

## Differential expression of inhibitory and activating CD94/NKG2 receptors on NK cell clones

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### Abstract

Natural killer cells are known to express a variety of surface receptors involved in HLA class I monitoring. It is thus of interest to investigate the clonal distribution and relative expression levels of activating versus inhibitory NK receptors. We have developed a quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) assay designed to determine specific and absolute mRNA levels for NKG2-A/B, -C, -E, -F, -H and NKG2-D. When analyzing NK cell clones derived from a single donor we found differential expression of inhibitory (NKG2-A/B) versus triggering (NKG2-C and potentially -E, -F, -H) NK receptor chains. The generation of the splice variants NKG2-E and -H seemed to occur at a constant ratio. We further compared NKG2 transcript levels to surface receptor expression as monitored by flow cytometric analysis and to NK cell cytotoxicity as detected by reverse ADCC: a clear correlation was observed. Thus, the data obtained reveal a substantial variability in the NKG2 repertoire among NK cell subpopulations, which is likely to affect the sensitivity and reactivity towards the ligand HLA-E. © 2002 Elsevier Science B.V. All rights reserved.

*Keywords:* Natural killer cell; NK receptor; NKG2; Real-time PCR; Transcript; Clonal expression

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*Abbreviations:* GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HUVEC, human umbilical vein endothelial cells; KIR, killer immunoglobulin-like receptors; rADCC, reverse antibody dependent cellular cytotoxicity; RT-PCR, reverse transcription-polymerase chain reaction.

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### 1. Introduction

Innate immune responses mediated by NK cells involve target cell recognition as well as activation of the cytotoxic machinery via a multitude of NK cell receptors (Moretta et al., 2000). Based on the “missing self-hypothesis”, NK cells are believed to detect and eliminate cells which have lost or severely reduced their expression of self-MHC class I proteins, for instance due to viral infection or malignant trans-

formation (Karre, 1997). The NKG2 receptor family is involved in monitoring class I expression (Navarro et al., 1999) and has been extensively characterized in human NK cells and more recently in rodents also (Disse et al., 1997; Lohwasser et al., 1999). NKG2 receptors are type II transmembrane proteins containing a C-type lectin-like domain and form disulfide-bonded heterodimers with the invariant CD94 chain (Lazetic et al., 1996; Brooks et al., 1997; Carretero et al., 1997). To date, seven family members have been described and designated NKG2-A, -B, -C, -D, -E, -F, -H (Houchins et al., 1991; Glienke et al., 1998; Hofer et al., 2001). Subsequent research demonstrated that NKG2-D indeed constitutes a separate type of lectin-like NK receptor forming homodimers which are involved in the recognition of MHC related molecules such as MICA (Bauer et al., 1999). All other family members were found to be highly homologous, with NKG2-A/B and NKG2-E/H being splice variants of the same genes (Houchins et al., 1991; Bellon et al., 1999).

A crucial contribution to the understanding of NKG2 receptor function was achieved by the discovery of its ligand HLA-E (Borrego et al., 1998; Braud et al., 1998; Lee et al., 1998). While previously characterized NK receptor families (such as Ly49 and KIR, killer Ig-like receptors) recognized a defined repertoire of different HLA class I alleles, NKG2 proteins were found to bind the non-classical HLA class I molecule HLA-E. HLA-E surface expression is mediated by peptide loading of leader sequences derived from other class I alleles and can thus be regarded as a general monitor of cellular class I expression (largely independent of allelic combinations) (Braud and McMichael, 1999). However, HLA-E is not only recognized by inhibitory NKG2 family members, thereby blocking NK cytotoxicity, but is similarly bound by activating family members (Llano et al., 1998; Vales-Gomez et al., 1999). While substantial homology is observed for the extracellular regions of NKG2 receptors, which argues for a common ligand, the transmembrane and intracellular sequences fall into distinct groups: Inhibitory family members (NKG2-A, -B) are characterized by the presence of immunoreceptor tyrosine-based inhibition motifs, which mediate tyrosine phosphorylation as well as subsequent recruitment of phosphatases SHP-1 and -2 (Carretero et al., 1998). In contrast,

activating family members (NKG2-C and potentially -E, -F, -H) non-covalently associate with the adapter molecule DAP-12 via a charged amino acid residue in their transmembrane domain (Lanier et al., 1998). DAP-12 contains an immunoreceptor tyrosine-based activation motif capable of transducing activation signals.

