

Identification of a Novel Signal Transduction Surface Molecule on Human Cytotoxic Lymphocytes

By Nicholas M. Valiante and Giorgio Trinchieri

From the Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104

Summary

In this study, we have used a newly generated monoclonal antibody (mAb C1.7) to identify a novel 38-kD signal-transducing surface molecule (p38) expressed by lymphocyte subsets capable of cell-mediated cytotoxicity. Virtually all CD16⁺/CD56⁺ natural killer (NK) cells and approximately half of CD8⁺ (T cell receptor [TCR] α/β^+) T cells and TCR- γ/δ^+ T cells express the p38 surface molecule. Stimulation of p38 on NK cells with mAb C1.7 activated cytotoxicity, induced lymphokine production, and initiated polyphosphoinositol turnover and $[Ca^{2+}]_i$ increases. Unlike other NK cell surface molecules that activate cytotoxicity, p38 stimulation did not result in the release of the granule enzyme *N*-carbobenzoxyl-L-thiobenzyl ester-esterase even under conditions in which mAb C1.7 induced NK cell-mediated redirected lysis of Fc γ R⁺ target cells. Activated (recombinant interleukin 2 [rIL-2], 5 d) CD8⁺ T cells mediated non-major histocompatibility complex (MHC)-restricted cytotoxicity, and the CD8⁺/p38⁺ subset contained the overwhelming majority of this activity. F(ab')₂ fragments of mAb C1.7 inhibited non-MHC-restricted cytotoxicity mediated by resting NK cells and rIL-2-cultured T cells but did not affect spontaneous cytotoxicity mediated by activated, cultured NK cells. Taken as a whole, our results suggest that p38 may have a direct role in the recognition, signal transduction, and/or lytic mechanisms of non-MHC-restricted cytotoxicity.

Cytotoxic lymphocytes are a critical effector arm of cell-mediated immune responses to intracellular parasitic (bacterial or viral) infections and tumors. These cytotoxic cells recognize target cells by at least two distinct mechanisms. The first mechanism, which is mediated by CTL, involves the interaction of the CTL with the target cell via recognition of MHC class I or, less frequently, class II and antigenic peptides by clonally distributed TCR (1). This recognition is highly specific, elicits memory, and has been termed MHC-restricted cytotoxicity. By contrast, the second mechanism is mediated primarily by CD3⁻, CD16⁺, and CD56⁺ NK cells, which recognize target cells through possibly multiple and heterogeneous interactions of still incompletely characterized receptor/ligand pairs that are not directly restricted by MHC recognition. Cytotoxic effectors mediating this type of recognition and lysis are termed non-MHC-restricted cytotoxic cells (2). Although non-MHC-restricted cytotoxic effectors are less specific than CTL and do not generate immunologic memory responses, their early activation during an immune response and their broad lytic abilities make them an important effector component of natural resistance, active as a first line of defense well before specific effector mechanisms have been elicited.

Resting NK cells express a number of surface molecules which, when stimulated, can activate their cytotoxic mechanism. The low affinity Fc receptor for IgG (Fc γ RIII or CD16)

is expressed by virtually all human NK cells as well as a minor population of T cells, and when triggered by IgG antibodies on a target cell surface induces antibody-dependent, cell-mediated cytotoxicity (3, 4). In addition, the CD2 molecule, which is expressed by 80–90% of NK cells and recognizes CD58 or CD59 ligands on target cells, can also activate NK cells cytolytically (5–7). Neither CD16 nor CD2 appear to be required for non-MHC-restricted cytotoxicity, as cytotoxic lymphocytes that lack either molecule can still mediate natural killing (3, 8, 9). Other surface molecules with unknown ligands have recently been identified on NK cells that can also activate cytotoxicity. NKRP1 was identified on rat NK cells and an antibody against this molecule (mAb 3.2.3) can induce redirected lysis of Fc γ R⁺ target cells as well as other activation and signal transduction events (10). The cloned cDNA for NKRP1 encodes a putative type II integral membrane protein with significant homology (22%) with members of the C-type lectin superfamily (11). The NK1.1 antigen expressed by murine NK cells can initiate many of the activation events observed with NKRP1 stimulation, and upon cloning of the cDNA coding for NK1.1 was found to be the murine equivalent of NKRP1 (12, 13). In humans, a family of related cDNAs (NKG2) was identified with restricted expression to NK cells and codes for putative polypeptides displaying homology with NKRP1 (14). Additional molecules, such as pNKR1 (15), the molecules reacting with the mAbs

5C6 and 6D3.2 (16–18), and the surface molecule identified by mAb PP35 (19), although not structurally related to the NKR1P1 family members, also appear to be activating structures on NK cells.

Although NK cell-mediated killing is not MHC restricted, it is not always MHC independent. On certain target cell types the level of MHC class I expression inversely correlates with the sensitivity of the target cell to NK cell lysis (20–24). One of the interpretations advanced to explain these findings is the missing self model, which hypothesizes that NK cells recognize and lyse target cells that are low or deficient in MHC class I expression or possibly target cells that express allogeneic class I (25). This model has been used to explain the phenomenon of murine hybrid resistance and the specificity of human NK cell alloreactive clones (26–28). Two alternative mechanisms have been proposed to account for the effects of class I on NK cell activity. MHC class I expression may mask the target cell antigen responsible for recognition by and activation of NK cells, or MHC class I recognition by the NK cell may deliver a negative signal to lysis. Recent studies (29) support the negative signal hypothesis and in fact one NK cell surface molecule has now been identified that appears to be inhibitory to NK cell cytotoxicity upon ligation. Ly-49 not only displays characteristics of an inhibitory receptor but also appears to be directly involved in MHC class I recognition (30). In addition, the molecules identified by the antibodies SW5E6 (31–33) or GL183/EB6 (34, 35) have also been proposed to recognize class I. Although it is now clear that the expression of class I by target cells can affect NK cell-mediated lysis, it is likely that non-MHC-restricted cytotoxicity can occur through both MHC-independent and -dependent mechanisms (36–39).

Here we report the identification of a novel 38-kD signal transduction surface molecule (p38) expressed by virtually all human (CD3⁻/CD16⁺/CD56⁺) NK cells and subpopulations of T cells comprising approximately half of CD8⁺ T cells and γ/δ ⁺ T cells. Perturbation of p38 with the anti-p38 antibody mAb C1.7 stimulated NK cell-mediated redirected cytotoxicity and under certain experimental conditions induced lymphokine (IFN- γ and IL-8) production and modulated proliferation. Upon crosslinking, mAb C1.7 induced polyphosphoinositide turnover and increases in [Ca²⁺]_i in purified, cultured NK cells. On rIL-2-cultured (5 d) CD8⁺ T cells p38 was shown to be a marker for non-MHC-restricted cytotoxicity activity as suggested by the observation that only p38⁺ and not p38⁻ T cells had the ability to lyse a variety of NK cell-sensitive and -resistant target cells. In addition, the findings that F(ab')₂ fragments of mAb C1.7 inhibited non-MHC-restricted cytotoxicity mediated by resting NK cells or rIL-2-activated T cells suggest that p38 may have a role in target cell recognition and cell-mediated cytotoxicity.

Materials and Methods

mAb C1.7 Preparation. 6-wk-old female BALB/c mice were immunized intraperitoneally with 20 × 10⁶ purified, cultured human NK cells, followed by four intraperitoneal injections of NK cells in a 5-mo period. The animals were then injected intravenously

with the same amount of NK cells and after 3 d the mice were killed and a spleen cell suspension was prepared and then fused with the BALB/c myeloma cell line PX63.Ag8.653. The fused cells were cloned by limiting dilution and the resultant hybridomas were labeled with ⁵¹Cr and screened for their ability to activate NK cell cytotoxicity in a standard 3-h chromium release assay. The C1.7 hybridoma producing the IgG1 C1.7 antibody was selected for further study on the basis of its ability to be efficiently lysed by the NK effector cells.

Cell Lines. The human Burkitt lymphoma-derived cell line Daudi, the EBV-transformed cell line RPMI-8866, the erythromyeloid leukemia cell line K562, the murine mastocytoma (Fc γ R⁺) cell line P815X2, and the murine antibody-secreting hybridomas were maintained in culture in RPMI-1640 medium (Flow Laboratories, Inc., McLean, VA) supplemented with 10% FBS (Flow Laboratories, Inc.). All cell lines were free of mycoplasma contamination on repeated testing.

