

# P58 Molecules as Putative Receptors for Major Histocompatibility Complex (MHC) Class I Molecules in Human Natural Killer (NK) Cells. Anti-p58 Antibodies Reconstitute Lysis of MHC Class I-protected Cells in NK Clones Displaying Different Specificities

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## Summary

Human CD3<sup>-</sup>16<sup>+</sup>56<sup>+</sup> natural killer (NK) cells have been shown to display a clonally distributed ability to recognize major histocompatibility complex (MHC) class I alleles. Opposite to T lymphocytes, in NK cells, specific recognition of MHC class I molecules appears to induce inhibition of cytolytic activity and, thus, to protect target cells. Since a precise correlation has been established between the expression of the NK-specific GL183 and EB6 surface molecules (belonging to the novel p58 molecular family) and the specificity of NK clones, we analyzed whether p58 molecules could function as receptors for MHC in human NK cells. NK clones displaying the previously defined "specificity 2" and characterized by the GL183<sup>+</sup>EB6<sup>+</sup> phenotype, specifically recognize the Cw3 allele and thus fail to lyse the FcγR<sup>+</sup> P815 target cells transfected with Cw3. On the other hand, NK clones displaying "specificity 1" and expressing the GL183<sup>-</sup>EB6<sup>+</sup> phenotype failed to lyse Cw4<sup>+</sup> target cells. Addition of the F(ab')<sub>2</sub> fragments of either GL183 or EB6 mAb as well as the XA141 mAb of IgM isotype (specific for the EB6 molecules) completely restored the lysis of Cw3-transfected P815 cells by the Cw3-specific NK clones EX2 and EX4. Similarly, both the entire EB6 mAb, its F(ab')<sub>2</sub> fragment and the XA141 mAb reconstituted the lysis of C1R, a FcγR<sup>-</sup> target cell expressing Cw4 as the only serologically detected class I antigen. Thus, it appears that masking of different members of p58 molecules prevents recognition of "protective" MHC class I alleles and thus the delivering of inhibitory signals. Further support to the concept that p58 molecules represent a NK receptor delivering a negative signal was provided by experiments in which the entire anti-p58 mAbs (of IgG isotype) could inhibit the lysis of unprotected FcγR<sup>+</sup> P815 target cells, thus mimicking the inhibitory effect of MHC class I molecules.

Recent data have substantially modified the general concept that human CD3<sup>-</sup>CD16<sup>+</sup>CD56<sup>+</sup> NK cells (1, 2) represent a homogeneous cytolytic lymphoid population displaying nonspecific, non-MHC-restricted functions. Thus, it has been shown that NK cells are capable of specific functions since they can discriminate among different normal allogeneic target cells (3, 4). This ability is clonally distributed as different NK clones derived from single individuals display unique cytolytic patterns against allogeneic target cells (5–7). Thus, five distinct groups of alloreactive clones (each displaying a different specificity) have been identified (7).

Genetic analysis (6, 7) and the use of cell transfectants (8) indicated that the target molecules recognized by NK clones are represented, at least in some instances, by HLA class I alleles (8–11). Whereas specific recognition of MHC molecules by alloreactive cytolytic T lymphocytes leads to triggering of their cytolytic function, in the case of NK lymphocytes, a given HLA allele (Cw3) was found to confer specific protection from lysis by a group of NK clones displaying a defined specificity (specificity 2) (8, 9). These data imply that NK cells are equipped with clonally distributed receptors specifically involved in MHC class I recognition (8,

9). A likely explanation for the MHC class I-mediated protection from lysis, could be that binding of NK-receptors (NK-R) to MHC molecules expressed on target cells, would induce a negative signal to the NK cells (9, 12, 13). A possible candidate to act as an NK-R for MHC is represented by the NK-specific p58 molecular family (5, 9, 14). Single members of this family have been identified by the GL183 (14) and EB6 (5) mAbs, and were found to be confined to subsets of human NK cells. The combined use of these mAbs allowed the identification of four different NK subsets (5). That the p58 molecular family may be involved in MHC recognition has been suggested by the strict correlation existing between the allospecificities recognized by NK clones and their GL183/EB6 surface phenotype (5, 7, 9). In addition, the possibility that p58 molecules could function as NK-R was suggested by their ability to regulate the cytolytic function of human NK cells against different allogeneic tumor target cells (5, 14).

In this report we show that mAb-mediated masking of p58 molecules leads to restoration of lysis of MHC class I protected target cells by NK clones recognizing defined (allo)specificities. This strongly suggests that anti-P58 mAbs are specific for NK-R involved in specific MHC class I recognition.

## Materials and Methods

**Antibodies.** mAbs OKT3 (IgG2a anti-CD3; Ortho Pharmaceuticals, Raritan, NJ), JT3A (IgG2a anti-CD3), HP2.6 (IgG2a anti-CD4), B9.4 (IgG2b anti-CD8, GL183 (IgG1 anti-p58), EB6 (IgG1 anti-p58), XA-141 (IgM anti-p58), KD1 (IgG2a anti-CD16), c127 (IgG1 anti-CD16), c288 (IgG1 anti-CD16), K218 (IgG1 anti-CD56), and A6-220 (IgM anti-CD56), were used in this study.

**Cloning of CD3<sup>-</sup>16<sup>+</sup>56<sup>+</sup> Lymphocytes.** PBL derived from normal donors were isolated on Ficoll-Hypaque gradients and cells were then incubated with a mixture of OKT3, HP2.6, and B9.4 mAbs followed by treatment with rabbit C for 1 h at 37°C as previously described (3, 6). Viable cells were isolated and cloned under limiting dilution conditions in the presence of irradiated feeder cells, 0.1% (vol/vol) PHA (Gibco Ltd., Paisley, Scotland), and exogenous IL-2 (rIL-2, Cetus Corp., Emeryville, CA), as previously described for both T and NK cell cloning (7, 15, 16).