Expression of the various inhibitory and activating NKG2 receptors was found to be largely restricted to NK cells and a subset of cytotoxic T lymphocytes (Yabe et al., 1993). Similar to what has been reported for other NK receptor families (Valiante et al., 1997), the available evidence suggests that the CD94/NKG2 receptors show diversity in clonal distribution (Toomey et al., 1999; Williams et al., 2000). Matching NK receptor pairs which recognize the same or highly similar ligands but deliver distinct inhibitory versus activating signals have been proposed to be an integral part of the ligand sensing system employed by NK cells to detect changes in MHC class I expression levels (López-Botet et al., 2000). In this respect, it is of major interest to further characterize the distribution of NKG2 receptors as well as to quantitate the respective expression levels in individual NK cells. However, such approaches are clearly hampered by the limited availability of antibodies specific for the various NKG2 members due to the high homology among those proteins. In contrast, the detection of different NKG2 transcripts by reverse transcription-polymerase chain reaction (RT-PCR) is more readily performed and should offer intriguing insights into various aspects of NK cell biology.

## 2. Materials and methods

### 2.1. Cell culture

NK cells were isolated from the PBL of a healthy donor by removal of adherent cells on plastic dishes, followed by positive selection with  $\alpha$ -CD56 mAb and magnetic beads (Dyna, Oslo, Norway). NK clones were prepared by limiting dilution and further cultured in RPMI 1640 medium supplemented with 10% FCS and 100 U/ml of human rIL-2 in the presence of irradiated feeder cells. The NK cell line NKL was generously supplied by M.J. Robertson (Robertson et al., 1996); NK92 cells (Gong et al., 1994) were

purchased from Immune Medicine (Vancouver, Canada). Both cell lines were grown in RPMI 1640 medium containing 10% heat-inactivated human serum, 1 mM sodium pyruvate and 50 mM  $\beta$ -mercaptoethanol. Cultures were supplemented with human rIL-2 at a final concentration of 1000 U/ml.

## 2.2. RNA isolation and cDNA synthesis

Total cellular RNA was isolated with TRIzol Reagent (Life Technologies, Paisley, UK) according to the manufacturer's instructions. A 1 mg of total RNA was reverse transcribed in a 20  $\mu$ l reaction containing 250 ng oligo(dT) and 125 ng of random hexamer primers as well as 200 U of Superscript II enzyme as specified by Life Technologies. Samples were diluted 1:10 before PCR analysis.

## 2.3. Real-time PCR

A 5  $\mu$ l of cDNA sample was subjected to PCR analysis using primer sets (Table 1) specific for the various NKG2 members or control genes at optimized oligonucleotide concentrations (Table 2). Reactions were performed in a total volume of 25  $\mu$ l including SYBR Green PCR core reagents as supplied by PE

Applied Biosystems (Foster City, CA): 1  $\times$  SYBR Green PCR buffer, 3 mM  $MgCl_2$ , 0.2 mM dATP/dCTP/dGTP, 0.4 mM dUTP, 0.6 U AmpliTaq Gold, 0.25 U AmpEraseUNG (to prevent carry-over of previous PCR products). Amplification cycles were as follows: 2 min at 50 °C (treatment with AmpEraseUNG), 10 min at 95 °C (activation of AmpliTaq Gold) and 40 cycles of 15 s at 95 °C followed by 1 min at 60 °C for product amplification. Incubation and on-line detection of PCR products were carried out with optical 96-well plates in the GeneAmp 5700 Sequence Detection System by PE Applied Biosystems.