Monoclonal Antibody. Antibodies used were OKT3 (IgG2a, anti-CD3), B36.1 (IgG2b, anti-CD5), 3G8 (IgG1, anti-CD16), B73.1 (IgG1, anti-CD16), B159.5 (IgG1, anti-CD56), B52.1 (IgM, anti-CD14), B66.6 (IgG1, anti-CD4), B116.1 (IgG2a, anti-CD8), OKT8 (IgG2a, anti-CD8), TCR- δ 1 (anti-TCR δ chain), TIA-2 (anti-CD3 ζ), B133.1 and B133.5 (both IgG1, anti-IFN- γ), Mc.a-NAP-1 (anti-IL-8), and Pc.a-NAP-1 (goat anti-human IL-8 antibody). Antibody 3G8-producing cells were kindly provided by Dr. J. Unkeless (Mount Sinai School of Medicine, New York, NY); OKT3, OKT4, and OKT8 cells were obtained from the American Type Culture Collection (Rockville, MD); antibody TCR- δ 1 was kindly provided by Dr. M. Brenner (Harvard University, Boston, MA), antibody TIA-2 was provided by Dr. P. Anderson (Dana Farber Cancer Institute, Boston, MA), and the anti-IL-8 antibodies were a generous gift from Dr. M. Ceska (Sandoz Inc., Vienna, Austria); all other antibodies were produced and characterized in our laboratory.

Cytokines and Cytokine Assays. Chinese hamster ovary cell-derived recombinant NK cell stimulatory factor/IL-12 (rNKSF/IL-12)¹ was kindly provided by Dr. S. Wolf (Genetics Institute, Boston, MA), and rIL-2 (10⁷ U/mg) was provided by Dr. T. Taguchi (Osaka University and Takeda Chemical Industry, Inc., Osaka, Japan). IFN- γ was measured by RIA as described (40) using mAbs B133.1 and B133.5. IL-8 was measured by ELISA using mAb Mc.a-NAP-1 and the alkaline phosphatase-conjugated goat polyclonal antibody Pc.a-NAP-1.

PBL Cultures. PBMC were obtained from peripheral blood by Ficoll-Hypaque density gradient centrifugation followed by a 1-h incubation in plastic flasks to partially deplete adherent monocytes. Cultured NK cells were prepared as described (41) with some modifications. Briefly, PBL were cultured in 24-well plates (Nunc, Roskilde, Denmark) at 2.5 × 10⁵ cells/ml in RPMI-1640 medium with 10% fetal bovine serum (FBS) at 37°C in a 9.5% CO₂ atmosphere together with the irradiated (50-Gy) RPMI-8866 B cell line (5 × 10⁴ cells/ml). On day 6 of culture, half of the medium was replaced with fresh RPMI-1640/10% FBS and all cultures were collected on days 8–10. NK cells (>98% CD16⁺/CD56⁺/CD3⁻) were purified from 8–10-d cultures by depletion of T cells and monocytes by antiglobulin rosetting with the mAbs OKT3, B36.1, and B52.1. Non-MHC-restricted cytotoxic CD8⁺ T cells were obtained from rIL-2 (200 U/ml)-cultured (5 d) PBL

¹ Abbreviations used in this paper: BLT, N-carbobenzoxyl-L-lysine-thiobenzyl ester; FBS, fetal bovine serum; G α MiG, goat anti-mouse Ig; IP, inositol phosphate; rNKSF/IL-12, recombinant natural killer cell stimulatory factor/IL-12; [³H]TdR, thymidine [methyl-³H].

that were depleted of NK cells and monocytes by antiglobulin rosetting using mAbs 3G8, B73.1, B159.5, and B52.1 at the start and end of cultures. At the end of culture, the NK-depleted PBL were subjected to two color immunofluorescence analyses and depleted of CD4⁺ cells or CD4⁺ and C1.7⁺ cells by antiglobulin rosetting with mAb B66.6 or with mAbs B66.6 and C1.7, respectively.

Two-color Immunofluorescence Assays. The surface phenotype of the indicated lymphocyte populations was determined by two-color immunofluorescence with mAbs using an EPICS Elite cytofluorograph (Coulter Corp., Hialeah, FL). mAbs were either biotinylated (biotin-*N*-hydroxysuccinimide ester; Calbiochem-Novabiochem Corp., La Jolla, CA) or directly conjugated to FITC (International Biological Supplies, Melbourne, FL) according to the manufacturer's instructions. Biotinylated reagents were detected with phycoerythrin Streptavidin (PE-Avidin; Coulter Corp.).

Generation of F(ab')₂ Fragments. mAbs C1.7 and 3G8 (1 mg/ml) purified by affinity chromatography on a protein G column (Pharmacia Fine Chemicals, Upsala, Sweden) were digested with pepsin (0.05%; Sigma Chemical Co., St. Louis, MO) for 18 h at 37°C in 0.1 M sodium acetate, pH 4.1. Pepsin digests were dialyzed against PBS and subjected to protein G column chromatography to remove undigested IgG. Commercially obtained goat anti-mouse Ig (GαMIg) F(ab')₂ fragments (Cappel Laboratories, Cochranville, PA) were also passed through a protein G column to remove possible contaminating IgG. All column effluents were confirmed to consist of pure F(ab')₂ fragments by SDS-PAGE followed by silver staining.

SDS-PAGE and Western Blotting. Purified, cultured NK cells were lysed with 1% NP-40, 5 mM PMSF, and 10 μg/ml leupeptin in PBS. The lysate equivalent of 2 × 10⁸ cells was loaded onto 5–20% continuous gradient polyacrylamide slab gels (1.0-mm thickness) and SDS-PAGE was performed as described (42) under non-reducing and reducing (2% β-mercaptoethanol) conditions. After electrophoresis, resolved proteins were transferred to nitrocellulose using a semi-dry transblotter (Bio-Rad Laboratories, Richmond, CA). The blots were cut into strips and probed with the indicated antibodies that were detected by ¹²⁵I-labeled, affinity-purified GαMIg. The strips were reassembled and subjected to autoradiography.

Cell-mediated Cytotoxicity Assays. Chromium release assays were performed in U-bottomed microtiter plates (Costar Corp., Cambridge, MA) using the indicated ⁵¹Cr-labeled target cells (10⁴ cells/well) with a 3-h incubation. All assays were performed in triplicate using four different effector cell concentrations. Results were quantitated by calculating the percentage of specific ⁵¹Cr release and in some assays the number of lytic units per 10⁷ cells at 45% specific lysis were calculated using the linear regression to a modified Van Krogh's equation (43). In antibody-redirected lysis assays, FcγR⁺ P815X2 cells were labeled with ⁵¹Cr and used as targets in the presence of medium alone or medium with 0.1 μg/ml of the indicated antibodies. In the hybridoma-redirected lysis assays, the PX63.Ag8.653 myeloma cell line or the indicated hybridomas were labeled with ⁵¹Cr and used as target cells. Non-MHC-restricted cytotoxicity assays were performed with either ⁵¹Cr-labeled Daudi or K562 target cells.

NK Cell Lymphokine Production. Purified, cultured NK cells were incubated with P815X2 cells at a ratio of 10:1 in U-bottomed microtiter plates for 18 h in the presence or absence of mAb C1.7 (0.1 μg/ml) and in medium alone or in medium supplemented with rIL-2 (100 U/ml) or rNKSF/IL-12 (1 ng/ml). After 18 h cell-free supernatants were collected and the concentrations of IFN-γ and IL-8 were determined by RIA and ELISA, respectively.

***N*-Carbobenzoxy-L-Thiobenzyl Ester-Esterase Assay.** *N*-Carbo-

benzoxy-L-thiobenzyl ester (BLT) esterase activity in cell-free supernatants was determined in a microtiter assay. Purified, cultured NK cells were incubated for 3 h with P815X2 cells at a ratio of 10:1 in the presence of medium alone or medium with 0.1 μg/ml of the indicated antibodies. NK cell effectors from the same donors were also assayed in parallel for antibody-redirected cytotoxicity of ⁵¹Cr-labeled P815X2 cells as described. After incubation, 50 μl of cell-free supernatant fluid was added to 100 μl of 0.1 mg/ml dithiobis-2-nitrobenzoic acid and 0.1 mg/ml *N*α-carbobenzoxy-L-Lys-thiobenzyl ester (Sigma Chemical Co.) in 0.1 M Tris and 1 mM MgCl₂, pH 7.5. BLT-esterase activity was determined by increased absorbance at 405 nm. Total BLT-esterase activity was determined from lysed (freeze/thaw three times), untreated NK cells. The percentage of specific BLT-esterase release was calculated as experimental BLT-esterase activity – spontaneous (untreated) BLT-esterase activity/total BLT-esterase activity × 100.