**Cytolytic Assays.** The cytolytic activity of cloned NK cells was assessed in a 4-h <sup>51</sup>Cr-release assay in which effector cells were tested against the murine P815 cell line (either transfected with the Cw3 gene or untransfected) (8) or the C1R human cell line (17). In other experiments, cloned NK cells were used against a series of human or murine tumor target cells including IGROV, Raji, U937, M12, and P3U1.

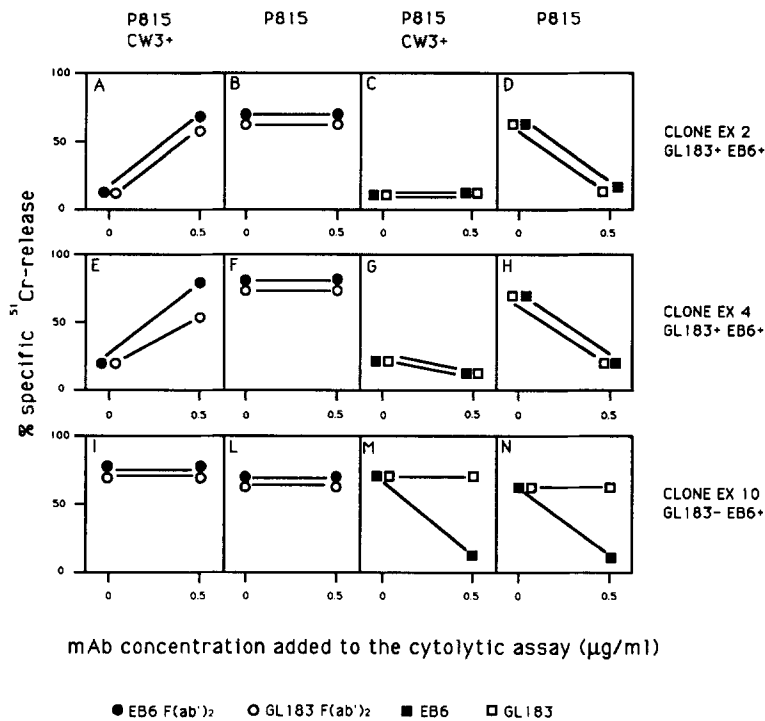
All these target cells were used at 5 × 10<sup>5</sup>/well and the final E/T ratio is indicated in the text. Percent specific lysis was determined as previously described (3, 6). mAbs were added at the onset of the cytolytic assay before adding target cells. The mAb concentrations used in the various assays are indicated in the text.

**Flow Cytometric Analysis.** Cells were stained with the appropriate mAb followed by fluoresceinated goat anti-mouse Ig. Control aliquots were stained with the fluoresceinated reagent alone. All samples were then analyzed on a flow cytometer (FACStar<sup>®</sup>, Becton Dickinson & Co., Mountain View, CA) gated to exclude nonviable cells (7).

**Preparation of F(ab')<sub>2</sub> Fragments of Anti-p58 mAbs.** This method was previously described (14). Briefly, 2 ml of DEAE-Sephacel (Pharmacia LKB Biotechnology, Uppsala, Sweden)-purified IgG1 GL183 mAb (1 mg/ml) was dialyzed overnight against acetate buffer (0.1 M, pH 3.8). Next, the mAb was digested with 50 μg of pepsin (Worthington Biochemical Corp., Freehold, NJ) for 6 h at 37°C, after which the digestion was stopped with 3 M Tris-HCl, pH 8.6, and by placing the reaction mixture on ice. This mixture was dialyzed overnight in 50 mM Tris-HCl, pH 8, and applied to a 10-ml DEAE-Sephacel column. 1-ml fractions were collected and assayed spectrophotometrically at 280 nm and by 7 and 11% SDS-PAGE under reducing and nonreducing conditions, respectively.

