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Inhibition of selective signaling events in natural killer cells recognizing major histocompatibility complex class I

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ABSTRACT **Many studies have characterized the transmembrane signaling events initiated after T-cell antigen receptor recognition of major histocompatibility complex (MHC)-bound peptides. Yet, little is known about signal transduction from a set of MHC class I recognizing receptors on natural killer (NK) cells whose ligation dramatically inhibits NK cell-mediated killing. In this study we evaluated the influence of MHC recognition on the proximal signaling events in NK cells binding tumor targets. We utilized two experimental models where NK cell-mediated cytotoxicity was fully inhibited by the recognition of specific MHC class I molecules. NK cell binding to either class I-deficient or class I-transfected target cells initiated rapid protein tyrosine kinase activation. In contrast, whereas NK cell binding to class I-deficient targets led to inositol phosphate release and increased intracellular free calcium ($[Ca^{2+}]_i$), NK recognition of class I-bearing targets did not induce the activation of these phospholipase C-dependent signaling events. The recognition of class I by NK cells clearly had a negative regulatory effect since blocking this interaction using anti-class I $F(ab')_2$ fragments increased inositol 1,4,5-trisphosphate release and $[Ca^{2+}]_i$ and increased the lysis of the targets. These results suggest that one of the mechanisms by which NK cell recognition of specific MHC class I molecules can block the development of cell-mediated cytotoxicity is by inhibiting specific critical signaling events.**

Natural killer (NK) cells are $CD3^-$, $CD16^+$ lymphocytes that mediate lysis of certain tumor cells and virus-infected cells without prior sensitization (1). While NK cells are not major histocompatibility complex (MHC)-restricted in their killing of targets, numerous studies *in vitro* and *in vivo* have demonstrated that target cell expression of class I molecules can inhibit NK cell-mediated killing of these targets (2–7). However, attempts to define the basis for this negative regulation have been complicated by multiple factors. First, there appear to be several families of MHC-recognizing receptors on NK cells and within each family there are multiple members. Different receptors on NK cells also appear to recognize different subsets of MHC class I molecules (3). In addition, depending on the NK clone, MHC recognition can be alternatively activating or inhibiting (e.g., kp43) (8). Therefore, depending on the repertoire of MHC-recognizing molecules on a given subpopulation of NK cells and on the specific expression of MHC class I molecules on a given target, the nature of the effector–target interaction can be quite complex. Moreover, even among a clonal population of effector cells, there will likely be cells in differing states of activation. In the context of all of this heterogeneity, it has been observed that most cloned human NK cell lines are only partially inhibited in their killing of MHC-bearing targets. Indeed, Litwin *et al.* (9) examined >200 human NK cell clones and found few cases where there was an absolute inhibition of NK cell-mediated cytotoxicity. In this setting of "quantitative" rather than "qualitative" inhibition, experimentation has been unable to identify a discernible correlation between NK recognition of MHC and a reduction in early signaling events in the NK cells (10).

To more clearly assess whether MHC recognition by NK cells could directly alter proximal signaling events, we sought to identify experimental systems where there was a more absolute (i.e., qualitative) inhibition. First, we found that NKL, a $CD3^-$ interleukin 2 (IL-2)-dependent NK cell line that can effectively kill class I-deficient cells, was unable to kill specific HLA-B27-bearing targets. Similarly, certain cloned populations of NK cells bearing the $GL183^-/EB6^+$ phenotype are unable to kill HLA-Cw4-bearing targets (11). In each of these experimental models where MHC recognition fully inhibited NK cell-mediated cytotoxicity, proximal signaling in the NK cells was also inhibited. These results suggest that ligand binding by MHC-recognizing receptors on NK cells can alter transmembrane signaling and the subsequent development of the cytotoxic response.

