Comparative analysis of human NK cell activation induced by NKG2D and natural cytotoxicity receptors

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NKG2D and natural cytotoxicity receptors (NCR) are essential recognition structures that mediate NK cell activation. NKG2D and NCR signaling is achieved through membrane association with signaling adaptors. The adaptors that associate with NCR — such as CD3ζ, FcRγ and KARAP/DAP12 — bear intracytoplasmic immunoreceptor tyrosine-based activation motifs that activate Syk protein tyrosine kinases. Human NKG2D associates with the DAP10 transmembrane adaptor, which bears a YxxM motif and activates the phosphatidylinositol 3-kinase pathway. In the mouse, a short NKG2D-S isoform, generated by *Nkg2d* alternative splicing, can associate with either DAP10 or KARAP/DAP12. Here, we report that neither short human NKG2D alternative transcripts nor NKG2D association with KARAP/DAP12 was detected in activated human NK cells. Despite these results, NK cell triggering by both recombinant soluble NKG2D ligands (MICA and ULBP-1) and anti-NCR cross-linking antibodies induced similar CD25 expression, NK cell proliferation and cytokine production. In contrast, NKG2D triggering by anti-NKG2D antibodies did not lead to any detectable activation signals. These data thus show that target recognition via NKG2D or NCR triggers all aspects of NK activation, and pave the way for further dissection of the signaling pathways induced by NK cell recognition of ULBP-1 and MICA.

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1 Introduction

NK cells are equipped with multiple activating and inhibitory cell surface receptors whose engagement by cognate ligands expressed on target cells determines NK cell tolerance or activation [1]. Among NK cell activating receptors, NKG2D is a lectin-like homodimeric receptor that recognizes a variety of stress-induced self-ligands, such as MICA, MICB and ULBP molecules in humans as well as H60, Rae1 and MULT molecules in the mouse [2]. Besides its expression on NK cells, NKG2D expression is also detected in $CD8^+$ T cells and γδ T cells, and can be up-regulated by IL-15 [2, 3]. TNF-α and IL-15 are also associated with induction of NKG2D expression in $CD4^+CD28^-$ autoreactive T cells from patients with rheumatoid arthritis [4]. Natural cytotoxicity receptors (NCR) belong to the Ig-superfamily, are almost exclusively expressed on NK cells and include NKp30, NKp44 and NKp46 [5]. NCR ligands remain to be identified on tumor cells, but it has been reported that viral hemagglutinins may serve as ligands for NKp44 and NKp46 on virus-infected cells [6, 7].

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Abbreviations: CFSE: Carboxyfluoresceine diacetate succinimidyl ester **ITAM:** Immunoreceptor tyrosine-based activation motif **NCR:** Natural cytotoxicity receptor **RLM 5'-RACE:** RNA-ligase-mediated rapid amplification of cDNA ends

A common feature of these activating NK receptors resides in the presence of a transmembrane, charged aa-residue that enables their non-covalent association with signaling adaptors. NCR associate with immunoreceptor tyrosine-based activation motif (ITAM)-bearing transmembrane polypeptides such as CD3ζ and FcRγ (for NKp30 and NKp46), or KARAP/DAP12 (for NKp44). Association of NCR with ITAM identifies these receptors as primary recognition structures that stimulate cytokine production and cytotoxicity [5]. In contrast, in human NK cells, $CD8^+$ T cells and γδ T cells, NKG2D associates with the DAP10 adaptor that lacks any ITAM [8]. DAP10 is a transmembrane signaling adaptor with an intracytoplasmic YxxM motif [9]. This signaling motif couples to phosphatidylinositol 3-kinase (PI-3K)-dependent pathways that also mediate signaling of co-stimulatory molecules, such as CD28, ICOS and CD19 [10]. Together with the fact that NKG2D can function as a co-stimulatory receptor for antigen-specific responses of $CD8^+$ T cells [11], these features support the view that NKG2D functions as a co-stimulatory molecule in both NK and γδ T cells.

In the mouse, the occurrence of alternative association of NKG2D with KARAP/DAP12 and DAP10 has recently been characterized. Indeed, two alternative spliced products of the mouse *Nkg2d* gene (m*Nkg2d*), mNKG2D-L and mNKG2D-S, co-exist in activated NK cells and T cells [12]. Whereas the protein encoded by mNKG2D-L selectively associates with DAP10, the short mNKG2D-S protein can associate with either DAP10 or KARAP/DAP12 [12].

In association with KARAP/DAP12, the NKG2D-S receptor was shown to function as a primary recognition structure leading to full mouse NK cell activation [12]. In particular, association of NKG2D-S with KARAP/DAP12 is important for NKG2D-mediated production of IFN-γ by mouse NK cells [12]. This role of KARAP/DAP12 in NK-cell-mediated cytokine secretion is supported by evidence that NKG2D–DAP10 complexes are insufficient to induce IFN-γ secretion by human NK cells [13]. The role of KARAP/DAP12 in NKG2D-mediated natural cytotoxicity is more complex, as it appears to be dependent on the status of NK cell activation as well as on the nature of the target cell. In particular, NKG2D-mediated natural cytotoxicity depends upon KARAP/DAP12 association in NK cells freshly isolated from poly-IC-injected mice [12]. Yet, stimulation with IL-2 overcomes this KARAP/DAP12-dependency over time [14]. Thus, NKG2D-mediated cytotoxicity can occur in IL-2-activated NK cells from mice that are deficient in Syk kinases, involving KARAP/DAP12-independent pathways [14]. Along this line, in humans, NKG2D–DAP10 complexes have been shown to be sufficient to trigger granule release following anti-NKG2D mAb cross-linking on human purified NK cells [13]. These data prompted us to further dissect the effect of NKG2D triggering on human NK cell activation and to compare it to that mediated through the engagement of ITAM-associated NKp30 and NKp46 NCR.