## 2.4. PCR standards and controls

Each PCR reaction was performed in triplicate. For absolute quantitation of transcript copy numbers, standard samples were included for each gene of interest. cDNAs of various NKG2 members were derived from  $\lambda$  phage clones (Houchins et al., 1991) and were either subcloned into the pBS vector (NKG2-C and -D) or supplied as purified PCR products (NKG2-A). Expression plasmids for NKG2-E (J1224), NKG2-F (B09555), NKG2-H and CD94 (LL195) have previously been described (Chang et

Table 1  
Primer pairs used in real-time PCR analysis

Primer	Sequence	Position	Exon
R5A	GGCATTGTTTGTCTTGTCTTGG	4	1
R3A	TCCCTTGAAAATCCTGAGAAGCT	327	2
R5CEH	CTCATGGATTGGTGTGTTTCGT	517(C) 558(E) 513(H)	5
R3C	CACTGTAAACGCAAATGCTTTACTTC	728	6
R3E	GCTAAATGGTACATGAGCACTCAGG	856	7
R3H	TGCAATCATAATATCATTTCTGTTTGAAC	771	6
R5F	CCTGAATAGAAGAATGCAGAAAGCA	500	3
R3F	AAATATTATGAAGTCAGTTGAATACTACACAGAC	818	4
R5D	GGCTTTTATCCACAAGAATCAAGATC	232	2
R3D	GTGCACGTCTACCGCAGAGA	325	3
R5CD94	GTGAACAGAAAACCTTGAACGAAA	314	4
R3CD94	AGGCGGTGTGCTCCTCACT	466	5
R5IFN	GCTCTGCATCGTTTTGGGTTCTCTTG	162	1
R3IFN	CATTCATGTCTTCCTTGATGGTCTCC	433	3
R5GAP	GAAGGTGAAGGTCGGAGTC	81	2
R3GAP	GAAGATGGTGATGGGATTC	306	4

Precise nucleotide sequence and position (with respect to published cDNAs) are given for each primer.

Exon numbering in the corresponding genomic sequence is indicated. The GenBank accession numbers of the respective cDNA sequences read as follows: NKG2-A (X54867), NKG2-C (X54869), NKG2-D (X54870), NKG2-E (L14542), NKG2-H (AF078550), NKG2-F (AJ001683), CD94 (U30610),  $\gamma$ -IFN (M29383), GAPDH (XM006959).

Table 2  
PCR conditions and specifications

Target cDNA	Primers	Optimized concentration (nM)	Amplification product (bp)	Sensitivity (1.5 × molecules)
NKG2-A/B	R5A	300	324	10 <sup>2</sup>
	R3A	300		
NKG2-C	R5CEH	300	212	10 <sup>1</sup>
	R3C	300		
NKG2-E	R5CEH	300	299	10 <sup>1</sup>
	R3E	300		
NKG2-H	R5CEH	300	259	10 <sup>1</sup>
	R3H	300		
NKG2-F	R5F	300	319	10 <sup>2</sup>
	R3F	300		
NKG2-D	R5D	300	94	10 <sup>0</sup>
	R3D	900		
CD94	R5CD94	300	153	10 <sup>1</sup>
	R3CD94	50		
γ-IFN	R5IFN	300	272	10 <sup>1</sup>
	R3IFN	50		

The optimal primer concentration, length of generated PCR products and sensitivity of detection (cDNA molecules per PCR reaction) are given for each target gene.

al., 1995; Glienke et al., 1998; Bellon et al., 1999). Furthermore, cDNA templates for γ-IFN and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were generated by standard PCR of NKL cDNA applying the primer pairs R5IFN/R3IFN and R5GAP/R3GAP, respectively. Plasmids were purified using the QIAGEN Plasmid Maxi Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions; PCR fragments were separated by gel electrophoresis and isolated via the QIAquick Gel Extraction Kit (QIAGEN). DNA concentrations were repeatedly measured at A<sub>260 nm</sub> and the cDNA copy number was calculated as follows: The average absorbance at 260 nm was multiplied by 50 to yield μg/ml of double-stranded DNA and was further divided by the basepair count of the respective plasmid/PCR fragment times 660 (daltons per basepair). To calculate molecules per μl, the figure was further multiplied by Losschmidt's number  $6.023 \times 10^{23}$  and subsequently divided by 10<sup>9</sup>. A standard dilution series of  $1.5 \times 10^7$ , 10<sup>5</sup>, 10<sup>3</sup> and 10<sup>2</sup> cDNA molecules was routinely run for every primer pair/gene of interest and was used to calculate absolute copy numbers in NK probes. Since standard samples were based on pure preparations of plasmid DNA or NKG2 cDNA fragments rather than total cDNA mixtures as obtained for NK clones, we performed a test dilution