MATERIALS AND METHODS

Cells. The NKL cell line was derived from the peripheral blood of a patient with a $CD3^-$, $CD16^+$, $CD56^+$ large granular lymphoproliferative disorder (12, 13). Clonal human NK cell lines were isolated and passaged as described (14). The HLA class I-deficient C1R cell line and the HLA-transfected C1R sublines were generously provided by Peter Cresswell (Yale University). C1R cells express no detectable HLA-A molecules, low levels of HLA-B35, and normal levels of HLA-Cw4 (15).

Antibodies. The p58-specific monoclonal antibodies (mAbs) GL183 and EB6 were kindly provided by Alessandro and Lorenzo Moretta (Genoa, Italy) (16). The anti-phosphotyrosine antibody 1G2 was the kind gift of A. R. Frackelton, Jr. (Brown University). MB40.5 (reactive with conserved, α_2 domain-associated, monomorphic determinants on HLA-A, -B, and -C, IgG1) and G28-5 (anti-CD40, IgG1) were obtained from the American Type Culture Collection. All mAbs were purified from ascites using protein A-agarose affinity chromatography (Bio-Rad). $F(ab')_2$ fragments of MB40.5 were generated by digestion with immobilized pepsin (Pierce), and purity of the fragments was confirmed by SDS/PAGE followed by silver staining.

Measurements of Inositol Phosphates, Intracellular Calcium, Cytotoxicity, Conjugate Formation, and Protein Phosphorylation. Inositol phosphate release, intracellular free calcium concentrations ($[Ca^{2+}]_i$), cell-mediated cytotoxicity, con-

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Abbreviations: $[Ca^{2+}]_i$, intracellular free calcium concentration; IP_3, inositol 1,4,5-trisphosphate; PLC, phospholipase C; PTK, protein-tyrosine kinase; MHC, major histocompatibility complex; NK, natural killer; mAb, monoclonal antibody; IL-2, interleukin 2; E:T, effector: target.

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jugate formation, and tyrosine phosphorylation were measured as described (10, 14, 17).

RESULTS

Class I Expression on Target Cells Inhibits NKL Cytotoxic Functions. The human cell line NKL was derived from the peripheral blood of a patient with a $CD3^-$, $CD16^+$, $CD56^+$ large granular lymphocyte proliferative disorder (12). Functionally, NKL demonstrates cytolytic activity similar to normal peripheral blood NK cells in that it mediates natural killing of K562 and Fc receptor-dependent cytotoxicity of antibody-coated P815 cells (12). Since class I MHC molecules expressed on tumor targets can inhibit NK cell-mediated cytotoxicity, we chose to examine NKL for its ability to kill the class I-deficient C1R cell line compared to C1R transfected with various class I molecules (Fig. 1*A*). C1R cells expressing transfected HLA-B27 (C1R-B27) consistently demonstrated a near-absolute resistance to lysis by NKL. In contrast, C1R-A3, C1R-Aw68, C1R-B7, and C1R-Bw58 showed intermediate resistance to NKL (Fig. 1*A*), and C1R-A2 was killed just as well as untransfected C1R cells (data not shown). These data support previous observations that different class I molecules can vary in their ability to inhibit NK cell-mediated cytotoxicity. Since C1R-B27 mediated the greatest inhibition of NKL cells, we chose to focus on this interaction for subsequent studies.

To confirm that the decreased lysis of C1R-B27 was due to the expression of class I on the target cell surface, we pretreated the targets with $F(ab)'_2$ fragments of the anti-class I mAb MB40.5. Addition of MB40.5 increased lysis of C1R-B27 in a dose-dependent manner (Fig. 1*B*), whereas addition of the isotype-matched control mAb G28-5 against CD40 (also expressed on the targets) did not. Moreover, revertant sublines of class I-transfected C1R cells that had decreased surface class I expression showed increased susceptibility to NK cell-mediated killing (data not shown). Therefore, the resistance of C1R-B27 was due to its surface MHC class I expression. In addition, these results support the concept that MHC recognition by NK cells can confer a negative regulatory influence on the activation of these effector cells.