Lymphocyte Proliferation Assays. Fresh PBL or purified, cultured (7 d) NK cells were cultured in flat-bottomed Linbro/Titertek plates (Flow Laboratories, Inc.) at 10⁵ cells/well for 6 or 3 d, respectively. PBL were cultured in the presence of rNKSF/IL-12 (0.1 ng/ml) alone or with 10, 100, or 1,000 U/ml of rIL-2. Cells were either untreated or treated with the indicated soluble antibodies (1 μg/ml). NK cells were cultured in medium alone or medium supplemented with 10, 100, or 1,000 U/ml of rIL-2 in wells that were treated with 0.1 M bicarbonate buffer, pH 9.5, alone or bicarbonate buffer with 5 μg/ml of the indicated antibodies. Before cells were added, the bicarbonate buffer was removed and the wells were washed twice with PBS to remove unadsorbed antibodies. Thymidine [methyl-³H] ([³H]TdR; New England Nuclear, Boston, MA) was added (1 μCi/well) during the last 6 h of culture. Cells from triplicate cultures were harvested on glass fiber filters and [³H]TdR incorporation was determined by liquid scintillation graphy.

Phosphoinositide Turnover. Determination of intracellular accumulation of inositol phosphates (IP) was performed as described (44). Briefly, purified NK cells (5–10 × 10⁶/ml) were incubated in inositol-free RPMI-1640 medium (Flow Laboratories, Inc.) with 5% FBS for 18 h at 37°C in the presence of myo-[³H]inositol (10 μCi/ml; Amersham Corp., Arlington Heights, IL). After incubation, cells were resuspended in HBSS (1 mM CaCl₂, 5.6 mM glucose, 20 mM Hepes, and 10 mM LiCl, pH 7.4) (GIBCO BRL, Gaithersburg, MD). C1.7 F(ab')₂ fragments (1 μg/ml) and GαMIg F(ab')₂ (20 μg/ml) fragments were added at the indicated times to duplicate samples of 10 × 10⁶ cells. The different IPs (IP₁ – IP₄) were measured as cell-incorporated counts per minute after anion exchange chromatography of each sample on a 1-ml Ag1-X8 column (Bio-Rad Laboratories). Columns were washed with a four-step discontinuous gradient of 0.1 M formic acid containing 0.2, 0.5, 0.8, and 1.0 M ammonium formate to elute IP₁, IP₂, IP₃, and IP₄, respectively. Radioactivity of each fraction was measured by liquid scintillation counting in a β counter.

[Ca²⁺]_i Measurement. [Ca²⁺]_i increases in NK cells were determined as previously described (44). Briefly, NK cells were labeled with the fluorescent Ca²⁺ indicator fura-2/AM (2 μM) (Calbiochem-Novabiochem Corp.). After fura-2 loading, cells were washed and resuspended in HBSS and fluorescence measurements were performed in a spectrofluorometer (Perkin Elmer Corp., Norwalk, CT) equipped with a thermostatic cuvette holder maintained at 37°C with continuous stirring. Each sample contained 5 × 10⁶ cells in 1.7 ml of HBSS. The C1.7 F(ab')₂ reagent (0.5 μg/ml final) and the GαMIg F(ab')₂ reagent (10 μg/ml final) were added at the indicated times and changes in fluorescence were recorded as a function of time. [Ca²⁺]_i was calculated as previously described (44).

Results

mAb C1.7 Reactivity with Leukocyte Subsets. Single-color indirect immunofluorescence analyses (flow cytometry) of human peripheral blood leukocytes (not shown) indicated that $24.4 \pm 2.9\%$ (mean \pm SE [$n = 12$]) of lymphocytes were reactive with mAb C1.7. Monocytes stained with much lower fluorescence intensity than lymphocytes with mAb C1.7, whereas granulocytes were negative for mAb C1.7 reactivity. Two-color immunofluorescence analyses were performed on PBL preparations from 10 donors. In all experiments mAb C1.7 reacted with virtually all CD16⁺ (Fig. 1 B) and CD56⁺ (not shown) NK cells and with $51.6 \pm 5.2\%$ (range: 29.9–73.2%; $n = 10$) of CD3⁺/CD8⁺ T cells (Fig. 1, C and E). mAb C1.7 reactivity was never observed on CD4⁺ T cells (Fig. 1 D). To analyze mAb C1.7 reactivity with fresh peripheral blood γ/δ^+ T cells, which in most donors comprise a very low percentage of lymphocytes, PBL from five donors were depleted of CD16⁺, CD56⁺, and CD4⁺ lymphocytes by antiglobulin rosetting and analyzed by two-color immunofluorescence. In these preparations TCR- γ/δ^+ cells represented $21.2 \pm 9.1\%$ ($n = 5$) of the total lymphocytes, and $62 \pm 6\%$ (range: 45.1–81.7%) of these T cells were mAb C1.7⁺ (Fig. 1, E and F). The intensity of mAb C1.7 staining and its reactivity with different lymphocyte subsets were unaffected by activation or culture conditions. Activated NK cells from 8-d culture with certain EBV-

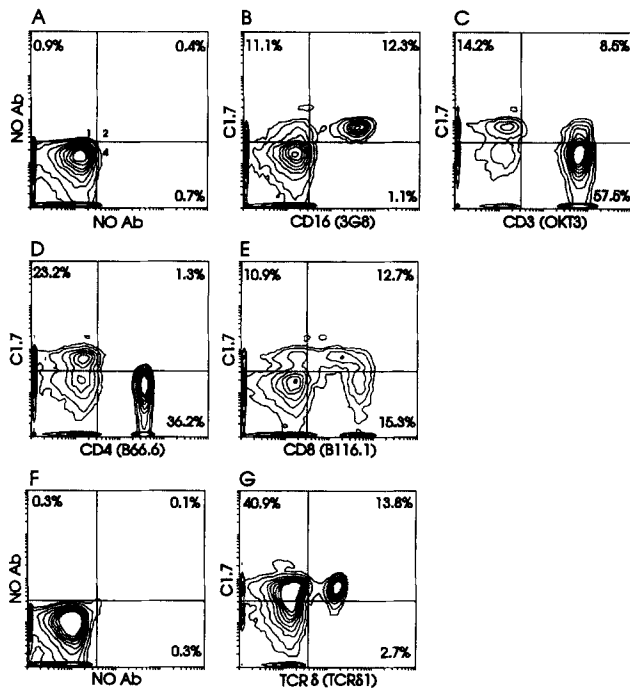


Figure 1. Two-color immunofluorescence (flow cytometry) analyses. PBL from a representative donor were stained with no antibody (A) or with biotinylated mAb C1.7 (detected with PE-avidin) and simultaneously with FITC-conjugated anti-CD16 (3G8) (B); anti-CD3 (OKT3) (C); anti-CD4 (B66.6) (D); or anti-CD8 (B116.1) (E) mAbs. The lymphocyte populations from a different donor shown in F and G were depleted of CD16⁺, CD56⁺, CD4⁺ cells before analysis and stained with no antibody (F) or with mAb C1.7 and anti-TCR- δ (TCR- δ 1) (G).

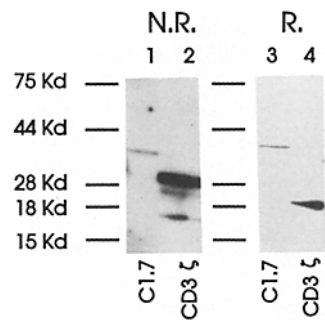


Figure 2. Western blots of NK cell lysates resolved under non-reducing (lanes 1 and 2) or reducing (lanes 3 and 4) conditions were probed with the following mAbs: C1.7 (lanes 1 and 3) and TIA-2 (anti-CD3 ζ) (lanes 2 and 4).

transformed B cell lines, activated γ/δ^+ T cells from similar cultures, or IL-2-cultured PBL all remained virtually unchanged in the level and distribution of mAb C1.7 staining (not shown).