2 Results

2.1 Expression of NKG2D protein and transcripts in resting and activated human lymphocytes

We first analyzed the cell surface distribution of the NKG2D receptor on human peripheral blood cells by flow cytometry using the ON72 anti-NKG2D mAb. As reported earlier, NKG2D was found to be expressed on all detectable NK cells, $CD8^+$ T cells and γδ T cells [9], but was not expressed on human peripheral blood $CD4^+$

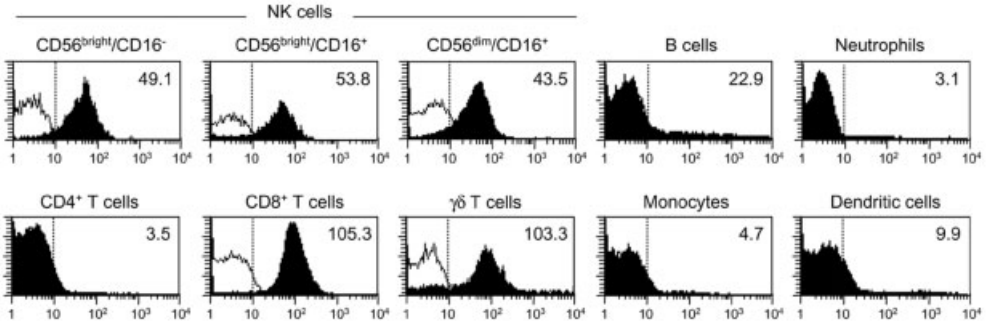

Fig. 1. Cell surface expression of NKG2D within blood lymphoid subsets. NKG2D expression was analyzed by flow cytometry using the ON72 anti-NKG2D-specific mAb on the following human PBMC subsets: $CD3^-CD56^+CD16^+$ and $CD3^-CD56^+CD16^-$ NK cell subsets further discriminated as $CD56^{bright}$ or $CD56^{dim}$ cells; $CD3^+CD8^+$ and $CD3^+CD4^+$ T cells; $CD3^+TCR\gamma\delta^+$ T cells; $CD19^+$ B lymphocytes; $CD14^+$ monocytes; $CD3^-TCR\alpha\beta^-CD19^-CD56^-HLA\text{-}DR^+$ dendritic cells; and $CD14^+$ neutrophils purified on dextran. The numbers displayed in each histogram are the mean fluorescence intensity. The control staining using isotype-matched negative control mAb is overlaid (open histograms).

T cells, B cells and neutrophils from normal donors (Fig. 1). Human NKG2D was also not detected at the surface of monocytes and dendritic cells freshly isolated from peripheral blood (Fig. 1), or on monocytes cultured for 72 h in the presence or absence of LPS (200 ng/ml) or IFN-α (1000 U/ml) (data not shown). The latter result contrasts with the reported cell surface expression of NKG2D on LPS- and IFN-α-stimulated mouse macrophages [15].

In the mouse, the NKG2D-L and NKG2D-S protein isoforms only differ by a 13-aa-long intracytoplasmic segment (Fig. 2A) [12]. The m*Nkg2d-S* isoform results from an exon 1 to exon 3 splice, thus skipping the ATG1 start codon that initiates NKG2D-L protein transduction (Fig. 2B). On the basis of these data, we examined whether comparable NKG2D splicing events occur in humans. Analysis of h*NKG2D* gene sequences (genomic location ref AC022075.29.1.168351, ensembl transcript ID ENST00000240618) reveals that two in-frame putative ATG start codons are present in h*NKG2D* [16]. The reported ATG start (ATG1) is found within h*NKG2D* exon 4, whereas an additional putative ATG (ATG2) is found within h*NKG2D* exon 5 (Fig. 2B). These observations are consistent with the possibility that alternative splicing of h*NKG2D* exon 4 leads to a short transcript that lacks the 5' ATG1 and uses the 3' ATG2 as a translation initiation start. In this hypothesis, a putative h*NKG2D-S* transcript would correspond to a splicing event using a donor site located upstream from h*NKG2D* exon 3 merging with an acceptor site within h*NKG2D* exon 5.

As an initial step to characterize h*NKG2D* alternative 5' transcriptional start sites, we used RNA-ligase-mediated rapid amplification of cDNA ends (RLM 5'-RACE), a method that allows selected amplification of capped transcripts. RLM 5'-RACE primers were designed within exon 6 (pE6D) and 5 (pE5D) of h*NKG2D*, 3' of the putative ATG2 start codon (Fig. 2B). Four distinct NKG2D transcripts were detected by RLM 5'-RACE using, as a template, the total RNA isolated from the following NKG2D$^+$ cells: resting purified NK cells as well as such cells activated for 3 or 18 days in IL-2; an NK cell line (NKL); a $\gamma\delta$ T cell clone (G12) [17]; as well as resting and IL-2-activated PBMC (Fig. 2C). Systematic sequencing of the RLM 5'-RACE PCR products revealed the presence of h*NKG2D* transcripts encompassing exons 3, 4 and 5 (transcripts 1 and 2) or exons 4 and 5 (transcript 3) (Fig. 2C). Therefore, both ATG are present in transcripts 1, 2 and 3 and do not exclude the ATG1 start codon.

Strikingly, the induction of a shorter h*NKG2D* transcript, transcript 4, correlated with IL-2 stimulation (Fig. 2C). Yet, transcript 4 only included a truncated exon 5 sequence that lacks the putative ATG2 (Fig. 2C). Therefore, the RLM 5'-RACE did not allow the detection of putative h*NKG2D-S* transcripts that correspond to a splice between exon 5 and an upstream exon distinct from exon 4.