Recognition of HLA-B27 Expression Inhibits Inositol Phosphate Release and Elevated $[Ca^{2+}]_i$ in NKL Cells. To evaluate mechanisms for class I-mediated inhibition of NK cell-mediated cytotoxicity, we analyzed proximal signaling events in NKL cells stimulated with either C1R or C1R-B27. While C1R cells stimulated a 2- to 6-fold increase in the generation of inositol phosphates in NK cells, C1R-B27 stimulation of NKL did not result in significant inositol 1,4,5-trisphosphate (IP_3) release (Fig. 2*A*). Moreover, treatment of C1R-B27 with $F(ab')_2$ fragments of MB40.5 (anti-class I), but not G28-5 (anti-CD40), restored the ability of C1R-B27 to activate IP_3 production (Fig. 2*B*). Therefore, the increased lysis of anti-class I-treated C1R-B27 cells correlates with restoration of IP_3 production in NKL cells upon recognition of these targets.

Parallel findings were observed for elevation of $[Ca^{2+}]_i$ in NKL upon stimulation with these targets. Using indo-1 AM-labeled NKL cells and flow cytometry, we found C1R cells stimulated increased $[Ca^{2+}]_i$ in NKL, whereas C1R-B27 failed to initiate any detectable increase (Fig. 3). Treatment of C1R-B27 with anti-class I Ab restored the ability of C1R-B27 to induce increased $[Ca^{2+}]_i$ (Fig. 3). Addition of either anti-class I or anti-CD40 mAb to NKL cells alone or NKL cells stimulated with C1R cells had no effect on IP_3 release or calcium signaling (data not shown). Taken together, these results suggest that the inhibition of NKL activation after the

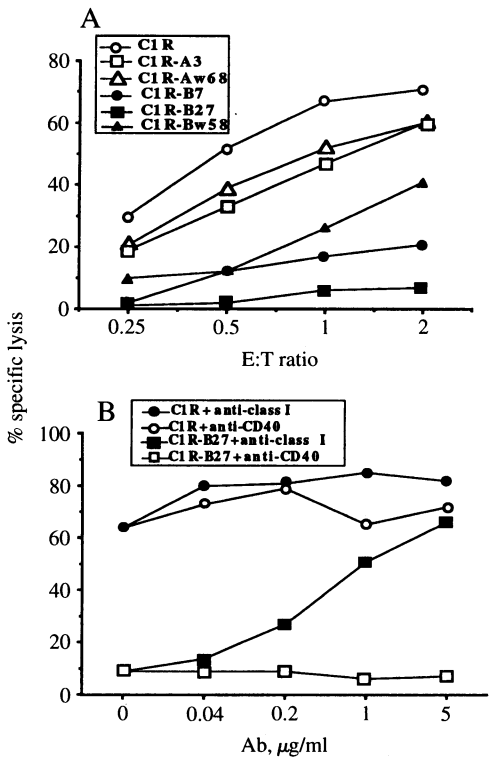

FIG. 1. HLA class I expression on target cells inhibits lysis by NKL cells. (*A*) NKL cells were used as effectors in a 4-hr ^{51}Cr release assay against class I-deficient C1R cells or stably transfected C1R cells expressing high levels of HLA class I molecules. (*B*) ^{51}Cr-labeled C1R cells or HLA-B27 transfected C1R cells (C1R-B27) were pretreated for 30 min with $F(ab')_2$ fragments of MHC class I-specific mAb (MB40.5) or an isotype-matched CD40-specific mAb (G28-5). NKL effector cells were then added at an effector:target (E:T) ratio of 1:1.