Detection of p38 in NK Cell Lysates by Western Blotting. 1% NP-40 lysates from purified, cultured NK cells were resolved by SDS-PAGE, transferred to nitrocellulose, and screened with various mAbs specific for NK cell proteins. Fig. 2 illustrates a representative blot from one donor. As shown in Fig. 2, mAb C1.7 (lanes 1 and 3) detected a single immunoreactive species of 38 kD (p38) under both nonreducing (lane 1) and reducing (lane 3) conditions. As shown, the anti-CD3 ζ (TIA-2) control (lanes 2 and 4) detected a 34-kD protein under nonreducing conditions (lane 2) which reduced to a single 17-kD immunoreactive species (lane 4).

Effect of mAb C1.7 on NK Cell Cytotoxic Activity. As depicted in Fig. 3, mAb C1.7 was able to activate NK cell cytotoxicity in different assay systems. Soluble mAb C1.7 was capable of substantially increasing both cultured (Fig. 3 A) and fresh (Fig. 3 B) NK cell-mediated antibody-redirection lysis against the Fc γ R⁺ P815X2 target cells. In all donors the levels of specific ⁵¹Cr release observed with mAb C1.7 were comparable to those observed with anti-CD16 (3G8) treatment and, as shown, markedly greater than those ob-

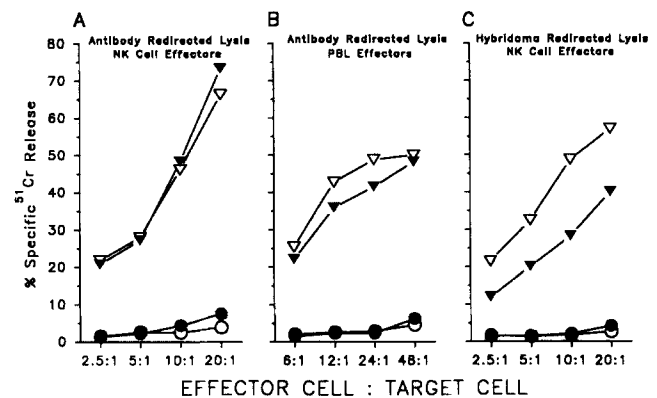


Figure 3. mAb C1.7-induced, NK cell-mediated cytotoxicity. Purified, cultured NK cells (A) or PBL (B) were used as effectors in standard 3-h ⁵¹Cr release assays against ⁵¹Cr-labeled Fc γ R⁺ P815X2 target cells at the indicated ratios. Assays were performed in the presence of medium alone (O) or medium with 0.1 μ g/ml of mAb B159.5 (anti-CD56) (●), mAb 3G8 (anti-CD16) (▽), or mAb C1.7 (▼). Purified, cultured NK cells (C) were used as effectors against the ⁵¹Cr-labeled myeloma fusion partner, PX63.Ag8.653 (O), or the hybridomas 3G8 (▽), B159.5 (●), or C1.7 (▲).

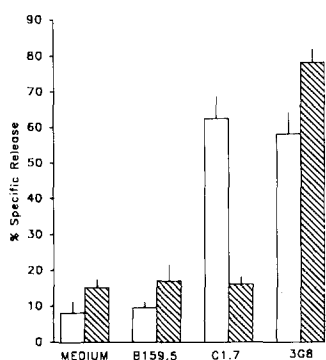


Figure 4. mAb C1.7-induced cytotoxicity and BLT-esterase release. Purified, cultured NK cells were incubated for 3 h with FcγR⁺ P815X2 target cells at a ratio of 10:1 in parallel assays for ⁵¹Cr release (open bars) and BLT-esterase release (striped bars) in the presence of medium alone or medium supplemented with 0.1 μg/ml of mAb B159.5, mAb C1.7, or mAb 3G8. Results are presented as the mean percentage of specific release ± SE (n = 4).

served with anti-CD56 (B159.5) treatment or medium alone. Fig. 3 C illustrates that the C1.7 hybridoma was sensitive to NK cell cytotoxicity, although in most experiments to a lesser extent than the anti-CD16 hybridoma (3G8). In these experiments, neither the anti-CD56 hybridoma (B159.5) nor the PX63.Ag8.653 myeloma fusion partner were significantly lysed by the NK cells. The decreased efficiency of the C1.7 hybridoma in inducing cytotoxicity compared with the 3G8 hybridoma could be due to the fact that the surface Ig expression of the C1.7 hybridoma was much lower than that observed for the 3G8 hybridoma (not shown).

Effect of mAb C1.7 Stimulation on BLT-Esterase Release from NK Cells. The ability of mAb C1.7 to induce the release of the granule enzyme BLT-esterase from cultured NK cells was determined using a variety of stimulation protocols. mAb C1.7 was: (a) immobilized on a plastic support, (b) bound to Sepharose 4B, (c) absorbed to plastic-bound GαMIg, or (d) presented to NK cells by the FcγR⁺ target cell P815X2. Under no conditions were detectable levels of BLT-esterase release observed above controls after mAb C1.7 stimulation. By contrast, anti-CD16 stimulation resulted in substantial increases in BLT-esterase levels with all four stimulation methods. Fig. 4 summarizes the results of four experiments in which cultured NK cells were incubated with P815X2 cells (E/T 10:1) in the presence of medium alone or the indicated

antibodies and assayed in parallel for antibody-redirected lysis (open bars) and BLT-esterase release (striped bars). Both mAb C1.7 and mAb 3G8 induced cytotoxic activity in the NK cells when compared with anti-CD56 (B159.5) and medium controls. However, only mAb 3G8 was capable of stimulating BLT-esterase release with no increase observed in C1.7-treated cultures.

Stimulation of NK Cell Lymphokine Production by mAb C1.7. Using the same stimulation protocols used to induce BLT-esterase release, the effect of mAb C1.7 on NK cell lymphokine production was examined. Specifically, the ability of mAb C1.7 to induce the production of IFN-γ, IL-8, and TNF-α was determined by RIA and ELISA assays of 18-h-stimulated cell-free supernatant fluids. The C1.7 antibody was capable of inducing significant increases in IFN-γ and IL-8 production from cultured NK cells only when soluble mAb C1.7 (0.1 μg/ml) was presented to the NK cells by FcγR⁺ P815X2 cells. As shown in Table 1, increases in IFN-γ production were observed in the presence of rIL-2 (100 U/ml) or rNKSF/IL-12 (1 ng/ml), whereas increases in IL-8 production occurred with mAb C1.7 alone or in the presence of rIL-2 or NKSF/IL-12. Under all stimulation conditions mAb C1.7 treatment had no effect on TNF-α production (not shown). Unlike mAb C1.7, anti-CD16 (3G8) treatment induced comparable lymphokine (IFN-γ and IL-8) production under all four stimulation protocols and anti-CD56 (B159.5) treatment had no effect on IFN-γ and IL-8 production under any of the conditions tested (not shown).

Effect of mAb C1.7 Treatment on Lymphocyte Proliferation. We analyzed the ability of both soluble and plastic-bound mAb C1.7 to affect [³H]TdR uptake of fresh PBL (Fig. 5 A) or purified, cultured (7 d) NK cells (Fig. 5 B) alone or in the presence of increasing doses of rNKSF/IL-12 or rIL-2. Fig. 7 A demonstrates that soluble C1.7 induced a two- to four-fold enhancement of PBL [³H]TdR incorporation in PBL cultured for 6 d in the presence of 0.1 ng/ml of rNKSF/IL-12 and increasing doses (10–1,000 U/ml) of rIL-2. This enhancement was greater than that observed with soluble anti-CD3 (OKT3) treatment, but OKT3 treatment resulted in

Table 1. mAb C1.7-induced Lymphokine Production

Treatment*	mAb C1.7	IFN-γ, mean U/ml ± SE (n = 10)	IL-8, mean pg/ml ± SE (n = 6)
Medium	–	5.2 ± 2.1	150.5 ± 50.8
Medium	+	5.5 ± 1.1	236.4 ± 81.3 [†]
rIL-2	–	61.3 ± 8.2	251.2 ± 75.2
rIL-2	+	208.7 ± 25.5 [‡]	550.9 ± 135.0 [‡]
rNKSF/IL-12	–	60.6 ± 12.2	243.4 ± 100.5
rNKSF/IL-12	+	247.8 ± 30.9 [‡]	496.9 ± 161.7 [‡]

* Purified, cultured NK cells were incubated for 18 h with P815X2 cells (E/T, 10:1) in the presence or absence of mAb C1.7 (0.1 μg/ml). Stimulation was performed in medium alone or medium supplemented with rIL-2 (100 U/ml) or rNKSF/IL-12 (1 ng/ml). IFN-γ and IL-8 concentrations were determined in the cell-free supernatant fluids by RIA or ELISA, respectively, as described. Significance was determined by Student's *t* test for dependent samples.