## Results and Discussion

**Effect of Anti-p58 F(ab')<sub>2</sub> on the Lysis of Cw3-transfected P815 Cells by NK Clones Displaying Specificity 2.** The murine P815 target cells are efficiently lysed by human NK clones regardless of their (allo)specificity (5, 8). However, transfection of P815 cells with the human class I allele Cw3 has been shown to confer selective protection from lysis by NK clones displaying specificity 2 (8, 9). P815 cells transfected with other class I alleles including A2, A3, and A24 were not protected from lysis by this group of clones (8, 9). In addition, other groups of clones (i.e., displaying specificities other than specificity 2) efficiently lysed Cw3-transfected P815 cells. As previously shown, all NK clones displaying specificity 2 are characterized by the simultaneous expression of the two members of the p58 molecular family defined by the GL183 and EB6 mAbs (7, 9). We assessed whether the p58 molecules expressed by these clones played a role in the recognition of the Cw3 allele in P815 transfectants. To this end, we used the F(ab')<sub>2</sub> fragments of the mAbs GL183 and EB6. The use of F(ab')<sub>2</sub> fragments was required since P815 target cells express FcγR which efficiently bind murine antibodies of the γ1 isotype, such as GL183 and EB6 mAbs (5, 14). As shown in Fig. 1, clones EX2 and EX4, both expressing the GL183<sup>+</sup>EB6<sup>+</sup> surface phenotype and displaying specificity 2, were highly cytolytic against untransfected P815 cells (Fig. 1, B and F). In agreement with our previous reports (8, 9), P815 cells transfected with Cw3, but not with other class I alleles, were resistant to lysis (Fig. 1, A and E). Addition of F(ab')<sub>2</sub> fragments of either GL183 or EB6 mAb resulted in lysis of Cw3-transfected P815 cells that was comparable in magnitude to that of untransfected cells by the same NK clones (Fig. 1, A and E). That this phenomenon was not due to an anti-p58 F(ab')<sub>2</sub>-induced stimulatory effect was shown by control experiments in which lysis of untransfected P815 cells was not modified by the addition of the anti-p58 F(ab')<sub>2</sub> fragments (Fig. 1, B and F, 14). Note that, as previously described (5, 14), the use of the entire anti-p58 mAbs strongly inhibited the lysis of untransfected P815 cells (Fig. 1, D and H). Accordingly, unlike the correspondent F(ab')<sub>2</sub> fragments, the entire mAb did not restore lysis of Cw3-transfected P815 cells (Fig. 1, C and G). Fig. 1 also shows that the control clone EX10 (GL183<sup>-</sup>EB6<sup>+</sup>), displaying specificity 1, (5, 7, 9) lysed both P815 and P815 transfected with Cw3 (Fig. 1, I and L). Whereas the F(ab')<sub>2</sub> fragments of anti p58 mAb had no effect (Fig. 1, I and L), the



**Figure 1.** Reconstitution of cytolytic activity by anti-p58 F(ab')<sub>2</sub> fragments in CD3<sup>-</sup>16<sup>+</sup> NK clones displaying specificity 2. Clones EX2 (A–D) and EX4 (E–H), characterized by the GL183<sup>+</sup>EB6<sup>+</sup> phenotype, are specifically inhibited by the Cw3-transfected P815 cells (A, C and E, G, respectively). Clone EX10 (I–N) expressing the GL183<sup>-</sup>EB6<sup>+</sup> phenotype is inhibited by Cw4<sup>+</sup> but not by Cw3<sup>+</sup> target cells (I, M) (see text). The various clones were tested either in the presence or absence of anti-p58 mAbs (□—□, GL183, ■—■, EB6) or of their F(ab')<sub>2</sub> fragments (○—○, GL183, ●—●, EB6) against <sup>51</sup>Cr-labeled P815 cells or P815 transfected with the Cw3 gene. The E/T ratio was, in all instances, 10:1, and the various mAbs were added at the onset of the cytolytic assay (final concentration, 0.5 µg/ml).

entire EB6 mAb completely abrogated lysis of both target cells (Fig. 1, M and N). Although not shown, the XA141 mAb, which reacts with the same p58 molecule recognized by the EB6 mAb but is of IgM isotype, could restore lysis of Cw3-protected cells. In addition, since IgM antibodies do not bind to FcR expressed by P815 cells, the XA141 mAb could not inhibit cytotoxicity in the redirected killing assay (Fig. 2). Controls included mAbs K218 (anti-CD56, IgG1) and A6-220 (anti-CD56, IgM). These mAbs neither restored lysis of Cw3-transfected target cells (data not shown) nor inhibited cytotoxic activity in the redirected killing assay (see Fig. 2, B). Taken together, these data indicate that masking of p58 molecules by F(ab')<sub>2</sub> fragments, leads to restoration of lysis of target cells protected by appropriate class I alleles. On the other hand, the crosslinking induced by the entire anti-p58 mAb, in the presence of the FcγR<sup>+</sup> P815 target cells, resulted in inhibition of lysis (Fig. 1, D, H, and N) (5, 14). These data strongly suggest that both p58 molecules defined by GL183 and EB6 mAb may be part of a receptor specific for a given class I allele (i.e., Cw3) in NK clones displaying specificity 2. Along this line, the inhibitory effect induced by entire anti-p58 mAbs (of IgG isotype) on the lysis of un-protected P815 cells can be interpreted as an antigen-like effect mimicking the binding of the NK-R to protective class I MHC alleles.

**Effect of Anti-p58 mAbs on the Lysis of the Cw4<sup>+</sup> C1R Cell Line by NK Clones Displaying Specificity 1.** In previous reports (5, 7), we demonstrated that, in most individuals, expression of the GL183<sup>-</sup>EB6<sup>+</sup> surface phenotype correlates with the previously defined specificity 1. In the case of clones displaying this specificity, a number of experimental evidences suggested that Cw4 or related C alleles (Cw2 and Cw6) (8,

18) could represent protective elements when expressed by target cells. Thus, in representative families, a cosegregation could be determined between the character resistance to lysis (by NK clones with specificity 1) and expression of the Cw4 allele (6, 7). In addition, analysis of cell variants suggested that the loss of expression of Cw4 is associated with the de novo acquisition of susceptibility to lysis by clones with specificity 1 (in the same experiments, the loss of Cw3 led to susceptibility to lysis by clones with specificity 2) (8). Therefore, we assessed whether p58 molecules could function as putative receptors for MHC molecules also in GL183<sup>-</sup>EB6<sup>+</sup> NK clones displaying specificity 1 (7).