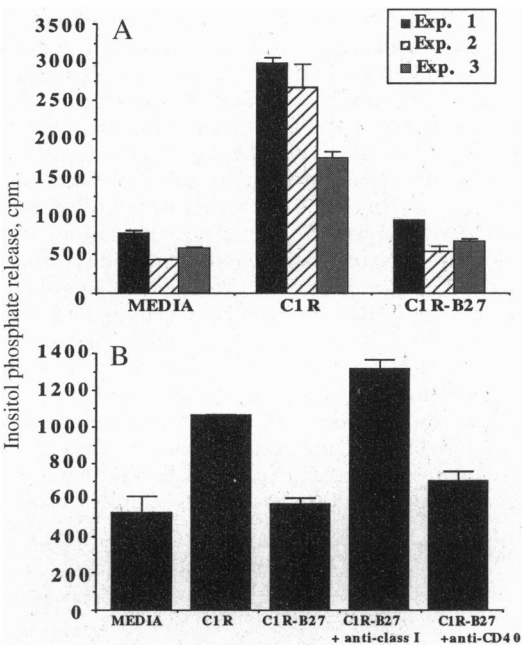

FIG. 2. Recognition of HLA-B27 expression on target cells inhibits inositol phosphate hydrolysis in NKL cells. (*A*) NKL cells were labeled with *myo*-[^{3}H]inositol, washed, and incubated for 30 min with medium alone, C1R cells, or C1R-B27 cells at an E:T ratio of 1:1. Total inositol phosphates were extracted, separated by anion-exchange chromatography, and quantitated by liquid scintillation spectroscopy. Three separate experiments, as indicated, showed similar results. Each bar represents the mean of triplicate samples in each experiment and the error bars indicate the SD. (*B*) C1R cells or C1R-B27 were pretreated with 10 μg of $F(ab')_2$ fragments of class I-specific or CD40-specific mAb per ml. Error bars represent the SD for triplicate samples. Data are representative of three separate experiments.

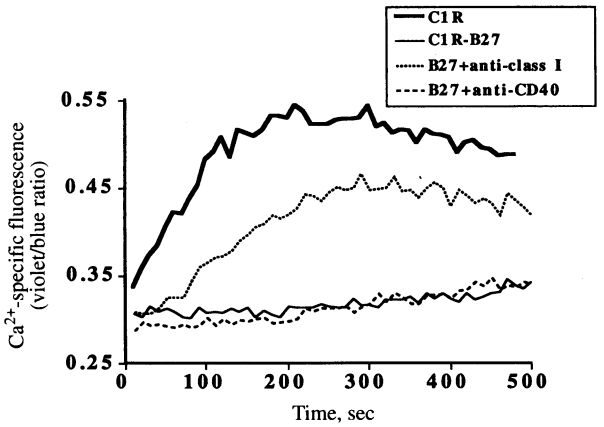

FIG. 3. Recognition of HLA-B27 expression on target cells inhibits calcium signaling in NKL cells. NKL cells were loaded with the calcium-sensitive dye indo-1 and stimulated with either C1R cells, C1R-B27 cells, or C1R-B27 cells pretreated with class I-specific or CD40-specific mAb. Changes in calcium-specific fluorescence were monitored by flow cytometry and expressed as the ratio of violet (390 nm) to blue (500 nm) fluorescence.

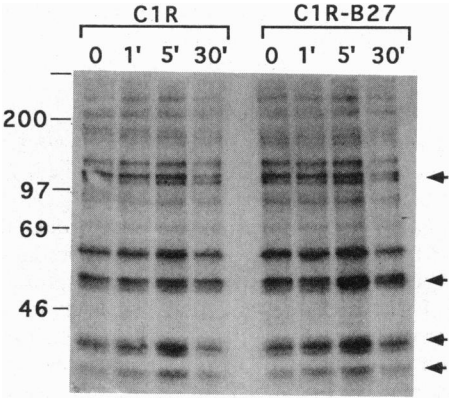

FIG. 4. C1R and C1R-B27 cells stimulate PTK activation in NKL cells. NKL cells were labeled with $^{32}P_i$, washed, and stimulated for the indicated time with either C1R or C1R-B27 cells at an E:T ratio of 1:2. Cells were then lysed and phosphotyrosine-containing proteins were immunoprecipitated with the mAb 1G2 linked to Sepharose. Lysates of unstimulated NKL cells (0 time) were mixed with lysates of an equivalent number of target cells to control for total protein content. Proteins were resolved on an SDS/8.5% polyacrylamide gel and transferred to an Immobilon-P membrane. Molecular masses (kDa) are indicated (left) and proteins with induced tyrosine phosphorylation are indicated by arrowheads (right).

recognition of HLA-B27 molecules is the result of an early block in production of phospholipase C (PLC)-derived second messengers.