series of NKG2-D cDNA plasmid in water as well as in HUVEC cDNA to verify that the sensitivity of real-time PCR assays was not affected by the presence of unrelated cDNA molecules. Transcript levels of NKG2 genes, CD94 or γ-IFN were normalized by GAPDH expression values obtained for each sample (to adjust for variations in RNA isolation or cDNA synthesis). The values given therefore refer to the number of specific transcripts detected per  $1 \times 10^6$  copies of GAPDH mRNA measured for the respective NK sample. To detect the amplification of false products or primer dimers (which are equally labeled by SYBR Green incorporation and would thus affect fluorescence readings), a melting profile, i.e. loss of fluorescence upon denaturation, was established for every generated PCR product and compared to the melting curve obtained for standard samples. Samples with a divergent melting profile were excluded from the analysis.

### 2.5. Flow cytometry

Cells were incubated with saturating amounts of mAb, followed by washing and labeling with PE-conjugated anti-mouse F(ab')<sub>2</sub> IgG. The monoclonal antibodies used included 3B1 (α-CD94) as previously described (Aramburu et al., 1990), Z199 (α-NKG2-A/

CD94) and P25 ( $\alpha$ -NKG2-A/C) kindly provided by Dr. A. Moretta and Dr. Pende (Sivori et al., 1996; Cantoni et al., 1998). Analysis was performed in comparison with the appropriate isotype controls on a Becton Dickinson FASCscan cytometer.

## 2.6. *rADCC*

Redirected lysis assays were performed as previously described (Bellon et al., 1999). Briefly,  $5 \times 10^3$   $^{51}\text{Cr}$ -labelled P815 target cells were incubated for 15 min at 37 °C with either  $\alpha$ -CD94 (3B1) or control mAb, before NK effector cells were added and the mixture further incubated for 4 h at 37 °C. Specific lysis was calculated as follows:  $\% \text{lysis} = [(E - C) / (T - C)] \times 100$  in which  $E$  is the chromium release (cpm) of targets incubated with effector NK cells,  $C$  equals the spontaneous chromium release from the targets in control medium only and  $T$  is the total release from targets exposed to 5% Triton X-100.

## 3. Results and discussion

### 3.1. Establishing a quantitative PCR assay for the specific detection of NKG2 receptor transcripts

We have set up a quantitative RT-PCR assay based on the incorporation of fluorescent SYBR Green and on-line detection of generated amplification products (“real-time PCR”) which permits specific and absolute measurements of transcripts for all NKG2 isoforms. While PCR-based methods have always been regarded to be at best semi-quantitative, the development of real-time PCR has led to a reliable quantitative assay suited to the measurement of gene transcripts (Orlando et al., 1998). To evaluate the expression of distinct NKG2 family members, primer pairs were designed (Table 1) based on small nucleotide differences among NKG2 genes and placed in two separate exons to allow specific detection of the various NKG2 transcripts. With respect to NKG2-C, -E and -H, a common 5' primer was chosen; the specificity of the PCR products was based on variation of the 3' primer. Furthermore, a primer pair was created for monitoring expression of the CD94 gene. Analysis of NKG2-D,  $\gamma$ -IFN (Kotake et al., 1996) and GAPDH (Revillion et al., 2000) was included for control pur-

poses. Concentrations were optimized for each set of primers in the real-time PCR analysis using a matrix test system of 50, 300 and 900 nM oligonucleotides (Table 2). Subsequently, the sensitivity and linearity of detection was assayed for each gene of interest; the detection limits usually ranged around 10 molecules/reaction (Table 2).