[†] *P* < 0.05.

[‡] *P* < 0.005.

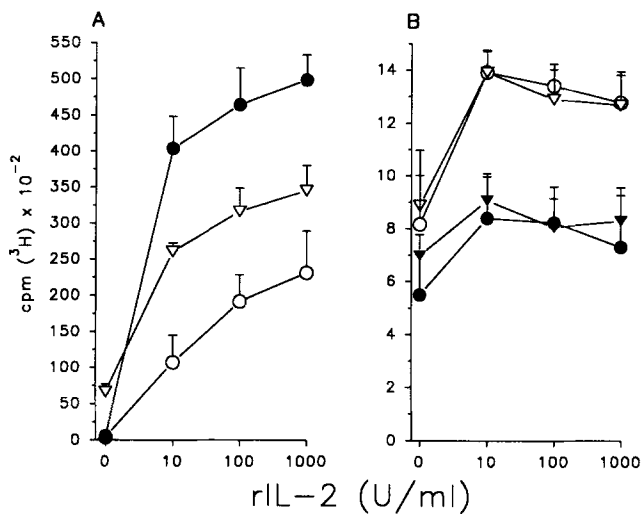


Figure 5. Effect of mAb C1.7 stimulation on lymphocyte proliferation. Fresh PBL (A) or purified, cultured (7 d) NK cells (B) were cultured for 6 or 3 d, respectively, as described. PBL (A) were cultured in the presence of 0.1 ng/ml of rNKSF/IL-12 and the indicated doses of rIL-2 in medium alone (O) or medium with 1 μ g/ml of soluble mAb C1.7 (●) or anti-CD3 antibody (OKT3) (▽). NK cells (B) were cultured with the indicated doses of rIL-2 in the presence of no antibody (O) or the following antibodies (5 μ g/ml) bound to plastic: mAb C1.7 (●), mAb 3G8 (▼), or mAb B159.5 (▽). In all experiments, NK cells incorporated far lower amounts of [3 H]TdR than PBL. Results are presented as mean + SE ($n = 4$).

enhanced [3 H]TdR uptake in the absence of either cytokine. Treatment of PBL with plastic-bound mAb C1.7 consistently resulted in moderately decreased [3 H]TdR uptake by the cells (not shown).

When purified, cultured (7 d) NK cells were treated in a similar fashion for 3 d, the effect of mAb C1.7 (soluble or plastic bound) was antagonistic to the effects of increasing doses of rIL-2 (Fig. 5 B) or rNKSF/IL-12 (not shown). In all experiments, anti-CD56 (B159.5) treatment had no effect on proliferation when compared with medium controls, and anti-CD16 (3G8) treatment, similar to what was observed in cultures treated with mAb C1.7, was antiproliferative (Fig. 5 B).

Signal Transduction through p38 on NK Cells. We measured the ability of mAb C1.7 stimulation to initiate polyphosphoinositol turnover and increases in [Ca^{2+}]_i in purified, cultured NK cells. Fig. 6 A shows that within 15–30 s after crosslinking with G α MiG F(ab')₂ (20 μ g/ml) the C1.7 F(ab')₂ (1.0 μ g/ml) induced substantial increases in the second messenger, inositol triphosphate (IP₃), followed by induction of inositol bisphosphate (IP₂) and inositol tetrakisphosphate (IP₄). Similar to the induction of polyphosphoinositol turnover, the F(ab')₂ of mAb C1.7 (0.5 μ g/ml) induced increases in [Ca^{2+}]_i after crosslinking with the G α MiG F(ab')₂ (10 μ g/ml) reagent (Fig. 6 B). In all donors anti-CD16 (3G8) stimulation resulted in low level generation of IPs and [Ca^{2+}]_i increases when the antibody was used alone and these levels were greatly increased upon crosslinking, consistent with our previously published results (44). Treatment of NK cells with the anti-CD56 antibody,

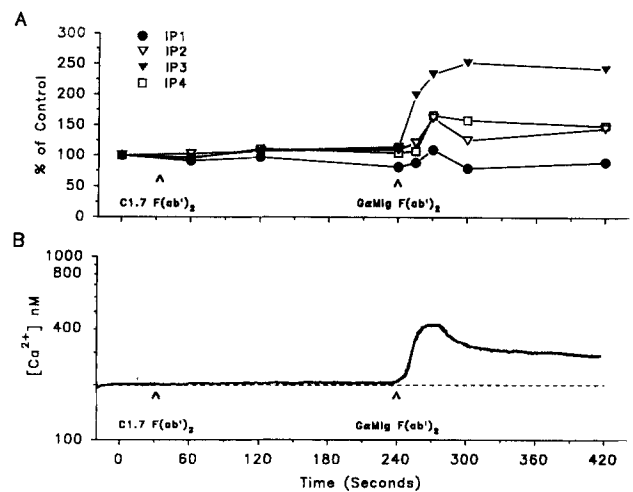


Figure 6. IP formation (A) and [Ca^{2+}]_i increase (B) in NK cells stimulated with mAb C1.7 F(ab')₂. (A) Purified NK cells were loaded with myo-[3 H]inositol as described in Materials and Methods. IP₁ (●), IP₂ (▽), IP₃ (▼), and IP₄ (□) were extracted from the cells (10×10^6 /duplicate samples) at the indicated times after addition of mAb C1.7 F(ab')₂ (1 μ g/ml) with or without F(ab')₂ G α MiG (20 μ g/ml). IP₁, IP₂, IP₃, and IP₄ basal cpm values were 405, 119, 79, and 55, respectively. (B) Purified NK cells were loaded with the fluorescent Ca^{2+} indicator fura2-AM and [Ca^{2+}]_i was determined as described. mAb C1.7 F(ab')₂ (0.5 μ g/ml) and G α MiG F(ab')₂ (10 μ g/ml) were added at the indicated times.

B159.5, had no effect on either of these signal transduction events with or without the addition of G α MiG (not shown).

Ability of p38⁺CD8⁺ T Cells to Mediate Non-MHC-restricted Cytotoxicity. Upon culture (3–6 d) with lymphokines such as IL-2 or NKSF/IL-12, cytotoxic T cells acquire the ability to lyse target cells in a non-MHC-restricted manner. Because p38 is expressed by ~50% of fresh CD8⁺ T cells, we examined whether p38⁺ and p38⁻ CD8⁺ T cells, obtained from PBL cultured for 5 d in the presence of rIL-2, differed in their ability to mediate non-MHC-restricted cytotoxicity. PBL were isolated from healthy donors, depleted of NK cells and monocytes by antiglobulin rosetting with mAb anti-CD16 (B73.1, 3G8), anti-CD56 (B159.5), and anti-CD14 (B52.1), and cultured for 5 d with 200 U/ml rIL-2. After culture, all of the NK-depleted (NK⁻) PBL were subjected to antiglobulin rosetting with anti-CD4 (B66.6) and the anti-CD16, anti-CD56, and anti-CD14 reagents, with half of the cells also being rosetted with mAb C1.7.

Fig. 7 A shows the results of two-color immunofluorescence analyses of the 5-d rIL-2 cultured NK-PBL obtained from a representative donor. Notably, there were no detectable CD16⁺ cells (Fig. 7 A, 1) in the cultured PBL and the percentage of C1.7⁺/CD8⁺ T cells (Fig. 7 A, 4) was consistent with that observed in fresh PBL. The small population (~4%) of p38⁺/CD3⁻ cells (Fig. 7 A, 2), present in this donor and some others, did not appear to affect the level of non-MHC-restricted cytotoxicity, because sorted (FACS[®]) CD3⁺ cells and total unsorted populations exhibited the same degree of non-MHC-restricted cytotoxic activity (not shown). Fig. 7 B summarizes the results from ^{51}Cr release