Tyrosine Kinase Activation in NKL Cells Recognizing HLA-B27. Previously, our laboratory and others have demonstrated that protein-tyrosine kinases (PTKs) in NK cells provide early and requisite signals for PLC-γ activation and the subsequent development of cytotoxic function (17–21). Here we examined the influence of MHC recognition on the tyrosine phosphorylations induced by binding to susceptible targets. ^{32}P-labeled NK cells were incubated with either C1R or C1R-B27 cells, and cell lysates from these reaction mixtures were then immunoprecipitated with anti-phosphotyrosine antibody. Subsequent analysis by SDS/PAGE and autoradiography demonstrated that C1R cells and the HLA-B27 transfectants induced in NKL cells the rapid tyrosine phosphorylation of similar (based on identical molecular masses) substrates (Fig. 4). In each case, the indicated substrates were maximally phosphorylated 5 min after stimulation, and densitometric evaluation of substrates with induced tyrosine phosphorylation (bands indicated by arrowheads) showed a 2- to 4-fold increase in C1R-stimulated NKL cells and a 2- to 8-fold increase in C1R-B27-stimulated NKL cells. This result indicates that although HLA-B27 recognition can interrupt PLC-γ activation and the subsequent development of the cytotoxic response, certain proximal PTK-catalyzed signaling events are initiated. More broadly, this result further refutes the concept that MHC expression on target cells masks NK cell recognition of triggering epitopes on the targets (target interference model). Rather, MHC recognition itself appears to modulate a subset of the signaling events initiated after NK cell recognition of susceptible targets (effector inhibition model) (22).

Engagement of p58 Molecules Also Blocks PLC-γ-Mediated Activation Events. The p58 family of receptor molecules on NK cells appears to preferentially recognize certain HLA-C alleles (11, 23). Elegant studies have shown that interaction between HLA-C and p58 leads to inhibition of NK cell-mediated cytotoxicity, and blocking this interaction with mAbs against either the p58 molecules or HLA-C prevents the inhibitory interaction and leads to lysis of the target (11, 24). Here we sought to determine the nature of the p58-mediated inhibitory effect. For this purpose, we took advantage of the normal level of HLA-Cw4 on C1R cells. While the recognition of HLA-Cw4 has no inhibitory effect on the majority of NK cells, NK clones phenotyped as GL183$^-$/EB6$^+$ are potently inhibited after HLA-Cw4 recognition (Fig. 5*A* and ref. 11). Importantly, this inhibition is absolute and qualitative, much like NKL and HLA-B27. We examined GL183$^-$/EB6$^+$ NK clones for generation of PLC-mediated signals upon interaction with resistant C1R cells or cells susceptible to either natural cytotoxicity or Fc receptor-dependent cell-mediated killing (K562 or the anti-FcγRIII-producing hybridoma, 3G8, respectively) (Fig. 5*A*). Whereas K562 and 3G8 hybridoma cells stimulated inositol phosphate release and elevated $[Ca^{2+}]_i$, C1R cells (HLA-Cw4$^+$) failed to stimulate the PLC-γ-generated signals (Fig. 5 *B* and *C*). In contrast, C1R cells, which can be effectively