To verify the specificity of the primer sets designed for the various NKG2 family members, each primer pair was tested with increasing concentrations of the other NKG2 cDNA templates. In general, cross-reactions were not observed (data not shown). Only in the case of the NKG2-C specific primer set, a minimal generation of “false products” based on very high concentrations ( $> 10^5$  copies) of the NKG2-A cDNA template was observed and this did not affect the analysis. Calculations of NKG2 transcript levels were based on pure standard preparations and numbers were always adjusted to  $1 \times 10^6$  detected GAPDH molecules per reaction. For instance, GAPDH values for NK cell lines NKL and NK92 usually ranged around  $2 \times 10^6$  molecules in 25 ng of total RNA applied in RT-PCR analysis, which corresponds to about 400 molecules per cell. It should be noted that GAPDH expression might differ between NK clones or especially between primary NK clones versus established NK lines according to growth and respiration rate. However, major emphasis was placed on the comparison of relative NKG2 receptor mRNA levels in each individual sample, and all NK clones were derived from a single donor to minimize the impact of differential GAPDH expression. When necessary, detection of  $\beta$ -actin mRNA could be included to avoid deviations of GAPDH expression due to distinct growth rates.

### 3.2. Quantitation of NKG2 transcripts in primary NK clones

Primary NK cells were isolated from a healthy volunteer and single cell clones were cultured for subsequent analysis of NK cell RNA. For comparison, NK cell lines NKL and NK92 were chosen. Negative controls included primary human endothelial cells (HUVEC) and the Jurkat T-helper cell line. Total cellular RNA was reverse transcribed and further tested in real-time PCR experiments for expression of the various NKG2 family members as well as