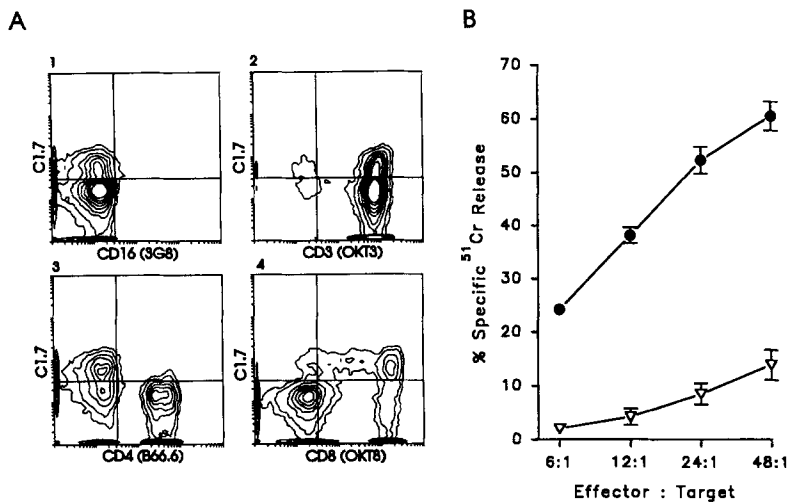


Figure 7. Spontaneous cytotoxic activity of p38⁺ and p38⁻ CD8⁺ T cells. A two-color immunofluorescence analysis was performed with the indicated antibodies on rIL-2-cultured (5 d), NK-depleted lymphocytes from a representative donor. (B) Cultured lymphocytes were depleted of CD4⁺ cells (●) or CD4⁺ and p38⁺ cells (▽) as described in Materials and Methods and used as effectors in 3-h chromium release assays against ⁵¹Cr-labeled Daudi targets at the indicated ratios. Results are presented as the mean percentage of specific ⁵¹Cr release ± SE (n = 4).

assays using CD8⁺ T cells from four separate donors as effector cells against Daudi target cells, and demonstrates that the overwhelming majority of the non-MHC-restricted cytotoxic activity of the rIL-2-cultured CD8⁺ T cells is contained within the p38⁺ subset. Experiments using K562, THP-1, Jurkatt, U937, and P815X2 target cells yielded similar results (not shown). The different non-MHC-restricted cytotoxic activity between p38⁺ and p38⁻ CD8⁺ rIL-2-cultured T cells was not restricted to tumor-derived targets, since virally infected (varicella zoster virus, HSV, and CMV) FS4 target cells were also lysed with much greater efficiency by p38⁺ than by p38⁻ effectors (Chehimi, J., N. M. Valiante, and G. Trinchieri, unpublished observations).

Effect of F(ab)₂ Fragments of mAb C1.7 on Non-MHC-restricted Cytotoxicity. To assess the ability of F(ab)₂ fragments of mAb C1.7 to alter non-MHC-restricted cytotoxicity, various populations of lymphocytes were used as effectors against ⁵¹Cr-labeled Daudi and K562 target cells in 3-h chro-

mium release assays. As shown in Fig. 8, 10 and 50 μg/ml of F(ab)₂ fragments of mAb C1.7 reduced non-MHC-restricted cytotoxicity of fresh PBL against K562 (Fig. 8 A) target cells and of NK-depleted rIL-2 (5 d) cultured PBL against K562 (Fig. 8 B) and Daudi target cells (Fig. 8 C). This inhibition appears specific because 50 μg/ml of anti-CD16 (3G8) F(ab)₂ had no effect on the lytic ability of the lymphocytes. In five out of five donors, C1.7 F(ab)₂ had no effect on the cytotoxic activity of purified, cultured NK cells against Daudi and K562 target cells (not shown).

Discussion

Using a newly generated mAb (C1.7), we have identified a novel 38-kD signal-transducing surface molecule (p38) expressed by all NK cells and subpopulations of CD8⁺ (α/β) T cells and γ/δ⁺ T cells, lymphocyte subsets capable of cell-mediated cytotoxicity. p38 expression identifies the subset of cytotoxic lymphocytes (NK cells) in fresh PBL capable of non-MHC-restricted cytotoxicity and a functionally similar subset in rIL-2-cultured CD8⁺ T cells. The shared expression of p38 by NK cells and non-MHC-restricted cytotoxic T cells suggests that p38 may be involved in a common cytotoxic mechanism utilized by these distinct effector cell populations.

A small number of surface molecules have been previously described that are able to trigger the cytotoxic mechanism of NK cells as measured by the ability of NK effectors to lyse hybridomas producing antibodies against these molecules or FcγR⁺ target cells presenting a soluble IgG mAb. The C1.7 antibody producing hybridoma was selected from a panel of hybridomas obtained by fusion of spleen cells of human NK-immunized mice on the basis of its ability to be efficiently lysed by NK cells. In addition, the soluble IgG1 antibody C1.7 also induced NK cell-mediated, antibody-redirected lysis of FcγR⁺ target cells, indicating that the p38 surface molecule recognized by mAb C1.7 appears to be an activating structure on NK cells. The best-described molecule with these characteristics on human NK cells is FcγRIIIA or CD16.

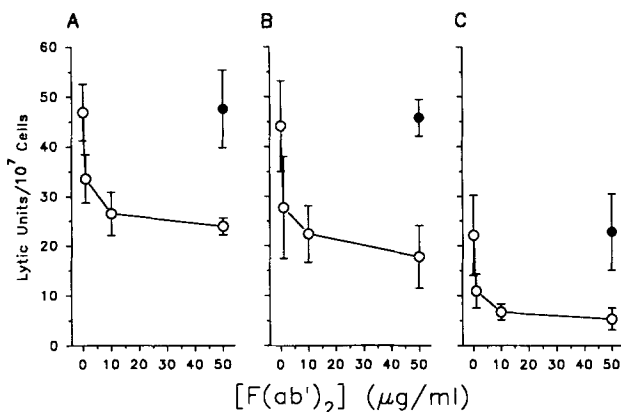


Figure 8. Inhibition of spontaneous cytotoxicity by mAb C1.7 F(ab)₂. PBL (A) or rIL-2-cultured (5 d), NK-depleted lymphocytes (B and C) were used as effectors in 3-h chromium release assays against ⁵¹Cr-labeled K562 (A and B) or Daudi (C) target cells. Assays were performed in the presence of mAb C1.7 F(ab)₂ (O) or mAb 3G8 F(ab)₂ (●) at the indicated concentrations. Results are presented as mean lytic units/10⁷ cells ± SE (n = 3).

Stimulation of NK cells with anti-CD16 mAb or CD16 ligands (immune complexes) results in activation of cell-mediated cytotoxicity as well as release of BLT-esterase and lymphokines. p38-mediated activation of NK cell cytotoxicity differs from CD16 activation in that p38 stimulation under conditions that induced NK cell cytotoxic activity equal to or greater than that observed for CD16 stimulation did not result in the release of the granule enzyme BLT-esterase. Because BLT-esterase release is associated with NK cell degranulation, the lack of BLT-esterase release from NK cells mediating mAb C1.7-redredirected lysis suggests that the cytotoxic mechanism induced by p38 stimulation may not involve extensive granule release. It has recently been demonstrated that non-MHC-restricted cytotoxicity mediated by resting and rIL-2-activated NK cells occurs through distinct mechanisms, in that rIL-2-activated, NK cell-mediated cytotoxicity appears independent of BLT-esterase release (45). Our results demonstrating that mAb C1.7-redredirected lysis does not involve BLT-esterase release may indicate that mAb C1.7-induced cytotoxicity occurs through a similar mechanism. Although mAb C1.7 stimulation did not induce BLT-esterase release, we have evidence that both mAb C1.7- and anti-CD16 (3G8)-induced redredirected lysis results in apoptotic events in P815X2 target cells as measured by decreased propidium iodide staining and fragmentation of target cell DNA (Weinhouse, G., N. M. Valiante, and G. Trinchieri, unpublished observations).

Investigation of the signal transduction pathways induced by p38 stimulation did not demonstrate any obvious difference between p38- and CD16-mediated signal transduction that would account for p38's inability to initiate BLT-esterase release. Both p38 and CD16 stimulation resulted in the rapid hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) and increases in [Ca²⁺]_i, as well as activation of phospholipase D (Valiante, N. M., M. Rengaraju, and G. Trinchieri, unpublished observations). Unlike CD16-mediated signal transduction, which is directly activated at low levels by soluble anti-CD16 antibodies and is substantially enhanced after cross-linking, p38-mediated signal transduction was observed only after crosslinking of F(ab')₂ fragments of mAb C1.7 with F(ab')₂ fragments of a G α MIg second antibody. We were unable to show signal-transducing activity of p38 on fresh and short-term cultured (rIL-2 or rNKSF/IL-12) T cells. However, leukemic T cell lines (TALL 103/2 [TCR- γ / δ ⁺] and TALL 104 [TCR- α / β ⁺]) that express p38 and exhibit non-MHC-restricted cytotoxic activity were shown to mediate mAb C1.7-redredirected lysis (Cesano, A., and N. M. Valiante, unpublished observations).