Table 1 shows that the GL183<sup>-</sup>EB6<sup>+</sup> NK clones EX10 and CEG53 failed to lyse the mutant B cell line C1R, which express Cw4 as the only serologically detected class I antigen (17). This target cell, was resistant to anti-1 NK clones (EX10 and CEG53) whereas it was efficiently killed by NK clones displaying specificity 2 such as clones EX2 and EX4 (Table 1), as well as by clones with other specificities (7, 8) (data not shown). On the other hand, the GL183<sup>-</sup>EB6<sup>+</sup> clones EX10 and CEG53 displayed a strong cytotoxic activity against a series of target cells negative for Cw4 or related C alleles (Cw2 and Cw6; 18) including Cw3-transfected or untransfected murine P815 cells (Fig. 1, I and L) and several EBV-transformed human cell lines (data not shown). Addition of either the entire EB6 mAb (or its F(ab')<sub>2</sub> fragment; Table 1) resulted in the de novo appearance of a strong cytotoxic activity of clones EX10 and CEG53 against C1R target cells. It should be noted that, unlike P815 cells, C1R cells do not function in a redirected killing assay with appropriate murine mAbs (e.g., anti-CD16 mAbs of γ1 or γ2a isotype, data not shown). As a consequence, the entire EB6 mAb can only

**Table 1.** Effect of Anti-p58 mAbs on the Cytolytic Activity of GL183<sup>-</sup>EB6<sup>+</sup> NK Clones Displaying Specificity 1 against the Cw4<sup>+</sup>, C1R Target Cells

Clone*	Phenotype	Antibody added to the cytolytic assay <sup>§</sup>				
		None	EB6	EB6 F(ab') <sub>2</sub>	XA141	K218
EX10	GL183 <sup>-</sup> EB6 <sup>+</sup>	8 <sup>  </sup>	92	75	89	6
CEG 53	GL183 <sup>-</sup> EB6 <sup>+</sup>	10	87	71	78	11
EX 2	GL183 <sup>+</sup> EB6 <sup>+</sup>	48	44	46	ND	49
EX 4	GL183 <sup>+</sup> EB6 <sup>+</sup>	67	60	63	ND	65

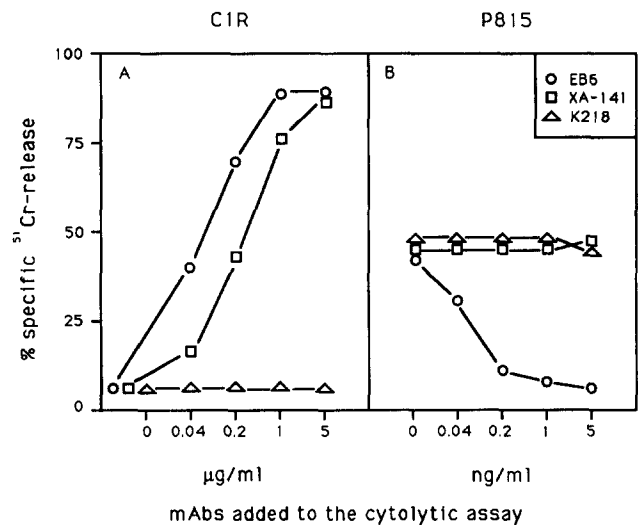
\* Clones EX 10 and CEG 53 are characterized by the previously described specificity 1 whereas clones EX 2 and EX 4 recognize the specificity 2.  
<sup>†</sup> <sup>51</sup>Cr-labeled C1R target cells are protected against the lysis mediated by clones displaying specificity 1 but not against clones displaying specificity 2.  
<sup>§</sup> In these experiments mAbs (0.5 μg/ml) were added at the onset of the cytolytic assay. EB6 (IgG1 anti p58 molecule), XA141 (IgM anti-p58, EB6 molecule), and K218 (IgG1 anti-CD56).  
<sup>||</sup> Data are expressed as a percent specific <sup>51</sup>Cr-release as previously described (15).

bind to the effector cells and prevent the interaction between P58 and MHC molecules.

Thus, also in the case of GL183<sup>-</sup>EB6<sup>+</sup>, NK clones displaying specificity 1, masking of p58 molecules restores lysis of protected target cells. It is noteworthy that the GL183<sup>-</sup>EB6<sup>+</sup> clones were strongly inhibited in their ability to lyse the FcγR<sup>+</sup> P815 cells by entire EB6 mAb (Fig. 1 N and Fig. 2 B [5]). Thus again, the mAb-induced crosslinking of p58 molecules (which occurs with target cells binding murine mAbs via surface FcγR) resulted in an antigen-like effect mimicking the interaction between the NK receptor and protective MHC molecules. No such effect occurred when either the F(ab')<sub>2</sub> fragment of EB6 mAb (Fig. 1 L) or the IgM mAb XA141 (specific for the EB6-defined p58 molecule) were used (Fig. 2 B). As shown in Fig. 2 A and Table 1, the XA141 mAb could restore the lysis of C1R target cells by clone CEG53, in a manner similar to EB6 mAb. Fig. 2 also shows that the amount of EB6 mAb needed for masking p58 molecules is higher than that required for inducing an inhibitory effect in a redirected killing assay. Similar differences in mAb concentrations required for triggering (in redirected killing assay) or masking have been previously detected in cytolytic T cells using anti-TCR mAbs (16, 19).