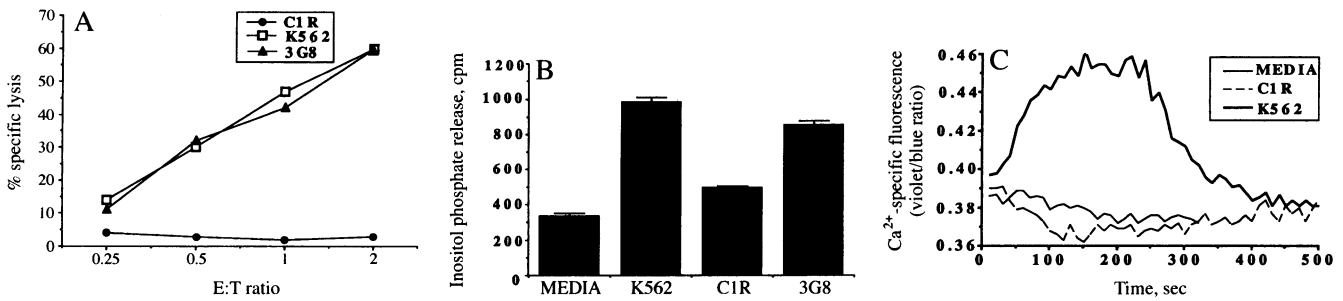

FIG. 5. Recognition of HLA-Cw4 expression on target cells inhibits the activation of GL183$^-$/EB6$^+$ NK cells. (*A*) GL183$^-$/EB6$^+$ cloned human NK cells were incubated with either C1R cells (HLA-Cw4$^+$), K562 cells, or anti-FcγRIII hybridoma cells (3G8) in a 4-hr ^{51}Cr release assay. Inositol phosphate hydrolysis (*B*) and calcium signaling (*C*) were separately analyzed for each effector–target combination. Results shown are representative of those obtained with five different EB6$^+$ NK clones.

killed by GL183$^-$/EB6$^-$ NK clones, did stimulate increased $[Ca^{2+}]_i$ and IP_3 release in GL183$^-$/EB6$^-$ NK cells (data not shown). These results are consistent with the notion that p58 recognition of HLA-Cw4 can prevent the generation of PLC-γ-derived second messengers.

Decreased Signaling After Recognition of MHC Class I$^+$ Targets Is Not Due to Decreased Conjugate Formation. The initial stage of NK cell-mediated cytotoxicity is the formation of stable conjugates between target and effector. One possible explanation for a lack of signaling events in NKL cells upon interaction with C1R-B27 would be if these cells did not effectively form conjugates. Although the presence of PTK activation after recognition of these class I-bearing targets suggests that functional effector–target conjugates are being formed, we sought, in separate analyses, to directly quantitate the influence of MHC expression on conjugate formation in this experimental system. Using two-colored flow cytometry, we found that C1R-B27 cells formed conjugates with NKL cells just as well as C1R cells (Table 1). Though we showed treatment of C1R-B27 with F(ab')$_2$ fragments of the anti-class I mAb, MB40.5, greatly increased NKL-mediated lysis (Fig. 1*B*), this treatment did not affect conjugate formation (Table 1). Additional studies of conjugate formation between EB6$^+$ NK clones and either C1R cells (resistant to lysis) or K562 (sensitive to lysis) also found no significant differences (Table 1). Therefore, the inhibition of proximal signaling events in NK cells interacting with class I$^+$ targets appears to follow normal conjugate formation.

Effects of Pretreatment with IL-2 on NK Cell Signaling and Cellular Cytotoxicity. Previously our laboratory and others have shown that for those NK cells that are partially inhibited in their killing of class I-bearing targets, pretreatment of the NK cells with IL-2 leads to increased lysis of these targets and proximal signaling events appear to remain intact (7, 10). In contrast, little is known about the influence of IL-2 on NK cells that are fully inhibited in their killing of class I-bearing targets. Comparative analysis in this study showed clearly divergent influences of IL-2 on these two effector–target groups. Specifically, whereas IL-2 pretreatment of either NKL cells or cloned NK cells enhanced killing of partially resistant targets, IL-2 pretreatment did not alter the killing of fully resistant targets (Fig. 6*A* and data not shown). Interestingly, MHC class I expression on the target cells also had divergent effects on the signaling occurring in these two effector–target groups. Specifically, whereas inositol phosphate release (Fig. 6*B*) and elevations in $[Ca^{2+}]_i$ (data not shown) remained intact in NK cells encountering partially resistant targets, these PLC-γ-dependent signaling events were inhibited after NK cells bound fully resistant targets. Taken together, these results suggest there are fundamentally different signaling events in NK cells that are quantitatively versus qualitatively inhibited after class I recognition.