NK cells are potent producers of lymphokines, and this activity can be induced by many stimuli that also activate their lytic mechanisms, such as perturbation of Fc γ RIII (CD16) (44, 46, 47). Stimulation with the C1.7 antibody resulted in production of IFN- γ and IL-8 by NK cells which, similar to CD16-induced lymphokine production, was maximal in the presence of other lymphokine-inducing stimuli such as rNKSF/IL-12 or rIL-2. Unlike anti-CD16-induced cytokine production, which is stimulated by antibodies presented on any solid support, p38-stimulated IFN- γ and

IL-8 production only occurred when mAb C1.7 was presented to the NK cells by an Fc γ R⁺ cell line. It is possible that mAb C1.7-induced lymphokine production requires accessory molecular interactions between other receptor-ligand pairs on the NK cells and presenting target cells which were not available when mAb C1.7 was presented on inert supports such as plastic surfaces or Sepharose beads. In addition, the fluidity of the presenting cell membrane in comparison to the rigidity of a solid support might also contribute to more efficient stimulation.

Treatment of fresh PBL with soluble mAb C1.7 resulted in enhanced thymidine uptake in the presence of rNKSF/IL-12 and rIL-2. However, we did not observe any consistent expansion of the number of C1.7⁺ cells in the cultures when compared with untreated controls. On cultured NK cells, mAb C1.7 treatment was antagonistic to rNKSF/IL-12- and rIL-2-induced proliferation, similar to that observed for anti-CD16 (mAb 3G8) treatment. The opposing effects of p38 stimulation on these lymphocyte preparations may indicate that p38 modulates proliferation differently depending on the cell type or level of activation.

Cultured (rIL-2; 5 d) CD16/CD56- and CD4-depleted CD8⁺ T cells displayed a high level of non-MHC-restricted cytotoxic activity. Our results, demonstrating that depletion of the p38⁺ population from these cultured CD8⁺ T cells almost completely abolishes this activity, suggest that p38 may be a marker for non-MHC-restricted cytotoxic T cells. Given that p38 is present on both fresh and activated CD8⁺ T cells, it is likely that other activation events are required for the generation of non-MHC-restricted cytotoxic T cells. However, taken as a whole, our results suggest that p38 may not only be a marker for non-MHC-restricted cytotoxic T cells, but also may be required for this activity.

The observation that F(ab')₂ fragments of mAb C1.7 substantially inhibited non-MHC-restricted cytotoxicity mediated by fresh PBL and rIL-2-activated T cells suggests that p38 may be involved in target cell recognition by these non-MHC-restricted cytotoxic cells. F(ab')₂ fragments of mAb C1.7 did not inhibit spontaneous cytotoxicity mediated by purified, cultured NK cells, suggesting that once activated, NK cells may utilize multiple or alternative mechanisms for cytotoxicity. The effects of F(ab')₂ fragments of antibodies directed against cytotoxic lymphocyte surface molecules can be either enhancing or inhibitory to cytotoxicity, depending on the type of surface molecule recognized by the antibody. It has been postulated that F(ab')₂ fragments of antibodies directed against activating structures or adhesion molecules inhibit cytotoxicity, whereas F(ab')₂ fragments of antibodies directed against inhibitory receptors enhance cytotoxicity, perhaps by blocking the delivery of a negative signal (48).

The p38 surface molecule identified by mAb C1.7 appears to be an activating structure on cytotoxic lymphocytes similar in this ability to other previously identified surface molecules, but distinct from these other molecules based on its unique leukocyte distribution and structure. The potential ligands for p38 have not been investigated. However, if p38 is involved in cell-mediated cytotoxicity, as is suggested by our

results, then it seems likely that these ligands would be expressed by a number of NK-sensitive targets. Although a few surface molecules have recently been identified that may have a role in spontaneous cytotoxicity, the mechanism by which NK cells and other non-MHC-restricted cytotoxic cells recognize target cells still remains poorly defined, and it is likely that multiple mechanisms may exist. The restricted expres-

sion of p38 to cells with non-MHC-restricted cytotoxic activity, its ability to activate these cells cytolytically, and the fact that F(ab')₂ fragments of the anti-p38 antibody C1.7 inhibit the spontaneous cytotoxic activity of some effector populations suggest that the p38 surface molecule may be directly involved in the mechanisms of non-MHC-restricted cytotoxicity.

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Address correspondence to Dr. Giorgio Trinchieri, The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104.

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References

1. Zinkernagel, R.M., and P. Doherty. 1974. Immunological surveillance against altered self components by sensitized T lymphocytes in lymphochoriomeningitis. *Nature (Lond.)* 251:547.
2. Lanier, L.L., J. Phillips, J. Hackett, M. Tutt, and V. Kumar. 1986. Natural killer cells: definition of a cell type than a function. *J. Immunol.* 137:2735.
3. Trinchieri, G. 1989. Biology of natural killer cells. *Adv. Immunol.* 47:187.
4. Perussia, B., G. Trinchieri, A. Jackson, N.L. Warner, J. Faust, H. Rumpold, D. Kraft, and L.L. Lanier. 1984. The Fc receptor for IgG on human natural killer cells: phenotypic, functional and comparative studies using monoclonal antibodies. *J. Immunol.* 133:180.
5. Siliciano, R.F., J.C. Pratt, R.E. Schmidt, J. Ritz, and E.L. Reinherz. 1985. Activation of cytolytic T lymphocyte and natural killer cell functions through the T11 sheep erythrocyte binding protein. *Nature (Lond.)* 317:428.
6. Anasetti, C., P.J. Martin, C.H. June, K.E. Hellstrom, J.A. Ledbetter, P.S. Rabinovitch, Y. Morishita, I. Hellstrom, and J.A. Hansen. 1987. Induction of calcium flux and enhancement of cytolytic activity in natural killer cells by cross-linking of the sheep erythrocyte binding protein (CD2) and the Fc-receptor (CD16). *J. Immunol.* 139:1772.
7. Hahn, W.C., E. Menu, A.L.M. Bothwell, P.J. Sims, and B.E. Bierer. 1992. Overlapping but nonidentical binding sites on CD2 for CD58 and a second ligand CD59. *Science (Wash. DC)* 256:1805.
8. Lanier, L.L., and J.H. Phillips. 1993. A map of the cell surface antigens expressed on resting and activated human natural killer cells. In *Leukocyte Typing II*. Vol. 3. E.L. Reinherz, editor. Springer Verlag New York Inc., New York. 157.
9. Lanier, L.L., J.J. Ruitenberg, and J.H. Phillips. 1988. Functional and biochemical analysis of CD16 antigen on natural killer cells and granulocytes. *J. Immunol.* 141:3478.
10. Chambers, W.H., N.L. Vujanovic, A.B. de Leo, M.W. Olszowy, R.B. Herberman, and J.C. Hiserodt. 1989. Monoclonal antibody to a triggering structure expressed on rat natural killer cells and adherent lymphokine-activated killer cells. *J. Exp. Med.* 169:1373.
11. Giorda, R., W.A. Rudert, C. Vavassori, W.H. Chambers, J.C. Hiserodt, and M. Trucco. 1990. NKR-P1, a signal transduction molecule on natural killer cells. *Science (Wash. DC)* 249:1298.
12. Karlhofer, F.M., and W.M. Yokoyama. 1991. Stimulation of murine natural killer (NK) cells by a monoclonal antibody specific for the NK1.1 antigen: IL-2-activated NK cells possess additional specific stimulation pathways. *J. Immunol.* 146:3662.
13. Ryan, J.C., J. Turck, E.C. Niemi, W.M. Yokoyama, and W.E. Seaman. 1992. Molecular cloning of the NK1.1 antigen, a member of the NKR-P1 family of natural killer cell activation molecules. *J. Immunol.* 149:1631.
14. Houchins, J.P., T. Yabe, C. McSherry, and F.H. Bach. 1991. DNA sequence analysis of NKG2, a family of related cDNA clones encoding type II integral membrane proteins on human natural killer cells. *J. Exp. Med.* 173:1017.
15. Frey, J.L., T. Bino, R.R. Kantor, D.M. Segal, S.L. Giardina, J. Roder, S. Anderson, and J.R. Ortaldo. 1991. Mechanism of target cell recognition by natural killer cells: characterization of a novel triggering molecule restricted to CD3- large granular lymphocytes. *J. Exp. Med.* 174:1527.
16. Evans, D.L., L. Jaso-Friedmann, E.E. Smith, Jr., A. St. John, H.S. Koren, and D.T. Harris. 1988. Identification of a putative antigen receptor on fish nonspecific cytotoxic cells with monoclonal antibodies. *J. Immunol.* 141:324.
17. Harris, D.T., L. Jaso-Friedmann, R.B. Devlin, H.S. Koren, and D.L. Evans. 1991. Identification of an evolutionarily conserved, function-associated molecule on human natural killer cells. *Proc. Natl. Acad. Sci. USA.* 88:3009.
18. Evans, D.L., D.T. Harris, J.H. Leary III, A.L. St. John, and L. Jaso-Friedmann. 1993. Identification of vimentin-like function associated molecule (FAM) on rat NK cells: evidence for receptor function. *Scand. J. Immunol.* 37:131.
19. Moretta, A., C. Bottino, G. Tripodi, M. Vitale, D. Pende, L. Morelli, R. Augugliaro, M. Barbaresi, E. Ciccone, R. Millo,

