immature NK cells (stage II and III) express NKG2D. During these stages of NK cell development, NKG2D expression rapidly increases and remains high through all later stages of maturation [127, 128].

Recently, the intracellular signaling components of IL-15R and NKG2D have been shown to be coupled [129]. IL-15 is an essential cytokine for the development and survival of NK cells [130, 131]. Mice deficient for IL-15, IL-15R β , or any of the components of the IL-15 signaling pathway (e.g., Jak3, STAT5) have severe defects in NK cell development [132–136]. Horng et al. [129] have created a transgenic mouse strain expressing a DAP10 protein fused with the monoubiquitin (DAP10-Ub), which directly targets this molecule for proteasomal degradation. These mice display completely abolished NKG2D expression on T cells and severely reduced expression of this receptor on NK cells, which also express the NKG2D adaptor molecule DAP12. They observed severe defects in NK cell development because of their failure to respond to IL-15 stimulation. Jak3 has been shown to be the essential kinase downstream of the IL-15 receptor that is responsible for DAP10 phosphorylation important for the activation of STAT5, a prime target of Jak3. In addition, it was found that DAP10 can associate with the IL-15R β and γ (Fig. 1). Interestingly, in contrast to DAP10-Ub mice, defects in NK

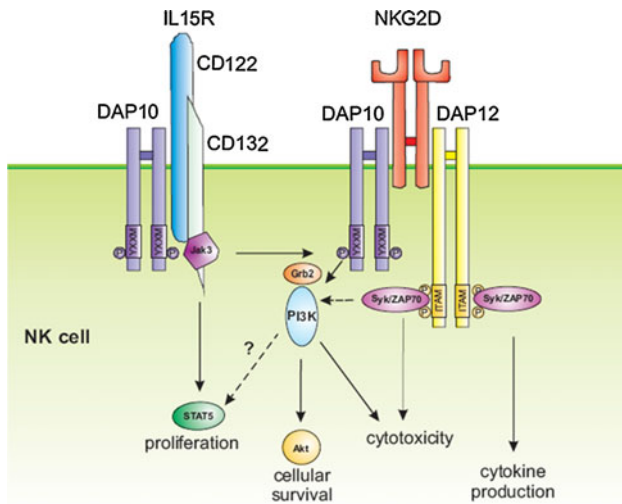


Fig. 1 Coupling of the IL-15R and NKG2D signaling components in NK cells. The figure roughly summarizes interactions between signaling components of the IL-15R and NKG2D, which was proposed by Horng et al. [129]. Jak3, as a part of canonical IL-15R signaling pathway, is essential for the phosphorylation of DAP10. Activated DAP10 recruits PI3K and Grb2, which control proliferation, survival, and cytotoxicity of NK cells. DAP10 also associates with IL-15R β and γ chains

cell development were not reported in *Dap10*^{-/-} mice [23]. This could be explained by redundancy of the system, since intact NKG2D–DAP12 complexes on the surface of NK cells could compensate the lack of DAP10 to a major extent, despite a reduction in NKG2D expression. Activation of Syk by DAP12 can regulate survival and cytotoxicity via activation of PI3K [137, 138].

Considering the above-mentioned findings, the question is whether and how NKG2D is involved in the development of NK cells. In transgenic mouse models with sustained expression of NKG2D ligands (MICA, Rael ϵ , Rael γ) and consequent downmodulation of NKG2D, impairments in the NK cell development were not observed [15, 95, 96]. In these models this may be the result of low-level expression of the receptor complexes on the cell surface and persistent NK cell stimulation.

To resolve this issue, recently two models of NKG2D-deficiency were reported [9, 139]. In the *Klrk1*^{-/-} mice generated by Guerra et al. [9], the authors did not find any major developmental defects, although they showed mild changes in some subpopulations of NK cells (i.e. Ly49A⁺ cells). In *Klrk1*^{-/-} mice generated in our lab, NK cell development was moderately affected [139]. In the absence of NKG2D we observed perturbations in the size of some developmental subsets of NK cells, increased proliferation of immature NK cells (mostly in stage II), their faster maturation and increased sensitivity to apoptosis. In addition, NK cell-mediated control of MCMV infection in these

mice was better than in littermate controls, which we ascribed to the dysregulation and faster maturation of NK cells in the absence of NKG2D.

Differences in targeting strategies may be responsible for the observed effects. Our targeting inserted the EGFP sequence in the third exon of the *Klrk1* locus, which could, as a foreign genetic element, cause non-specific effects. Therefore, we generated *Klrk1* ^{Δ/Δ} mice in which the EGFP cassette is not present (Fig. 2a). These mice were obtained from the breeding of *Klrk1*^{fl ox /fl ox} mice (our unpublished data) and Cre ‘deleter’ mice, which resulted in the elimination of the floxed second and third exon of the *Klrk1* gene in vivo, a mutation that was further propagated as *Klrk1* ^{Δ/Δ} . After we confirmed that NK cells from *Klrk1* ^{Δ/Δ} mice do not express NKG2D (Fig. 2b), we analyzed the various stages of NK cell development in *Klrk1*^{-/-} versus *Klrk1* ^{Δ/Δ} mice in comparison to *wt* animals (Fig. 3a). *Klrk1* ^{Δ/Δ} mice displayed alterations in cell surface marker expression and maturation stages on NK cell subsets that were very similar to those seen in *Klrk1*^{-/-} animals. In addition, both mouse strains controlled early MCMV replication in a highly comparable manner (Fig. 3b). Thus, these data strongly argue that the previously observed phenotype of NK cells in our *Klrk1*^{-/-} mice [139] is the consequence of the desired *Klrk1* mutation rather than of a genetic deregulation caused by the EGFP sequence.