Taken together, these data strongly suggest that the p58 molecules are directly involved in the mechanism of specific MHC recognition by human NK cells. Thus they may be part of NK receptors, different in different groups of NK clones, which mediate specific binding to distinct MHC class I alleles. To explain the MHC class I recognition by NK cells, two alternative hypotheses have been considered: (a) the delivering of a negative signal to NK cells after interaction with protective MHC class I molecules (9, 13, 20, 21); and (b) the "target interference" hypothesis postulating that class I antigens and/or their associated peptides sterically mask recognition of an NK target structure (responsible for NK cell activation) on the target cells (9, 20, 21). Our data are compatible with the hypothesis that the protective class I alleles,

upon interaction with appropriate NK receptors, deliver a negative signal that prevents lysis of protected target cells (9, 12, 13). In line with this concept, our data suggest that masking of the putative NK receptor prevents binding of the receptor itself to the specific MHC molecules expressed on target cells, thus inhibiting the delivering of the negative signal. According to the hypothesis that P58 molecules may be part



**Figure 2.** Reconstitution of cytolytic activity or antigen-like effect exerted by anti-p58 mAbs in CD3<sup>-</sup>16<sup>+</sup> clones displaying specificity 1. Clone CEG53 (GL183<sup>-</sup>EB6<sup>+</sup>), which is specifically inhibited by target cells expressing Cw4 allele, was assessed for cytolytic activity against the Cw4<sup>+</sup> cell line C1R in the presence of graded amounts of EB6 (○—○, IgG1) or XA-141 (□—□, IgM) mAbs (both specific for the same p58 molecule). Control antibodies included the anti-CD56 mAb K218 (△—△, IgG1). (B) The same clone was assessed against the FcγR<sup>+</sup> murine cell line P815 (which does not express Cw4). Note that in this redirected killing assay the IgG, but not the IgM mAb directed to the p58 molecule, inhibited the cytolytic activity.

of a NK receptor complex, it is likely that these molecules represent the actual specific receptor responsible for the recognition of different MHC alleles. On the other hand, it is unlikely that the p58 molecules may function as coreceptor structures (such as CD4 or CD8 in T lymphocytes). This is suggested by the finding that anti-p58 mAbs, in addition to their ability to interfere with the negative signal induced by protective MHC elements, also mimic the functional effect of protective MHC molecules in unprotected target cells (such as the FcγR<sup>+</sup> P815 cells) (Fig. 3). Further evidence supporting this hypothesis is provided by the precise correlation existing between the GL183/EB6 surface phenotype of NK clones and their specificity for different class I alleles (5, 7, 9).

Thus, interaction between MHC class I molecules and specific receptors is a crucial event in the function of both cytolytic T lymphocytes and NK cells. However, whereas in T cells such interaction results in triggering of cytotoxicity, in NK cells it would result in inhibition. These opposite functional effects also occur when anti-receptor mAbs are used. Anti-TCR mAbs (anti-α/β or -γ/δ) have been shown to trigger the cytotoxic function of CTLs in a redirected killing assay against P815 (16), thus mimicking the effect of MHC (e.g., alloantigen) recognition (this phenomenon is commonly referred to as an "antigen-like effect" [22, 23]). On the contrary, under the same experimental conditions, anti-p58 mAbs inhibit the cytotoxic activity of NK cells, thus mimicking the protective effect of MHC molecules. mAb-mediated masking of TCR prevents MHC recognition and T cell-mediated specific cytotoxicity (24). On the other hand, masking of p58 molecules would prevent MHC recognition and delivering of the negative signal (i.e., it reconstitutes target cell lysis).

Regarding the possible molecular structure of the NK

receptor for class I molecules, a likely interpretation is that two members belonging to the p58 family compose a surface heterodimer which dictates the specificity for given class I alleles. In accordance with this interpretation, in GL183<sup>+</sup>/EB6<sup>+</sup> clones, mAbs directed to either one of the p58 chains restored lysis of protected target cells. In addition, the finding that EB6<sup>+</sup>/GL183<sup>-</sup> clones display an MHC specificity different from double positive clones (5, 7, 8) can be interpreted by the existence, in these clones, of a heterodimer composed by the EB6 molecule and a still undefined p58 chain (EB6<sup>+</sup>/x<sup>+</sup>). According to this interpretation, EB6 molecules, possibly existing also in uncomplexed form, would not be able to recognize specific class I molecules. It is of note that recent data indicate that indeed, in double positive clones, GL183 and EB6 molecules are physically and functionally associated (Moretta, A., M. Vitale, C. Bottino, R. Augugliaro, E. Ciccone, and L. Moretta, manuscript submitted for publication).

We previously showed that appropriate anti-p58 mAbs could increase the lysis mediated by NK clones only against certain human tumor target cells. For example, the ovarian carcinoma cell line IGROV was relatively resistant to GL183<sup>+</sup>, EB6<sup>+</sup> clones displaying specificity 2 (14). Addition of GL183 mAb (or the corresponding F(ab')<sub>2</sub> fragment) resulted in a remarkable increase of lysis (note that IGROV cells do not express FcγR and thus are unable to function in redirected killing in the presence of appropriate murine mAbs; Table 2). On the other hand, IGROV cells were highly susceptible to lysis by NK clones expressing different GL183/EB6 phenotypes (and thus different specificities). A likely interpretation of these data can be that IGROV cells express a protective class I allele specifically recognized by GL183<sup>+</sup>/EB6<sup>+</sup> clones.