- and L. Moretta. 1992. Novel surface molecules involved in human NK cell activation and triggering of the lytic machinery. *Int. J. Cancer*. 7:6.
20. Storkus, W.J., D.N. Howell, R.D. Salter, J.R. Dawson, and P. Cresswell. 1987. NK susceptibility varies inversely with target cell class I HLA antigen expression. *J. Immunol.* 138:1657.
 21. Storkus, W.J., J. Alexander, J.A. Payne, P. Cresswell, and J.R. Dawson. 1989. The $\alpha 1/\alpha 2$ domains of class I HLA molecules confer resistance to natural killer. *J. Immunol.* 143:3853.
 22. Storkus, W.J., J. Alexander, J.A. Payne, J.R. Dawson, and P. Cresswell. 1989. Reversal of natural killing susceptibility in target cells expressing transfected class I HLA genes. *Proc. Natl. Acad. Sci. USA.* 86:2361.
 23. Öhlén, C., M.-T. Bejarano, A. Grönberg, S. Torsteinsdottir, L. Franksson, H.-G. Ljunggren, E. Klein, G. Klein, and K. Kärre. 1989. Studies of sublines selected for loss of HLA expression from an EBV-transformed lymphoblastoid cell line. *J. Immunol.* 142:3336.
 24. 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.
 25. Ljunggren, H., and K. Kärre. 1990. In search of the "missing self": MHC molecules and NK cell recognition. *Immunol. Today.* 11:237.
 26. Bellone, G., N.M. Valiante, O. Viale, E. Ciccone, L. Moretta, and G. Trinchieri. 1993. Regulation of hematopoiesis *in vitro* by alloreactive natural killer cell clones. *J. Exp. Med.* 177:1117.
 27. Moretta, L., E. Ciccone, A. Moretta, P. Höglund, C. Öhlén, and K. Kärre. 1992. Allorecognition by NK cells nonself or no self? *Immunol. Today.* 13:300.
 28. Öhlén, C., G. King, P. Höglund, M. Hansson, G. Scangos, C. Bieberich, G. Jay, and K. Kärre. 1989. Prevention of allogeneic bone marrow graft rejection by H-2 transgene in donor mice. *Science (Wash. DC).* 246:666.
 29. Kaufman, D.S., R.A. Schoon, and P.J. Leibson. 1993. MHC class I expression on tumor targets inhibits natural killer cell-mediated cytotoxicity without interfering with target recognition. *J. Immunol.* 150:1429.
 30. Karlhofer, F.M., R.K. Ribaud, and W.M. Yokoyama. 1992. MHC class I alloantigen specificity of Ly-49⁺ IL-2-activated natural killer cells. *Nature (Lond.).* 358:66.
 31. Sentman, C.L., J. Hackett, Jr., V. Kumar, and M. Bennett. 1989. Identification of a subset of murine natural killer cells that mediates rejection of Hh-1^d but not Hh-1^b bone marrow grafts. *J. Exp. Med.* 170:191.
 32. Yu, Y.Y.L., V. Kumar, and M. Bennett. 1993. Murine natural killer cells and marrow graft rejection. *Annu. Rev. Immunol.* 10:189.
 33. Sentman, C.L., V. Kumar, and M. Bennett. 1991. Rejection of bone marrow cell allografts by natural killer cell subsets: 5E6⁺ cell specificity for Hh-1 determinant 2 shared by H-2d and H-2f. *Eur. J. Immunol.* 21:2821.
 34. Moretta, A., C. Bottino, D. Pende, G. Tripodi, G. Tambussi, O. Viale, A. Orengo, M. Barbaresi, A. Merli, E. Ciccone, and L. Moretta. 1990. Identification of four subsets of human CD3⁻ CD16⁺ natural killer (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.
 35. Ciccone, E., D. Pende, O. Viale, A. Than, C. DiDonato, A.M. Orengo, R. Biassoni, S. Verdiani, A. Amoroso, A. Moretta, and L. Moretta. 1992. Involvement of class I alleles in natural killer (NK) cell-specific functions: expression of HLA-CW3 confers selective protection from lysis by alloreactive NK clones displaying a defined specificity (specificity 2). *J. Exp. Med.* 176:963.
 36. Leiden, J.M., B.A. Karpinsky, L. Gottschalk, and J. Kornbluth. 1989. Susceptibility to natural killer cell-mediated cytotoxicity is independent of the level of target cell class I HLA expression. *J. Immunol.* 142:2140.
 37. Holscher, M., A.L. Givan, and C.G. Brooks. 1991. The effect of transfected MHC class I genes on sensitivity to natural killer cells. *Immunology.* 73:44.
 38. Stam, N.J., W.M. Kast, A.C. Voordouw, L.B. Pastoors, F.A. van der Hoeven, C.J.M. Melief, and H.L. Ploegh. 1989. Lack of correlation between levels of MHC class I antigen and susceptibility to lysis of small cellular lung carcinoma (SCLC) by natural killer cells. *J. Immunol.* 142:4113.
 39. Chervenak, R., and R.M. Wolcott. 1988. Target cell expression of MHC antigens is not (always) a turn-off signal to natural killer cells. *J. Immunol.* 140:3712.
 40. Cuturi, M.C., M. Murphy, M.P. Costa-Giomi, R. Weinmann, B. Perussia, and G. Trinchieri. 1987. Independent regulation of tumor necrosis factor and lymphotoxin production by human peripheral blood lymphocytes. *J. Exp. Med.* 165:1581.
 41. Perussia, B., C. Romani, I. Anegón, M.C. Cuturi, J. Faust, and G. Trinchieri. 1987. Preferential proliferation of natural killer cells among peripheral blood mononuclear cells cocultured with B lymphoblastoid cell lines. *Nat. Immun. Cell Growth Regul.* 6:171.
 42. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680.
 43. Trinchieri, G., M. De Marchi, W. Mayr, M. Savi, and R. Cepellini. 1973. Lymphocyte antibody lymphocyte interaction (LALI) with special emphasis on HLA. *Transplant. Proc.* 5:1631.
 44. Cassatella, M.A., I. Anegón, M.C. Cuturi, P. Griskey, G. Trinchieri, and B. Perussia. 1989. Fc γ R (CD16) interaction with ligand induces Ca²⁺ mobilization and phosphoinositide turnover in human natural killer cells: role of Ca²⁺ in Fc γ R (CD16)-transcription and expression of lymphokine genes. *J. Exp. Med.* 169:549.
 45. Clément, M., S. Legros-Maida, A. Soulie, J. Guillet, and M. Sasportes. 1993. Dissociation of natural killer and lymphocyte-activated killer cell activities in human CD3⁻ large granular lymphocytes. *Eur. J. Immunol.* 23:697.
 46. Anegón, I., M.C. Cuturi, G. Trinchieri, and B. Perussia. 1988. Interaction of Fc γ receptor (CD16) with ligands induces transcription of IL-2 receptor (CD25) and lymphokine genes and expression of their products in human natural killer cells. *J. Exp. Med.* 167:452.
 47. Smyth, M.J., C.O. Zachariae, Y. Norihisa, J.R. Ortaldo, A. Hishinuma, and K. Matsushima. 1991. IL-8 gene expression and production in human peripheral blood lymphocyte subsets. *J. Immunol.* 146:3815.
 48. Yokoyama, W.M. 1993. Recognition structures on natural killer cells. *Curr. Opin. Immunol.* 5:67.