Table 1. Decreased killing after recognition of MHC class I$^+$ targets is not due to decreased conjugate formation

Effector cell	Target	Susceptibility to killing	Ab added	% NK cells in conjugate
NKL	C1R	+	None	15.5
		+	Anti-class I	16.7
		+	Anti-CD40	17.2
NKL	C1R-B27	−	None	12.1
		+	Anti-class I	12.9
		−	Anti-CD40	13.9
NK clone FZ3 (EB6$^+$)	C1R	−	None	27.6
	K562	+	None	26.9
NK clone FZ8 (EB6$^+$)	C1R	−	None	22.9
	K562	+	None	21.9

Sulfofluorescein diacetate-stained NK cell effectors were incubated with hydroethidine-stained targets. For Ab treatment, targets were preincubated for 10 min with F(ab')$_2$ fragments of either MHC class I-specific mAb (MB40.5) or CD40-specific mAb (G28-5). Conjugate formation was quantitated by two-color flow cytometry.

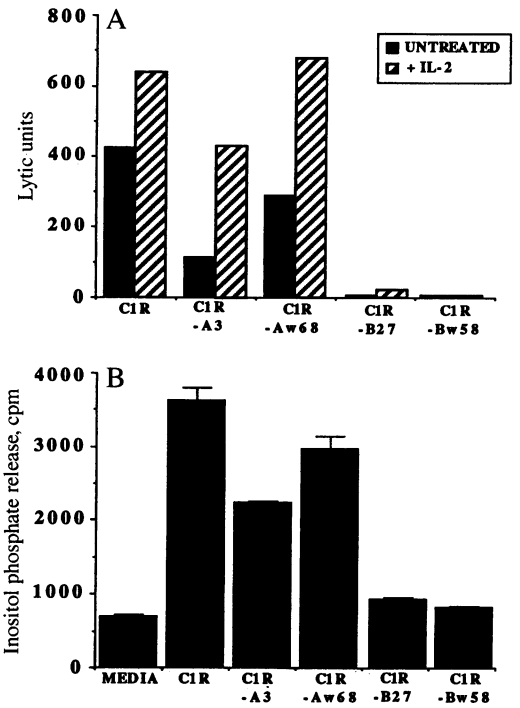

FIG. 6. Effects of IL-2 on EB6$^-$ NK cell signaling and cellular cytotoxicity. (*A*) Clonal EB6$^-$ NK cells were incubated for 18 hr in medium either without IL-2 or with 20 units of IL-2 per ml. These cells were then used as effectors against C1R cells or cells transfected with the gene for the indicated HLA molecules. (*B*) Inositol phosphate release was measured in *myo*-[^{3}H]inositol-labeled EB6$^-$ NK cells stimulated for 30 min with the indicated targets.

DISCUSSION

Studies that initially demonstrated an inverse correlation between the susceptibility of target cells to NK cell-mediated cytotoxicity and their level of class I expression lead to the proposal of two alternative models to explain the nature of the interaction between NK cells and MHC: the "target interference model" and the "effector inhibition model" (22). Subsequent findings have largely fulfilled the predictions made by the effector inhibition model (3). This model hypothesizes the presence of NK cell "inhibitory receptors" that specifically recognize class I molecules. Engagement of these receptors results in a "negative signal" that down-modulates NK cell-mediated killing of the class I$^+$ target. Indeed, several families of receptors have now been found on NK cells that specifically interact with certain class I molecules (2, 3, 25–27). Moreover, different subpopulations of NK cells within an individual vary in the family of MHC-recognizing receptors they express and in the MHC class I molecules they recognize (2, 3, 9). This differential expression creates a heterogeneous repertoire of NK cell reactivity against class I-bearing targets.