**Table 2.** Effect of Anti-p58 mAbs on the Cytolytic Activity of GL183<sup>+</sup>, EB6<sup>+</sup> Clones against Human or Murine Tumor Cells

Clone	Target cells	% Specific <sup>51</sup> Cr-release in the presence of the following mAbs					
		None	KD1 (anti-CD16) IgG2a	c127 (anti-CD16) IgG1	c288 (anti-CD16) IgG1	GL183 (anti-p58) IgG1	EB6 (anti-p58) IgG1
134T49*	IGROV	7.2†	6.4	8.6	9.8	75.5	63.4
F6.1	IGROV	11.3	ND	10.1	ND	68.4	51.3
134T49	Raji	12.9	10.2	13.4	12.6	71.6	56.8
F6.1	Raji	13.4	10.7	12.8	18.5	83.1	63.4
134T49	U937	18.6	68.4	22.3	16.4	78.1	72.5
F6.1	U937	19.3	89.8	29.4	19.4	93.2	80.4
134T49	P815	36.4	89.6	85.2	82.2	3.4	3.7
F6.1	P815	45.6	98.3	91.4	ND	7.8	9.1

\* Clones 134T49 and F6.1, which were assessed for cytolytic activity, were GL183<sup>+</sup>EB6<sup>+</sup>. The E/T ratio in this assay was 2:1 against the murine P815 cell line and 5:1 against the three different human cell lines.

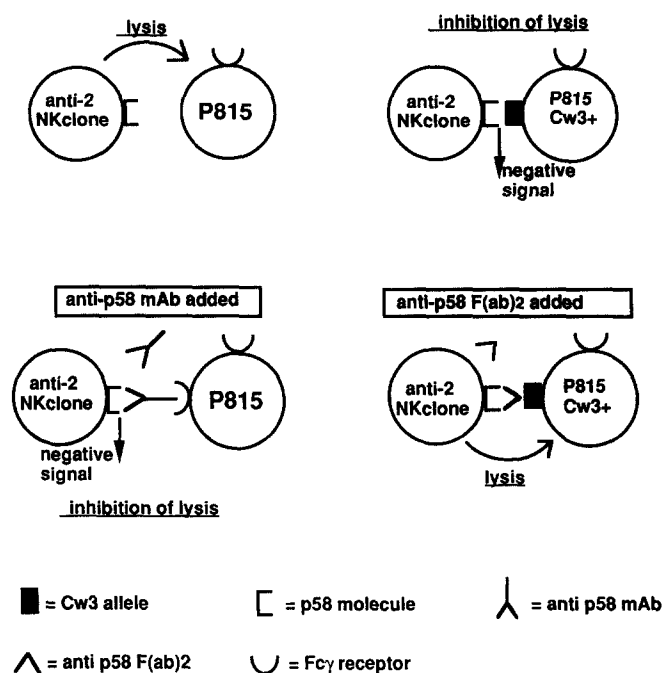
† The various mAbs were added at the onset of the cytolytic assay at a final dilution of 0.5 μg/ml.

In this context, IGROV cells, different from K562, constitutively express HLA class I antigens codified by the C locus (Ciccone, E., unpublished data).

In addition, we showed (5, 14) that in murine tumor target cells expressing surface FcγR, the anti-p58 mAbs had an opposite effect (i.e., they induced inhibition of lysis). These murine target cells are efficiently lysed in a redirected killing assay in the presence of anti-CD16 mAbs of IgG isotype. For example, the P815 cell line has been described to express FcR for IgG1, IgG2a, and IgG2b (25). The P815 cells used in our study expressed receptors for (at least) IgG1 and IgG2a as shown by the effect in redirected killing assays of anti-CD16 mAbs belonging to these subclasses (Table 2). Although not shown, NK-mediated lysis of FcγR<sup>-</sup> murine target cells was neither inhibited nor incremented by anti-p58 mAbs.

In the case of human tumor target cells, the functional effect of anti-p58 mAbs apparently did not seem to correlate with the surface expression of FcγR. For example, the lysis of both Raji (a Burkitt lymphoma) and U937 (a myelomonocytic leukemia) by NK clones was increased in the presence of anti-p58 mAbs. However, as shown in Table 2, the Raji cells could not mediate redirected killing with murine mAbs since neither IgG2a nor IgG1 anti-CD16 mAbs could increase target cell lysis. Thus, operationally, Raji target cells function as the FcγR<sup>-</sup> IGROV cell line. In the case of the U937 cell line available in our laboratory, an efficient redirected killing could be detected by using an anti-CD16 mAb of IgG2a isotype. On the other hand, two different anti-CD16 mAbs of the IgG1 subclass (i.e., the same subclass of GL183 and EB6 mAbs) failed to mediate a substantial redirected killing (Table 2). It is noteworthy that both anti-CD16 mAbs of IgG1 isotype could induce a strong increase of cytolytic activity against the FcγR<sup>+</sup> murine P815 target cells. These data suggest a likely explanation for the increases of cytolytic activity detected against some FcγR<sup>+</sup> human tumor target cells. Thus, in the absence of an efficient crosslinking due to the low affinity of human FcγR for murine IgG1 subclass, the increases of target cell lysis could again be consequent to a mAb-mediated masking of the NK receptor. In agreement with this interpretation, the XA141 mAb (IgM) also enhanced the lysis of the above human tumor target cells by GL183<sup>+</sup>EB6<sup>+</sup> NK clones (data not shown). Finally, in some human tumor cell lines anti-p58 mAbs failed to induce an increase in cytotoxicity by double positive clones. In general, these tumor cells were already characterized by a high susceptibility to lysis by double positive clones. This may reflect the absence of expression of appropriate protective elements. As a consequence, these tumor cells would be similar to unprotected target cells such as the class I-negative K562 (14), the Cw4<sup>+</sup> C1R (Table 1), or the FcγR<sup>-</sup> murine cell lines.