The donor site of m*Nkg2d* that generates the alternative mNKG2D-S transcripts is located within exon 1 of the m*Nkg2D* gene (Fig. 2B). Alignment of the genomic region encompassing m*Nkg2d* exon 1 revealed a significant level of homology with h*NKG2D* exon 3 sequences (data not shown). To identify similar splicing events between h*NKG2D* exon 3 and exon 5, we generated a 5' primer corresponding to h*NKG2D* exon 3 (pE3D; Fig. 2B). This primer was used in conjunction with pE5D or a primer encompassing the h*NKG2D* stop codon in exon 10 (Fig. 2B, D). In addition, Genscan analysis of public genome databases predicts a putative h*NKG2D* transcript containing *NKG2F* exon 3 but lacking h*NKG2D* exon 4 (Ensembl Genscan prediction sequence ID AC022075.29.1.1.168351.2.8249.146525). This prompted us to also generate a 5' primer within the *NKG2F* exon 3 (pE3F; Fig. 2B).

Corresponding RT-PCR fragments were generated using RNA extracted from resting and IL-2-activated NK cells, NKL cells, as well as resting and IL-2-activated PBMC; the fragments were cloned and systematically sequenced. None of the sequences obtained using these combinations of primers corresponds to an h*NKG2D* splice that lacks exon 4 and retains exon 5 (Fig. 2D). RT-PCR analysis performed with RNA from NK-enriched PBMC or polyclonal, activated NK cells that were stimulated for 3 days with IL-2 and IL-12, IL-2 and IL-15 or IFN-α and IL-12, also did not allow detection of a splicing isoform lacking exon 4 (data not shown). We thus conclude from these experiments that h*NKG2D* transcripts that exclude the ATG1 codon could not be detected in any of the populations tested: resting NK cells; NK cells activated with IL-2 (for 3 or 18 days) or the above-described combinations of cytokines for 3 days; NKL cells; resting PBMC or PBMC activated with IL-2 for 3 days.

2.2 NKG2D associates with DAP10 but not with KARAP/DAP12 in human NK cells

Despite the lack of hNKG2D-S transcripts, it is noteworthy that the human NKG2D protein is characterized by a short intracytoplasmic sequence (51 aa), closer to the length of mouse NKG2D-S (50 aa) than that of mouse NKG2D-L (63 aa) (Fig. 2A). It is still unclear whether the primary sequence or the length of the intracytoplasmic sequence is involved in the capacity of mNKG2D-S to associate with both DAP10 and KARAP/DAP12.