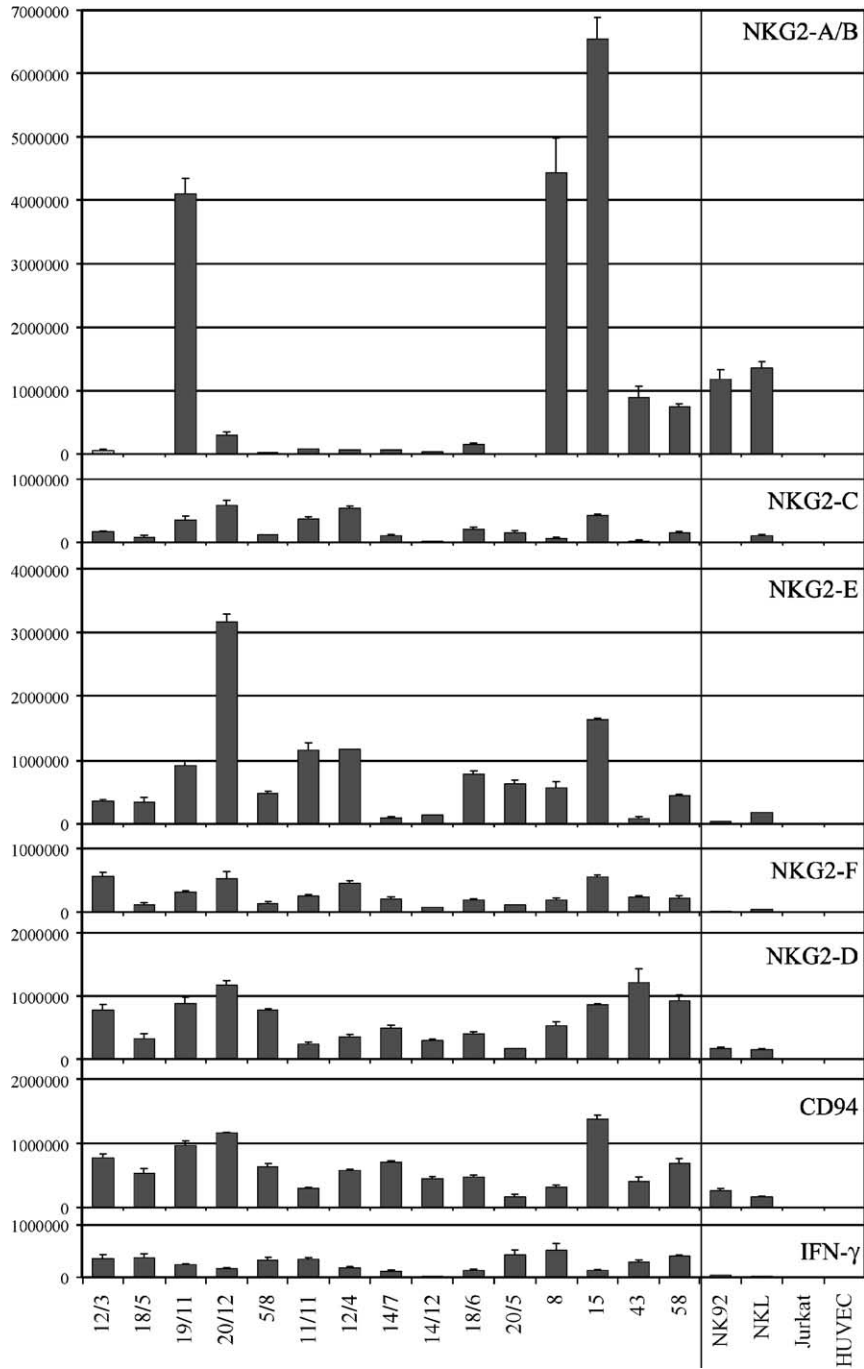


Fig. 1. Expression of mRNA for the various NKG2 and CD94 proteins. Primary NK cell clones as well as the NK cell lines NKL and NK92 were analyzed for transcript levels. HUVEC and Jurkat cells were used as negative controls. Total RNA was reverse transcribed and assayed by real-time PCR technology using specific primer sets for the various NKG2 family members as well as CD94 and  $\gamma$ -IFN. mRNA levels are given in copy numbers per  $10^6$  molecules of detected GAPDH transcripts.

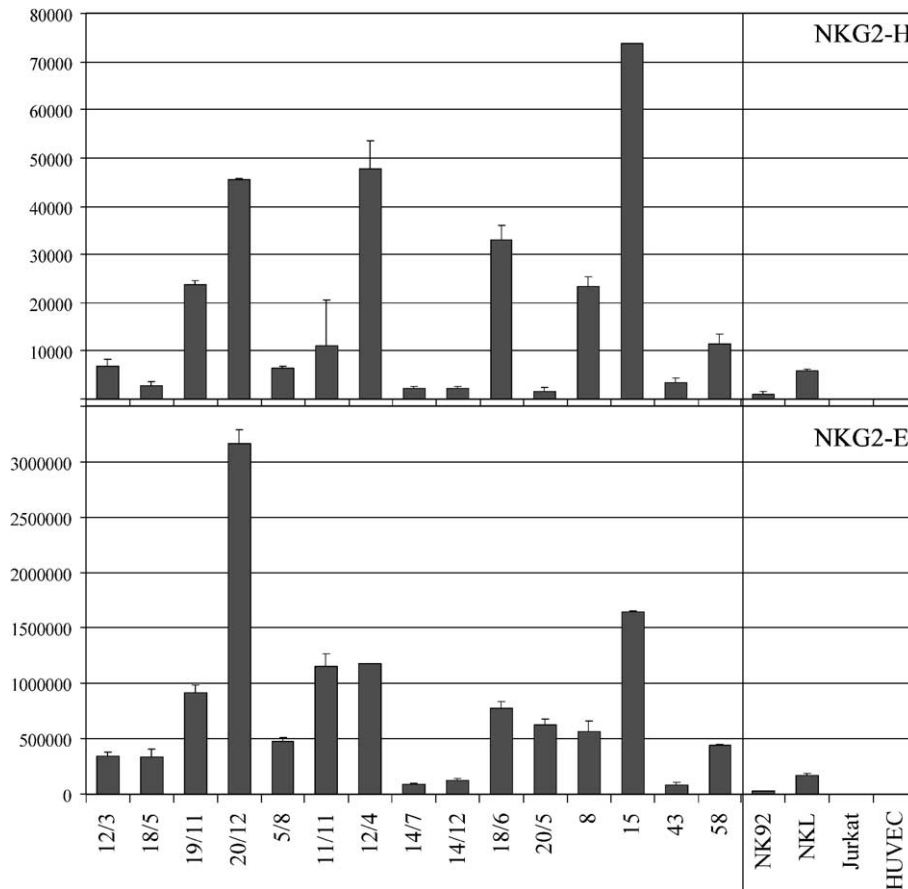


Fig. 2. Expression of mRNA splice variants NKG2-E and NKG2-H. Oligonucleotides specific for the differentially spliced exons were used in real-time PCR. Cells were processed and analyzed as specified in Fig. 1.