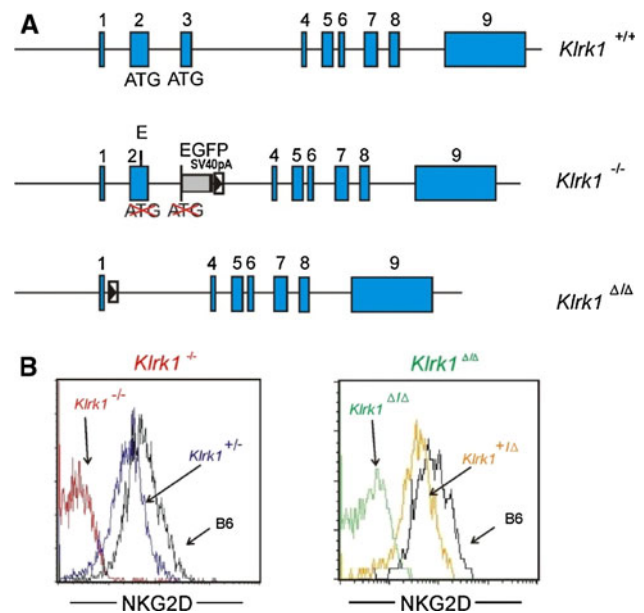


Fig. 2 Comparison of *Klrk1*^{-/-} and *Klrk1* ^{Δ/Δ} mice. **a** Different mutations of the *Klrk1* locus (upper row) in *Klrk1*^{-/-} (middle row) and *Klrk1* ^{Δ/Δ} (lower row) mice are shown. *Klrk1* ^{Δ/Δ} mice were obtained from the breeding of *Klrk1*^{fl ox /fl ox} and Cre deleter mice. **b** NKG2D expression on CD3⁻CD19⁻NK1.1⁺ cells isolated from the spleen of *Klrk1*^{-/-} (left panel) and *Klrk1* ^{Δ/Δ} (right panel) mice is shown

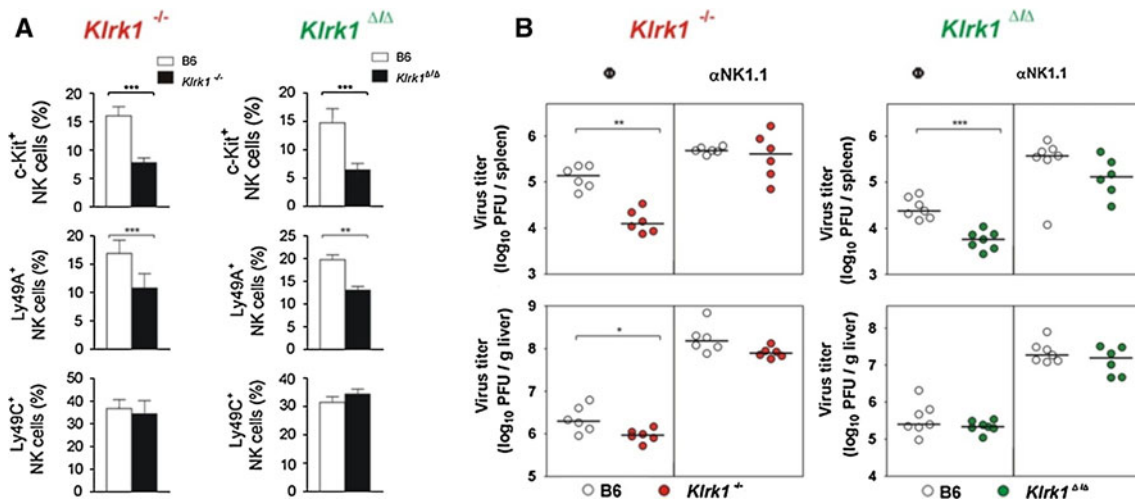


Fig. 3 Comparative analysis of NK cell phenotype and function between wild type, *Klrk1*^{-/-} and *Klrk1*^{ΔΔ} mice. **a** NK cells isolated from the spleens of 9 weeks old *Klrk1*^{-/-} and *Klrk1*^{ΔΔ} mice were analyzed by flow cytometry. *Klrk1*^{+/+} littermates (B6) were used as controls. The data represent mean values of at least five mice per group. Error bars are

indicated. **b** Indicated groups of *Klrk1*^{-/-}, *Klrk1*^{ΔΔ} and their littermate controls were infected intravenously with 5×10^5 PFU of $\Delta m157$ MCMV. The groups of mice were either NK cell depleted (PK136) or treated with PBS. Each symbol represents individual virus titers in indicated organs

In conclusion, NKG2D appears to play an important role in NK cell development. It seems that its role in the early development of NK cells is different than during the mature stages where NKG2D is responsible for activation of effector functions. “Unleashed” proliferation of immature NK cells and their increased sensitivity to apoptosis in the absence of NKG2D suggest that this receptor plays rather a regulatory role at this stage, most probably in concert with IL-15R and perhaps some other receptors (Fig. 1), controlling proliferation and survival of NK cells. Future research must reveal whether this function is limited to NK cells or whether other cellular subsets that constitutively express NKG2D (most notably NKT and $\gamma\delta$ T cells) are also affected.

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