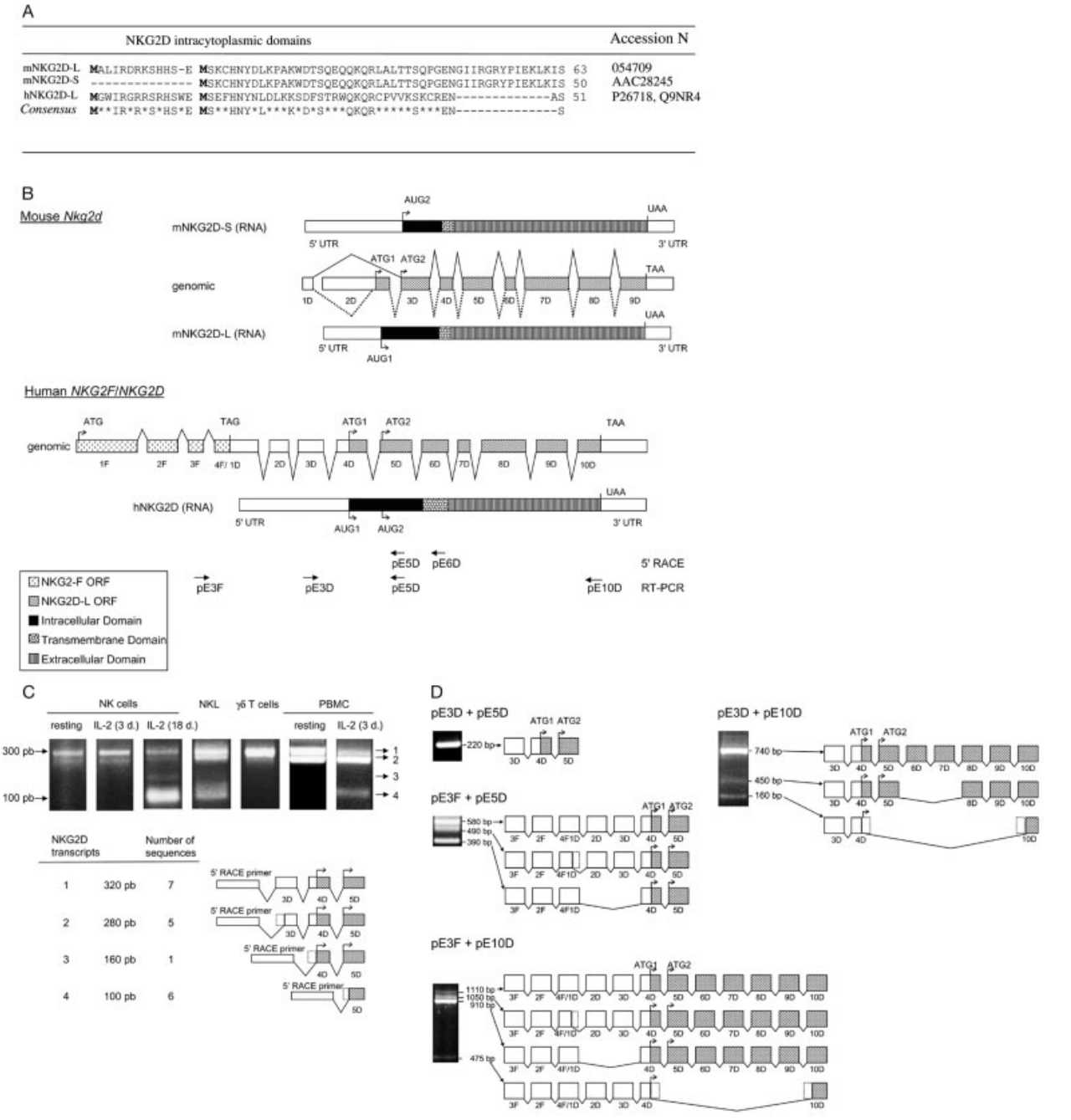

Fig. 2. Detection of NKG2D splice variants in activated and resting blood lymphocytes. (A) Protein alignment of mouse and human NKG2D N-terminal intracytoplasmic regions. Methionine residues that initiate translation of mNKG2D-L and -S proteins are aligned in bold. (B) Exon-intron organization and alternative splicing of murine and human NKG2D genes. The splicing pattern of mouse *NKG2D-L* has been revised based on comparisons to genomic sequences that indicate that this isoform is formed by the splicing together of exons 1, 2 and 3, rather than initiating in exon 2. The positions of the primers used to identify alternative transcripts of the hNKG2D gene using RT-PCR or RLM 5'-RACE are schematically indicated. (C) Detection of 5' alternatively spliced hNKG2D transcripts. RLM 5'-RACE was performed using total RNA from the indicated cells. Alternative transcripts corresponding to cloned sequences are represented on the lower panel. (D) RT-PCR analysis of hNKG2D transcripts. Representative agarose gel analysis of RT-PCR fragments obtained after amplification from IL-2-activated PBMC. Similar results were obtained with the following cell preparations: resting PBMC, IL-2-activated (for 3 days) polyclonal NK cells, and the NKL cell line. NKG2D transcripts corresponding to cloned sequences are represented on the right of representative electrophoresis agarose gels.

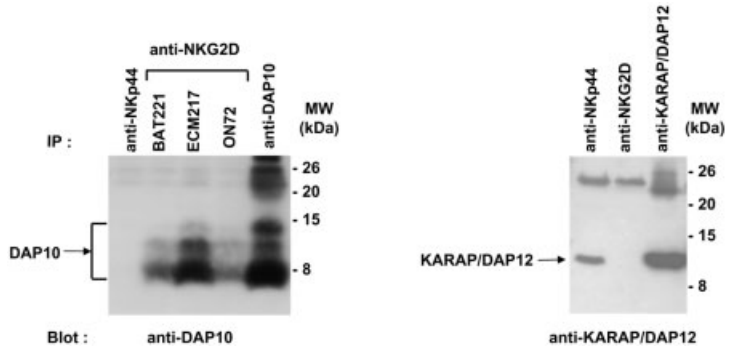

Fig. 3. Analysis of NKG2D complexes in human NK cells. Cell lysates, prepared using 1% digitonin, from a polyclonal NK cell population were immunoprecipitated with Z231 (anti-NKp44), BAT221, ECM217, or ON72 (anti-NKG2D) mAb, and anti-DAP10 or anti-KARAP/DAP12 antisera. Samples were analyzed onto 14% SDS-PAGE under reducing conditions and, after blotting, membranes were probed with anti-DAP10 (left panel) or anti-KARAP/DAP12 (right panel) antibodies. Anti-DAP10-reactive bands contain more than one protein and are indicated with a bracket.

Therefore, the length of the human NKG2D intracytoplasmic sequence may be compatible with a promiscuous association with DAP10 and KARAP/DAP12. Alternatively, it is also possible that the alternative splicing events involving the 5' untranslated region of the h*NKG2D* gene that we detected (Fig. 2D, 910 bp and 390 bp transcripts) affect the use of ATG1 or ATG2 as translation initiation starts.

We thus revisited whether NKG2D was able to associate with KARAP/DAP12 or was only detected in association with DAP10 in human NK cells. To this end, cell lysates prepared from polyclonal, activated NK cells were immunoprecipitated using three different NKG2D-specific mAb (BAT221, ECM217 and ON72). Samples were analyzed by SDS-PAGE and, after blotting, membranes were probed with either anti-DAP10 or anti-KARAP/DAP12 rabbit antisera. NKp44 (that specifically associates with KARAP/DAP12 polypeptides), DAP10 or KARAP/DAP12 immunoprecipitates were used as controls. As shown in Fig. 3 (left panel), DAP10 association was detected in NKG2D immunoprecipitates. In contrast, we could not reveal NKG2D association with KARAP/DAP12 in normal NK cells (Fig. 3, right panel) and in NK cell lines such as NKL and NK-92 (data not shown).

2.3 Induction of CD25 expression and NK cell proliferation upon NKG2D and NCR engagement

We then took advantage of the selective association of NCR and NKG2D with distinct signaling adaptors, to compare the NK cell activation programs initiated by DAP10- and KARAP/DAP12-dependent pathways. Available soluble recombinant NKG2D ligands, ULBP-1 (ULBP-1–Fc) and MICA (bac-MICA and MICA–Fc), were used together with specific anti-NKG2D mAb to evaluate NKG2D-mediated signaling. As NCR ligands are still not available, anti-NCR mAb were used to engage these receptors.

Incubation of PBMC with MICA recombinant proteins (bac-MICA and MICA–Fc), anti-NKp30 or anti-NKp46 mAb led to CD25 up-regulation on NK cells (Fig. 4A), as well as NK cell proliferation in the presence of IL-2 (Fig. 4B). Similar results were obtained when NK cells were triggered using ULBP-1–Fc recombinant proteins (Fig. 4B and data not shown). In contrast, incubation with control recombinant proteins (IgG1–Fc and bac-DR4) or isotype-control mAb did not induce CD25 expression or NK cell proliferation. In addition, treatment with bac-MICA or MICA–Fc had similar outcomes, ruling out the involvement of FcR stimulation in the effect observed using recombinant Fc proteins (Fig. 4 and data not shown). Notably, PBMC stimulation with anti-NKG2D mAb (soluble or plate-bound, at 1–10 μg/ml) did not result in inducing NK cell proliferation or CD25 expression (Fig. 4).