CD94 and  $\gamma$ -IFN (Fig. 1). Transcript levels of NKG2-A/B, -C, -E and -F showed a substantial degree of variability ranging from no detectable expression to remarkable levels of  $4\text{--}6 \times 10^6$  copies in 25 ng of RNA. Thus, the established NK clones displayed diversity in the absolute amount of the different NKG2 transcripts. Interestingly, expression levels of NKG2-C, -E and -F showed some degree of correlation, while NKG2-A/B transcript regulation was clearly distinct, which is in accordance with our recently published report on promoter structures (Glienke et al., 1998; Sobanov et al., 1999; Brostjan et al., 2000; Bull et al., 2000): Potential promoter sequences of the NKG2-C, -E and -F genes are highly homologous, whereas NKG2-A preferentially employs a different upstream promoter yielding transcripts

with an additional 5' untranslated exon. Analysis of the splice variants NKG2-E and NKG2-H (Fig. 2) showed that processing of the common mRNA precursor occurs at an average ratio of 50:1 (range 20:1 to 100:1) in most NK clones. However, in the case of clone 20/5 generation of NKG2-H was significantly reduced leading to an NKG2-E versus -H transcript ratio of 400:1.

Furthermore, it is intriguing that CD94, while being the invariant chain of all NKG2 heterodimers and essential for receptor surface expression (Brooks et al., 1997), displayed moderate transcript levels ( $1.5 \times 10^5\text{--}1.5 \times 10^6$ ) in all clones tested which usually did not exceed the quantities of NKG2 mRNA. Translation might, however, be more efficient for CD94 than for NKG2 transcripts to yield non-

limiting amounts of protein required for heterodimer formation. Similar to CD94, cellular concentrations of NKG2-D mRNA ranged around  $1 \times 10^5$  to  $1 \times 10^6$ , i.e. were comparable for all tested clones, which is in agreement with published studies demonstrating a broad expression on NK and T-lymphocytes (Yabe et al., 1993).

### 3.3. Comparison of NKG2 transcript levels with receptor surface expression and cytotoxic activity of NK clones

Several NK clones were chosen for further characterization of NKG2 receptors at the protein and functional levels. Flow cytometric analysis of NK cells stained with mAb directed against CD94, NKG2-A or NKG2-A/-C confirmed the results obtained by real-time PCR: CD94 surface expression could be demonstrated for all NK clones examined. NKG2-A protein was not detectable in cases where transcript levels were low ( $< 10^5$ ) but was clearly stained on NK clones 8, 15, 43 and 58 with transcript levels ranging from 1 to  $6 \times 10^6$ . In addition, surface expression of NKG2-C could be demonstrated via the bi-specific antibody P25 directed against NKG2-A/-C for clone 11/11 which was negative for the NKG2-A protein and transcript but showed substantial levels of NKG2-C mRNA ( $4 \times 10^5$ ). The correlation observed for NKG2 mRNA and protein levels thus argues for the importance of transcriptional regulation in balancing activating and inhibitory NKG2 receptor signals. In this context, it is noteworthy that murine Ly49 receptors show strong regulation at the post-transcriptional level, leading to significant adaptation of surface expression in response to self-ligand concentrations of NK cells and surrounding tissue (Held and Raulet, 1997; Andersson et al., 1998). In contrast, human KIR receptors do not adjust to expression levels of matching class I ligands in transgenic mice (Cambiaggi et al., 1997).

When further combined with data obtained from reverse antibody-dependent cellular cytotoxicity (rADCC) assays, a matching pattern of NKG2 receptor expression and NK cell function became apparent: P815 target cells harboring Fc $\gamma$ -receptors were pre-incubated with  $\alpha$ -CD94 mAb and subsequently exposed to NK cells to trigger redirected lysis. All tested clones with high expression ( $7 \times 10^5$ – $7 \times 10^6$ )

of NKG2-A transcript and protein (8, 15, 43, 58) showed inhibition of lysis even at substantial levels of potentially activating NKG2 members. Clones with little or no NKG2-A ( $< 2 \times 10^5$ ) were further divided into two subgroups: In cases where mRNA of NKG2-C and/or-E was strongly detectable, NK clones (11/11, 12/4, 18/6, 20/5) displayed activating potential in redirected lysis. In contrast, NK clones with NKG2-C, -E transcript levels around  $10^5$  or below (14/7 and 14/12), did not show any effect in rADCC assays. Table 3 illustrates the three categories observed for NK cell reactivity and NKG2 receptor expression by summarizing mRNA, protein and rADCC data obtained for two representative clones per category. In contrast to what is known for NKG2-C (Houchins et al., 1997), it is still unclear to what extent NKG2-E, -F and -H actually convey activating potential to NK