It is interesting that recent data on mice have suggested that the Ly49 antigen may be part of a NK receptor for MHC recognition (26). Expression of Ly49 molecules (or lack of expression) correlates with the specificity of murine NK cells for given MHC class I alleles. Moreover, similar to our present data, F(ab')<sub>2</sub> fragments of anti-Ly49 mAb reversed the inhibition of NK cell-mediated lysis of H2D<sup>d</sup>-transfected



**Figure 3.** Schematic representation of the effect of F(ab')<sub>2</sub> fragments of anti-p58 mAbs on the lysis of class I protected target cells by alloreactive NK clones. The figure refers to a NK clone displaying specificity 2, as effector, and to the murine P815 cells transfected (or not) with Cw3 (i.e., the protective element for anti-2 clones), as targets. Since clones displaying specificity 2 express two members of the p58 molecular family which are identified by the GL183 and by the EB6 mAbs, respectively, the corresponding F(ab')<sub>2</sub> fragments have been used. Note that anti-2 NK clones lyse untransfected P815 cells (*top left*) but not P815 that have been transfected with Cw3 (*top right*). Addition of F(ab')<sub>2</sub> fragments of either GL183 or EB6 mAbs completely restores the lysis of Cw3-transfected cells (*bottom right*). These data can be interpreted as a masking effect of F(ab')<sub>2</sub> fragments preventing the interaction of p58 molecules with their Cw3 ligand on target cells. Addition of the entire mAbs (which bind to FcγR<sup>+</sup> P815 cells) results in inhibition of lysis of untransfected cells (*bottom left*). Thus, the anti-p58 mAbs bound on target cells protect them from lysis, i.e., they mimic the effect of Cw3 molecules in delivering an inhibitory signal upon crosslinking of p58 molecules expressed by anti-2 NK clones.

FcγR<sup>+</sup> target cells. However, at the present, Ly49 molecules do not resemble p58 for either biochemical characteristics or cellular distribution. In addition, it would be of interest to define whether mAbs to Ly49 molecules could mimic the effect of MHC molecules and deliver a negative signal in a redirected killing assay against FcγR<sup>+</sup> target cells.

Therefore, it appears that the cytolytic activity mediated by both human and mouse NK cells may be regulated by specific receptors for MHC (p58 in human and Ly49 in mouse) which turn off the lytic machinery when engaged by appropriate MHC alleles. Molecules different from these receptors would be responsible for NK cell activation (27–29) upon binding to appropriate ligands expressed on target cells. According to this interpretation, the resistance to NK-mediated lysis of a given target cell would depend upon either the expression of protective MHC class I alleles or the lack of appropriate ligands for molecules involved in NK cell activation.

How could the receptor for MHC regulate the NK cell activation under physiological conditions? Since NK cells are continuously exposed to protective self-MHC class I molecules both in vivo and in vitro, one may speculate that the negative signal delivered via the NK-R could be polarized at the site of interaction between effectors and protected target

cells. Alternatively, the negative signal could function for a short period of time. Either one of these proposed mechanisms could explain why NK cells are able to lyse unprotected target cells in a self-environment. Experiments aimed at clarifying these mechanisms are in progress in our laboratory.

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## References

1. Trinchieri, G. 1989. Biology of natural killer cells. *Adv. Immunol.* 47:187.
2. Herberman, R.B., and J.R. Ortaldo. 1981. Natural killer cells: the role in defence against disease. *Science (Wash. DC)*. 214:24.
3. Ciccone, E., O. Viale, D. Pende, M. Malnati, R. Biassoni, G. Melioli, A. Moretta, E.O. Long, L. Moretta. 1988. Specific lysis of allogeneic cells after activation of CD3<sup>-</sup> lymphocytes in mixed lymphocyte culture. *J. Exp. Med.* 168:2403.
4. Bender, J.R., R. Pardi, and E. Engelman. 1990. T cell receptor negative natural killer cells display antigen-specific cytotoxicity for microvascular endothelial cells. *Proc. Natl. Acad. Sci. USA.* 87:694.
5. Moretta, A., C. Bottino, D. Pende, G. Tripodi, G. Tambussi, O. Viale, A.M. Orengo, M. Barbaresi, A. Merli, E. Ciccone, and L. Moretta. 1990. Identification of four subsets of human CD3<sup>-</sup>CD16<sup>+</sup> NK cells by the expression of clonally distributed functional surface molecules. Correlation between subset assignment of NK clones and ability to mediate specific alloantigen recognition. *J. Exp. Med.* 172:1589.
6. Ciccone, E., D. Pende, O. Viale, G. Tambussi, S. Ferrini, R. Biassoni, A. Longo, J. Guardiola, A. Moretta, and L. Moretta. 1990. Specific recognition of human CD3<sup>-</sup>CD16<sup>+</sup> NK cells requires the expression of an autosomic recessive gene on target cells. *J. Exp. Med.* 172:47.
7. Ciccone, E., D. Pende, O. Viale, C. Di Donato, G. Tripodi, A.M. Orengo, J. Guardiola, A. Moretta, and L. Moretta. 1992. Evidence of natural killer (NK) cell repertoire for (allo)antigen recognition: definition of five distinct NK-determined allospecificities in humans. *J. Exp. Med.* 175:709.
8. Ciccone, E., D. Pende, O. Viale, C. Di Donato, A.M. Orengo, R. Biassoni, S. Verdiani, A. Amoroso, A. Moretta, and L. Moretta. 1992. Involvement of HLA class I alleles in NK cell specific function: expression of HLA-Cw3 confers selective protection from lysis by alloreactive NK clones displaying a defined specificity (specificity 2). *J. Exp. Med.* 176:963.
9. Moretta, L., E. Ciccone, A. Moretta, and K. Karre. 1992. Allorecognition by NK cells: nonself or no self? *Immunol. Today.* 13:300.
10. Storkus, W.J., R.D. Salter, J. Alexander, F.E. Ward, R.E. Ruiz, P. Cresswell, and J.R. Dawson. 1991. Class I-induced resistance to natural killing: identification of nonpermissive residues in HLA-A2. *Proc. Natl. Acad. Sci. USA.* 88:5989.
11. Ciccone, E., M. Colonna, O. Viale, D. Pende, C. Di Donato, D. Reinherz, A. Amoroso, M. Jeannet, J. Guardiola, A. Moretta, et al. 1990. Susceptibility or resistance to lysis by alloreactive NK cells is governed by a gene in the human major histocompatibility complex Bf and HLA-B. *Proc. Natl. Acad. Sci. USA.* 87:9794.
12. Raulet, D.H. 1992. A sense of something missing. *Nature (Lond.)*. 358:21.
13. Ljunggren, H., and K. Kärre. 1990. In search of the "missing self": MHC molecules and NK cell recognition. *Immunol. Today.* 11:237.
14. Moretta, A., G. Tambussi, C. Bottino, G. Tripodi, A. Merli, E. Ciccone, G. Pantaleo, and L. Moretta. 1990. A novel surface antigen expressed by a subset of human CD3<sup>-</sup>CD16<sup>+</sup> natural killer cells. Role in cell activation and regulation of cytolytic function. *J. Exp. Med.* 171:695.
15. Moretta, A., G. Pantaleo, L. Moretta, M.C. Mingari, and J.C. Cerottini. 1983. Direct demonstration of the clonogenic potential of every human peripheral blood T cells. Clonal analysis of HLA-DR expression and cytolytic activity. *J. Exp. Med.* 157:743.
16. Bottino, C., G. Tambussi, S. Ferrini, E. Ciccone, M.C. Mingari, P. Varese, L. Moretta, and A. Moretta. 1988. Two subsets of human T lymphocytes expressing  $\gamma/\delta$  antigen receptor are identified by monoclonal antibodies directed to two distinct molecular forms of the receptor. *J. Exp. Med.* 168:491. 1988.
17. Storkus, W.J., D.N. Howell, D.R. Salter, J.R. Dawson, and P. Cresswell. 1987. NK susceptibility varies inversely with target cell class I HLA antigen expression. *J. Immunol.* 138:1657.
18. Colonna, M., T. Spies, J.L. Strominger, E. Ciccone, A. Moretta, L. Moretta, D. Pende, and O. Viale. 1992. Alloantigen recognition by two human natural killer cells is associated with HLA-C or a closely linked gene. *Proc. Natl. Acad. Sci. USA.* 89:7983.
19. Moretta, A., G. Pantaleo, M.C. Mingari, L. Moretta, J.C. Cerottini. 1984. Clonal heterogeneity in the requirement for