2.4 NK cell redirected killing of P815 target cells by anti-NKG2D or anti-NCR mAb

Our demonstration that anti-NKG2D mAb are not sufficient to induce CD25 expression and NK cell proliferation suggests that triggering of NKG2D by specific mAb may require the concomitant cross-linking of additional triggering receptors. Yet, anti-NKG2D mAb induced potent NK cell mediated cytotoxicity in redirected killing assays (Fig. 5), as previously described [18].

However, it is well known that during this type of assay, additional receptor–ligand interactions occur, including

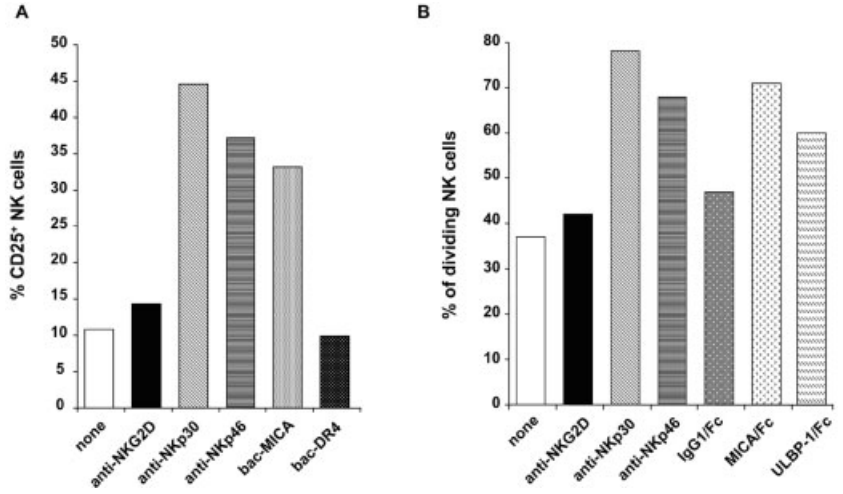

Fig. 4. Induction of NK cell CD25 expression and proliferation upon engagement with NKG2D or NCR. Resting PBMC were cultured in the presence or absence of plate-bound recombinant proteins or soluble mAb for 4 days (A) or 6 days (B) and then assessed for CD25 expression (A) or cell proliferation [CFSE staining; (B)] by cytofluorimetric analysis after gating on $CD56^+CD3^-$ cells. Results are expressed as % of NK cells ($CD3^-CD56^+$) undergoing proliferation. Data are representative of five independent experiments.

those involved in cell adhesion (*e.g.* between LFA-1 and ICAM molecules) and in cell activation (*e.g.* triggering receptors that recognize their ligands on target cells). Thus, it is possible that under these experimental conditions, the efficient NK cell response to NKG2D stimulation might be sustained by additional receptor–ligand interactions. A classical target cell that is commonly used for redirected killing analysis is represented by the murine mastocytoma P815. As shown previously, the spontaneous NK-mediated killing of these target cells is at least in part dependent upon NKp46 engagement by murine cell ligand(s) expressed on P815 [19]. Indeed, blocking of this receptor by the use of anti-NKp46 mAb of the IgM isotype was shown to down-regulate NK-mediated killing of P815 cells by NK clones or polyclonal NK cells [19]. Thus, it is conceivable that during redirected-killing experiments in which NK cells are stimulated by IgG mAb specific for one or another triggering receptor, the interaction between NKp46 and its putative murine ligand(s) may provide activating signals that cooperate with the stimulatory mAb.

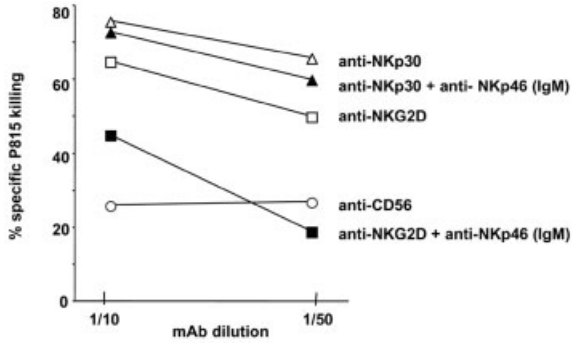

Fig. 5. Anti-NKG2D mAb-induced redirected killing of P815 mastocytoma cells. Polyclonal activated NK cells were assessed in a redirected killing assay against P815 murine target cells either in the presence of anti-NKG2D mAb or anti-NKp30 mAb alone, or in combination with anti-NKp46 (KL247, IgM) [19]. IgG1 mAb were used at the indicated dilution of hybridoma culture supernatant whereas the IgM mAb was used as undiluted culture supernatant. The E:T ratio used in this experiment, representative of three independent experiments, was 3:1. The SD of the mean of the triplicates was below 5%.

To evaluate whether the triggering of redirected cytotoxicity by NKG2D mAb could be dependent on concomitant NK cell stimulation via the NKp46 receptor, cytotoxicity assays were performed in the absence or in the presence of anti-NKp46 mAb of the IgM isotype. As shown in Fig. 5, blocking of NKp46 partially inhibited the triggering capability of anti-NKG2D mAb whereas, under the same conditions, virtually no inhibition could be detected on the triggering capability of anti-NKp30 mAb. These data indicate that the induction of cytotoxicity by anti-NKG2D mAb requires the engagement of ITAM-associated triggering NK receptors such as NKp46. On the other hand, in line with previous data, NK cell triggering via the NKp30 receptor did not appear to require the simultaneous engagement of NKp46.