Table 3

Comparison of NK cytotoxicity in rADCC assays with mRNA and protein expression of NKG2 receptors as measured by real-time PCR and flow cytometric analysis of primary NK cell clones

Category	Clone	rADCC	Receptor	mRNA	Protein			
1	8	–	NKG2-A	+++	+			
			NKG2-C	–	na			
			NKG2-E	++	na			
			CD94	++	+			
	58	–	NKG2-A	++	+			
			NKG2-C	+	na			
			NKG2-E	+	na			
2	11/11	+	NKG2-A	–	–			
			NKG2-C	++	+			
			NKG2-E	++	na			
			CD94	++	+			
	20/5	+	NKG2-A	–	–			
			NKG2-C	+	–			
			NKG2-E	++	na			
			CD94	+	+			
			3	14/7	=	NKG2-A	–	–
						NKG2-C	–	–
NKG2-E	–	na						
14/12	=	CD94		++	+			
		NKG2-A		–	–			
		NKG2-C		–	–			
			NKG2-E	–	na			
			CD94	++	+			

Activation (+), inhibition (–) or unchanged (=) cytotoxic activity is indicated. Transcript levels as given in Fig. 1 are symbolized by –, +, ++ and +++. When available, protein expression was analyzed with monoclonal antibodies and cell surface staining is represented by – or + (na=not available).

cells, particularly in the case of NKG2-F which yields a truncated protein without a functional carbohydrate recognition domain (Plougastel and Trowsdale, 1997; Glienke et al., 1998). However, it should be noted that expression levels of NKG2-E were significantly higher than for NKG2-C, and generation of the splice variant NKG2-H seemed to occur at a conserved ratio of about 50:1 (E:H) in most NK clones, thus arguing for the importance of NKG2-E gene products in NK cytotoxicity.

We have thus presented a specific and efficient method of quantifying transcript levels of NKG2 isoforms and have demonstrated substantial heterogeneity in cellular expression. A correlation between NKG2 mRNA levels, protein surface expression and NK cytotoxic potential was seen by analysis of several NK cell clones. At present, we can only speculate on the significance of the different expression levels of NK receptors. It is conceivable that a higher ratio of inhibitory to activating isoforms as observed for several NK clones, is needed to sense efficiently a decrease in HLA-E/peptide complexes and induce killing. In this case, activating isoforms are predicted to possess a higher ligand affinity and to compete for limiting amounts of HLA-E ligands. Alternatively, it is possible that the associated peptides may play an important role. The activating isoforms might turn the balance towards activation in the presence of certain peptide combinations that could originate during infections or cellular transformations. In the case of low concentrations or absence of inhibitory NKG2 members, it is likely that KIR molecules will play a dominant inhibitory role in the control of NK cytotoxicity. Using the method described, it will be possible to explore further the clonal distribution of the various receptors and to acquire additional information on expression levels, which possibly change during different activation states of the cell. This might be of particular interest in the field of NK cell ontogeny, since NKG2 genes seem to be activated early during NK cell development in a stochastic way as recently demonstrated in mouse studies of NKG2-A, -C and -D mRNA (Toomey et al., 1999; Williams et al., 2000). Furthermore, it might prove useful in the characterization of pathological NK- and T-cell alterations. NK cell leukemia involving EBV infection has recently been shown to be characterized by a substantial upregulation of NKG2-C mRNA in transformed versus normal NK cells (Seo

et al., 2000). Comparably, the role of NKG2 receptors in T-cell function is currently a focus of investigation and has been analyzed in multiple myeloma (Besostri et al., 2000) as well as melanoma (Vetter et al., 2000). In this case, the ratio of activating (NKG2-C/-E) and inhibitory (NKG2-A/-B) family members seems to be distinct in primary versus metastatic tumor lesions.

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