Despite the identification of MHC recognizing inhibitory receptors on NK cells, little is known about the mechanism(s) by which interaction between NK cells and class I$^+$ targets inhibits the development of the cytotoxic response. To evaluate this issue, we identified and characterized two experimental models in which class I recognition fully inhibited NK cell-mediated cytotoxicity. First, a recently isolated human NK

cell line, NKL, was unable to kill HLA-B27-transfected C1R cells. Similarly, $GL183^-/EB6^+$ NK cell lines were unable to lyse the HLA-$Cw4^+$ C1R target cells. For each of these effector populations, while susceptible targets elicited inositol phosphate hydrolysis and increased $[Ca^{2+}]_i$, the class I^+, NK-resistant targets did not trigger these signaling events. Blocking the interaction between MHC-recognizing receptors on the NK cells and MHC class I molecules on the targets, using anti-class I $F(ab')_2$ fragments, led to the restoration of the proximal PLC-γ-dependent signaling events and the subsequent generation of the cytotoxic response. These results strongly suggest that recognition of specific MHC class I molecules on target cells can potently inhibit the generation of pharmacologically active, inositol phosphate-derived second messengers critical to development of NK cell-mediated killing.

The mechanism by which MHC recognition inhibits the rapid activation of PLC-γ in NK cells remains unknown. Our demonstration that multiple proteins in NK cells are tyrosine phosphorylated after NK cell binding to class I^+ targets suggests that the inhibition is not global. However, this rapid PTK activation may or may not involve the specific proximal tyrosine kinases (i.e., lck, ZAP-70, and syk) that have been implicated in the tyrosine phosphorylation of PLC-γ in activated NK cells (28–31). Similarly, although MHC-recognizing receptors have been reported to be physically associated with certain subunits of trigger receptors (i.e., association of p58 molecules with ζ chains) (32), the functional consequence of these associations remains unclear. Theoretically, the sequestering of critical signaling elements could dramatically alter the normal activating response.

It should be emphasized that the inhibitory effects of MHC recognition on NK cell signal transduction were observed here in two systems where MHC conferred complete resistance to lysis. In contrast, partially resistant targets did not demonstrate reduced inositol phosphate release or calcium signaling (Fig. 6, ref. 10). Several factors could account for these differences. First, since clonal subpopulations of NK cells differ in the kinds of MHC-recognizing molecules expressed on their surface (3, 9), receptor-initiated signaling mechanisms are likely to differ between clones. In addition, depending on the NK clone, MHC recognition can be alternatively activating or inhibiting (8). Superimpose on this heterogeneity the fact that MHC-induced inhibition is qualitative in some cases and quantitative in others (9). The assay systems used here to measure signaling events may be sensitive to qualitative differences, but insufficiently sensitive to detect smaller quantitative changes. Specifically, the measurements of inositol phosphate release and elevated $[Ca^{2+}]_i$ used here are based on the average response of a whole population of cells. Interpretation of these kinds of assays is uncomplicated as long as there is a uniform response from each cell in the population. However, in the case of partial or intermediate responses by the cells, the generation of proximal signals in responding cells may mask signal inhibition in nonresponding cells. Identification of the MHC-recognizing receptors utilized in each of these experimental systems will help to differentiate between these alternative explanations.

Clearly, regulation of NK cell-mediated cytotoxicity is a multifactorial process. In addition to the inhibitory receptors, there are other "triggering receptors" and adhesion molecules that are important for NK cell activity (33). Furthermore, the absence of "triggering epitopes" on certain targets can make them resistant to NK cell-mediated killing (14, 17). Further studies are needed to determine not only the signaling pathways initiated by individual receptors but also how the multiple signals are integrated for regulation of NK cell responses.

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