2.5 NK cell cytokine production upon NKG2D and NCR engagement

We next analyzed whether NK cell incubation with anti-NCR, anti-NKG2D mAb or NKG2D ligands (ULBP-1 and MICA) stimulated NK cell cytokine production (*i.e.* IFN-γ and GM-CSF production). Under these experimental conditions, it is possible to evaluate the NK cell responses to stimuli acting on a single type of receptor, thus avoiding the complex interactions that are generated between different NK receptors and their cellular ligands during the type of redirected cytolytic assay described above. Incubation of PBMC (Fig. 6A, left panel) or polyclonal, activated NK cells (Fig. 6A, right panel) with anti-NKp30 or anti-NKp46 mAb led to IFN-γ production by NK cells, whereas incubation with anti-NKG2D mAb had no effect (Fig. 6A). Thus, unlike NK cell stimulation via NKp30 or NKp46, stimulation of NKG2D

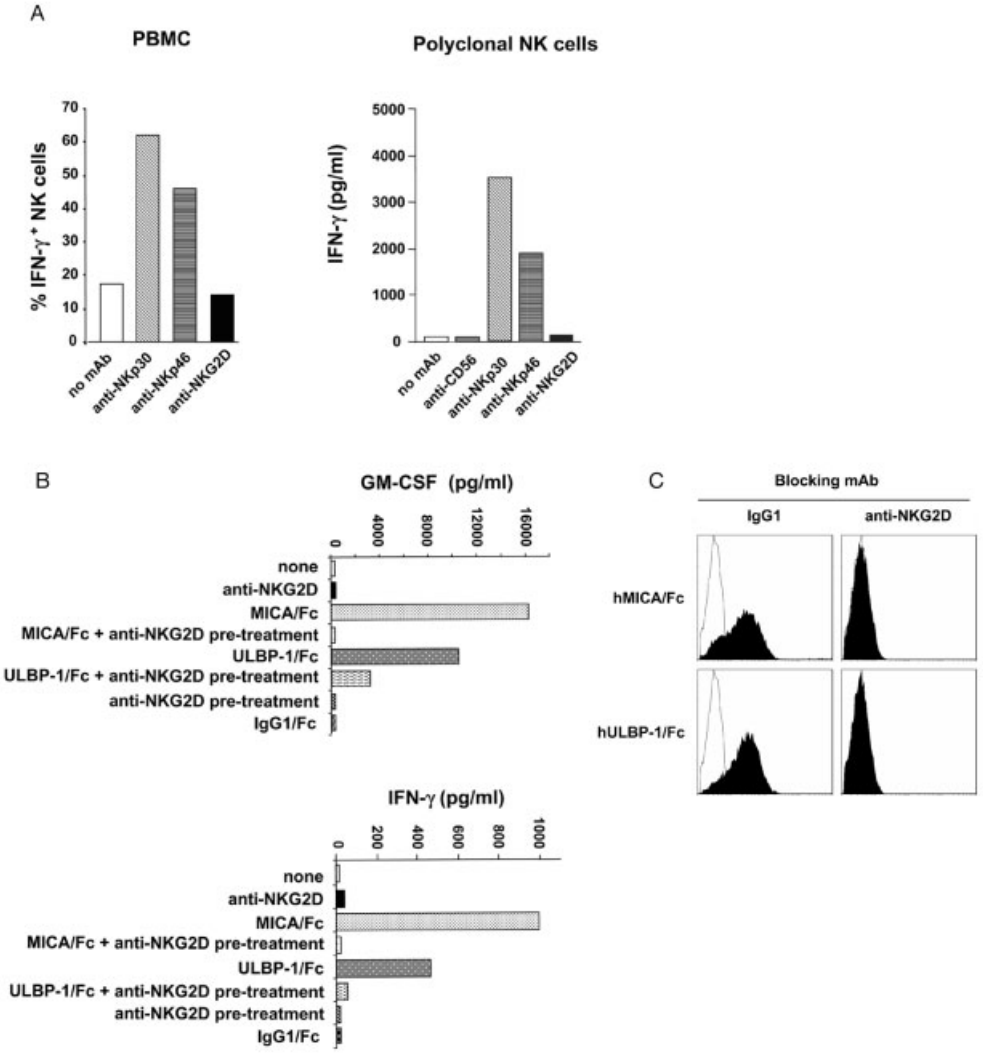

Fig. 6. Cytokine production by NK cells stimulated via NCR or NKG2D. (A) Resting PBMC (left panel) were incubated with indicated soluble mAb (10 μg/ml), and intracellular IFN-γ staining of NK cells (gated on $CD3^{-}CD56^{+}$) was assessed by flow cytometry at 96 h. Polyclonal activated NK cells (right panel) were stimulated for 18 h with the indicated plastic-bound purified mAb (10 μg/ml) and culture supernatants were assessed for IFN-γ production by ELISA. Data are representative of three independent experiments (SD <5%). (B) Polyclonal activated NK cells were stimulated for 18 h with indicated plastic-bound recombinant molecules (10 μg/ml). The amounts of GM-CSF and IFN-γ were quantified by ELISA. Anti-NKG2D pre-treatment consisted of pre-incubating activated NK cells with BAT221 supernatant for 30 min at room temperature. Data are representative of three independent experiments (SD <5%). (C) The indicated mAb (10 μg/ml) were added to polyclonal, activated NK cells for 30 min at 4°C and washed extensively prior to the addition of indicated recombinant proteins (10 μg/ml) for 30 min at 4°C. Filled histograms show staining with recombinant NKG2D ligands; open histograms show staining with IgG1–Fc.

by specific mAb is not sufficient to generate stimuli capable of inducing cytokine production. These results are in line with those reported above on the induction of NK cell proliferation and cytolytic activity.