- T3, T4, and T8 molecules in human cytolytic T lymphocyte function. *J. Exp. Med.* 159:921.
20. Janeway, A.J., Jr. 1991. To thine own self be true. *Curr. Biol.* 1:239.
  21. Kärre, K. 1992. An unexpected petition for pardon. *Curr. Biol.* 2:613.
  22. Meuer, S.C., J.C. Hodgdon, R.E. Hussey, J.P. Protentis, S.F. Schlossman, and E.L. Reinherz. 1983. Antigen-like effect of monoclonal antibodies directed at receptors on human T cell clones. *J. Exp. Med.* 159:988.
  23. Moretta, A., G. Pantaleo, M. Lopez-Botet, M.C. Mingari, L. Moretta. 1985. Anti-clonotypic monoclonal antibodies induce proliferation of clonotype positive cells in peripheral blood human T lymphocytes: evidence for a phenotype (T4/T8) heterogeneity of the clonotype-positive proliferating cells. *J. Exp. Med.* 162:1393.
  24. Reinherz, E.L., S.C. Meuer, and S.F. Schlossman. 1983. The human T cell receptor: analysis with cytotoxic T cell clones. *Immunol. Rev.* 74:83.
  25. Van de Griend, R., R.L.H. Bolhuis, G. Stoter, and R.C. Roozmond. 1987. Regulation of cytolytic activity in CD3<sup>-</sup> and CD3<sup>+</sup> killer cell clones by monoclonal antibodies (anti-CD16, anti-CD2, anti-CD3) depends on subclass specificity of target cell IgG-FcR. *J. Immunol.* 138:3137.
  26. Karlhofer, F.M., R.K. Ribaldo, and W.M. Yokoyama. 1992. MHC class I alloantigens specificity of Ly49<sup>+</sup> IL-2 activated natural killer cells. *Nature (Lond.)* 358:66.
  27. Moretta, A., E. Ciccone, G. Pantaleo, G. Tambussi, C. Bottino, G. Melioli, M.C. Mingari, and L. Moretta. 1989. Surface molecules involved in the activation and regulation of T or natural killer lymphocytes in humans. *Immunol. Rev.* 111:145.
  28. Moretta, A., A. Poggi, D. Pende, G. Tripodi, A.M. Orengo, N. Pella, R. Augugliaro, C. Bottino, E. Ciccone, and L. Moretta. CD69-mediated pathway of lymphocyte activation. 1991. Anti-CD69 mAbs trigger cytolytic activity of different lymphoid effector cells with the exception of cytolytic T lymphocytes expressing TCR $\gamma/\delta$ . *J. Exp. Med.* 174:1393.
  29. Moretta, L., E. Ciccone, D. Pende, G. Tripodi, C. Bottino, and A. Moretta. 1992. Human Natural Killer cells: clonally distributed specific functions and triggering surface molecules. *Lab. Invest.* 66:138.