Considering the differential effects observed on PBMC by the use of anti-NKG2D mAb *vs.* NKG2D ligands for CD25 expression and cell proliferation, we further evaluated the ability of two distinct ligands, MICA–Fc and ULBP-1–Fc, to induce production of IFN-γ or GM-CSF by polyclonal, activated NK cells. We show that both recombinant NKG2D ligands stimulated GM-CSF and IFN-γ production by NK cells, while such an effect was not detectable after treatment of NK cells with IgG1–Fc control proteins (Fig. 6B). We further confirmed the specificity of the effect of NKG2D-ligands in inducing GM-CSF and IFN-γ production by blocking-experiments using anti-NKG2D mAb. As shown in Fig. 6B, anti-NKG2D mAb fully abrogated the MICA-induced production of cytokines by NK cells, and partially counteracted that induced by ULBP-1–Fc. Altogether, these data indicate that triggering of human NK cells by anti-NCR mAb or NKG2D ligands, but not by anti-NKG2D mAb, induces potent NK cell activation resulting in both proliferation and cytokine production. Yet, the activation events induced by anti-NCR mAb and recombinant NKG2D ligands were detected in polyclonal activated NK cells or in NK cells within PBMC, but were not evident using resting purified NK cells (Data not shown).

3 Discussion

In the present study, we show that the effects of NCR or NKG2D engagement induced by cross-linking anti-NCR mAb or recombinant NKG2D protein ligands are similar, as evaluated by cell surface expression of CD25, NK cell proliferation and cytokine secretion (IFN-γ and GM-CSF). These results have several implications for the understanding of NK cell activating pathways.

Although their tumor ligands are still not characterized, NCR have been identified as major recognition structures for NK cell cytotoxicity against a wide range of tumor target cells [1]. Interestingly, the surface density of NCR was shown to directly correlate with the magnitude of cytolytic activity mediated by NK cells against several target cell types [20]. Along this line, down-regulation of NKp30 expression by exposure of NK cells to TGF-β1 resulted in markedly reduced NK-mediated cytotoxicity against several different target cell types [21]. Moreover, low NCR surface density on NK cells of HIV-1-infected individuals and AML patients was associated with the inability of NK cells to kill target cells [20, 22]. NCR are also expressed at significantly low levels in NK cells from patients with NK cell expansions, suggesting that these receptors may not be involved in the expansion process [23].

Yet, our present demonstration that NCR engagement results in NK cell proliferation suggests that these receptors might indeed drive NK cell expansions upon binding to as-yet-undefined cell surface ligand(s). In particular, it is possible that, in addition to cytokines, NCR engagement contributes to the NK cell expansions that often occur during the early phases of viral infection [24]. Our data thus complement the identification of NCR as primary recognition structures on NK cells and further emphasize the remarkable similarity between NCR in NK cells and TCR or BCR in T or B cells, respectively, with regard to their oligomeric architecture as well as the effector functions coupled to their engagement.

As for NKG2D, we show that various features distinguish the human and mouse receptors. First, in contrast to data reported in the mouse [15, 25], NKG2D cell surface expression was not detected on cells of myeloid origin.

Secondly, although alternatively spliced, truncated NKG2D transcripts are reproducibly generated in activated human lymphocytes, sequencing of transcripts isolated from activated NK cells or $\gamma\delta$ T cells did not allow characterization of a short hNKG2D transcript skipping exon 5 that would encode a NKG2D-S isoform corresponding to that observed in the mouse. Indeed, all open reading frames identified in resting and activated human NK cells retain the 5' ATG1 translation initiation codon. Yet, alternative splicing excluding the 5' non-coding exon 2 and 3 of h*NKG2D* were reproducibly detected, and we cannot exclude that these alternative splicing events provide a peculiar RNA structural context that favors the initiation of translation from h*NKG2D* ATG2. An impact of alternative splicing of the 5' non coding region in the control of the alternative usage of ATG translation initiation codons has been demonstrated for other genes as a tuning mechanism for protein isoform production [26]. Alternatively, since the kinetics of induction of NKG2D-S transcripts in mice are restrained to a narrow time-window during the course of NK cell activation [12], we cannot exclude that our search for transcripts in activated human NK cells and $\gamma\delta$ T cells missed the time-points at which a putative, short human NKG2D transcript could be detected. Despite these limits in our study, no association between human NKG2D and KARAP/DAP12 could be detected in various NK cell preparations (NK cell lines and polyclonal activated NK cells).

Thirdly, the consequences of NKG2D triggering by anti-NKG2D mAb are drastically different in the human and

the mouse. In mouse NK cells, NKG2D triggering using anti-NKG2D mAb leads to IFN-γ secretion [15], whereas we and others could not detect any production of IFN-γ by human NK cells upon anti-NKG2D mAb stimulation (Fig. 6A) [13, 21].

We also show that PBMC cultured in the presence of anti-NKG2D mAb do not undergo NK cell proliferation or expression of CD25, and that the stimulatory activity of anti-NKG2D mAb in redirected killing assay is largely dependent upon signals provided via the engagement of the NKp46 receptor. All these findings would suggest that the NKG2D molecule acts as a coreceptor rather than as a primary receptor during human NK cell activation. In contrast, we demonstrate that human NK stimulation with soluble recombinant NKG2D ligands (*i.e.* ULBP-1 and MICA) induces CD25 expression, cytokine secretion and proliferation. Previous studies also reported the effect of soluble recombinant ULBP on the induction of GM-CSF and IFN-γ production by activated NK cells [3, 27].

The mechanisms that underlie the differential potential of NKG2D ligands and anti-NKG2D mAb to activate NK cell proliferation and cytokine production remain unclear. Considering the highly heterogeneous structure of NKG2D ligands [2], one could argue that NKG2D epitopes targeted by mAb and ligands might be distinct. Distinct modes of NKG2D engagement might then differentially affect putative NKG2D conformational changes or, more likely, NKG2D redistribution in membrane rafts, leading to the initiation of distinct signaling events. The recent description of anti-CD28 super-agonistic mAb that can induce TCR-independent full T cell activation, in contrast to CD28 ligands and most anti-CD28 mAb, would represent an opposite example of such epitope-dependent receptor triggering [28]. Yet, the fact that several distinct anti-NKG2D mAb fail to induce cytokine production by human NK cells in different studies, including this one, do not argue in that direction.

Alternatively, the observed effects could signify that MICA and ULBP-1 bind other NK cell activating receptors that are distinct from NKG2D. Indeed, the binding of MICA and ULBP-1 to NKG2D–DAP10 and to an activating receptor complex that includes ITAM-bearing adaptors would reconcile the fact that triggering of the NKG2D–DAP10 complex by specific mAb is insufficient to induce IFN-γ secretion in human and mouse NK cells [12–14, 21], while stimulation with NKG2D ligands induces IFN-γ secretion in human NK cells [3, 27]. Yet, one should take into account that anti-NKG2D mAb abolish the binding of MICA–Fc and ULBP–Fc to NK cells (Fig. 6C) [3], and severely impair NKG2D-ligand-induced cytokine production by NK cells (Fig. 6B). Thus, if NKG2D ligands co-engage NKG2D and another NK cell activating receptor, this receptor has a low affinity for NKG2D ligands and/or cooperates with NKG2D in the interaction with common ligands. Altogether, investigating the pathways by which NKG2D ligands may achieve full activation of NK cell function remains a challenging issue for further dissection.

4 Materials and methods

4.1 Cell preparation and culture conditions

PBMC were isolated by Ficoll-Hypaque density gradient centrifugation (Amersham Pharmacia Biotech) from cytopheresis or whole blood samples obtained from normal healthy volunteer donors (Etablissement Français du Sang, Marseilles, France).

PBMC were cultured in the presence of 50 U/ml rIL-2 (Proleukin®, Chiron) and of 1–10 μg/ml of the following soluble or plate-bound purified IgG1 mAb: AZ20 anti-NKp30 [29], BAB281 anti-NKp46 [19] or ON72 anti-NKG2D [18]. Culture medium and IL-2 were replaced on day 3. Recombinant bac-MICA and control bac-DR4 human proteins were purified from baculovirus as described previously [30]. Purified ULBP-1–Fc, MICA–Fc and IgG1–Fc were purchased from R&D systems.

Polyclonal, activated NK cell populations were generated from PBMC as described earlier [19].

4.2 Flow cytometric analysis

Conjugated mAb and human and mouse secondary antibodies were purchased from Beckman Coulter. Cells were stained in PBS, containing 2% FCS, with the appropriate mAb, and samples were analyzed on an XL-MCL cytometer with the $EXPO_{32}$ program (Beckman Coulter). Analysis was performed within viable lymphocytes determined on the basis of forward and side-angle light scatter.

4.3 Analysis of NKG2D transcripts

Total RNA was extracted from distinct cellular subsets using Rneasy minikit (QIAGEN). 5'-RACE experiments were performed as described by manufacturer (AMBION). The 5'-RACE adaptor primer was 5'-GCTGATGGCGATGAATGAACACTGCGTTTGCTGGCTTTGATGAAA.

Human NKG2D-specific 3' primers used for the nested PCR were: PE5D, 5'-TTGACTACTGGACATCTTTGCTTTTG; and PE6D, 5'-GAATAATGAGTTTAGGAATACAGC. PCR products were obtained after 35 cycles of amplification (30 sec at 94°C, 30 sec at 60°C, and 1 min at 70°C). The cDNA synthe-

sis was performed using Superscript II (Invitrogen). In addition to pE5D, the primers used in RT-PCR experiments were: pE3F, 5'-AGTACTGGAGCAGAACAGTT; pE3D, 5'-GAATCAAGATCTTCCCTCTCTGAGCAG; and pE10D, 5'-CTTTACACAGTCCTTTGCATGCA. PCR products were analyzed after electrophoresis on a 1.5% agarose gel, purified and subcloned in pGEM-T easy vector (PROMEGA) in order to be sequenced.

4.4 Biochemical analysis of the NKG2D complexes

A normal polyclonal NK cell population was lysed in 1% digitonin and immunoprecipitated with antibodies coupled to sepharose-CnBr or sepharose-PA (Pharmacia Biotech), as previously described [31]. Samples were analyzed in discontinuous SDS-PAGE, and the rabbit anti-DAP10 and anti-DAP12 antisera used in this study have been described elsewhere [12, 32].

4.5 Cytotoxicity

NK cells were tested for cytolytic activity against the (FcRγ^+) P815 murine mastocytoma cell line in a 4-h [^{51}Cr]-release assay as previously described [19].

4.6 Cell division

PBMC were labeled prior to culture with the intracellular fluorescent dye 5-(and -6)-carboxyfluoresceine diacetate succinimidyl ester ([CFSE]; Molecular Probes, Eugene, OR, USA). Briefly, cells were labeled (10^7/ml) in RPMI-1640 containing 2% FCS and 1 μM CFSE for 8 min at 37°C, washed and then cultured as described above. Cultures were harvested at day 6. Cell division of CFSE-labeled PBMC subsets was analyzed by flow cytometry.

4.7 GM-CSF and IFN-γ production

IFN-γ production from PBMC was assessed by cell surface and intracytoplasmic staining, and analysis by flow cytometry after 4 days in culture. Briefly, brefeldin A (Sigma Aldrich) was added at a final concentration of 5 μg/ml for the last 4 h of culture. The cells were then incubated with anti-CD3 and anti-CD56 mAb prior to permeabilization (IntraPrep™; Beckman Coulter) and stained with PE–anti-IFN-γ or PE–IgG1 (Pharmingen).

GM-CSF and IFN-γ production from polyclonal activated NK cells was measured in supernatants using ELISA (GM-CSF DuoSet Elisa, R&D Systems, Minneapolis, MN, USA; IFN-γ OptE1A set, Pharmingen).

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