Differential Role of p38 and c-Jun N-Terminal Kinase 1 Mitogen-Activated Protein Kinases in NK Cell Cytotoxicity

Rossana Trotta, Katia Fettucciari, Livio Azzoni, Bekele Abebe, Kristin A. Puorro, Laurence C. Eisenlohr, and Bice Perussia

The serine-threonine mitogen-activated protein kinase (MAPK) family includes extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38 kinases. In NK cells, spontaneous or Ab-mediated recognition of target cells leads to activation of an ERK-2 MAPK-dependent biochemical pathway(s) involved in the regulation of NK cell effector functions. Here we assessed the roles of p38 and JNK MAPK in NK cell-mediated cytotoxicity. Our data indicate that p38 is activated in primary human NK cells upon stimulation with immune complexes and interaction with NK-sensitive target cells. FcγRIIIA-induced granule exocytosis and both spontaneous and Ab-dependent cytotoxicity were reduced in a dose-dependent manner in cells pretreated with either of two specific inhibitors of this kinase. Target cell-induced IFN-γ and FcγRIIIA-induced TNF-α mRNA accumulation was similarly affected under the same conditions. Lack of inhibition of NK cell cytotoxicity in cells overexpressing an inactive form of JNK1 indicates that this kinase, activated only upon FcγRIIIA ligation, does not play a significant role in cytotoxicity. These data underscore the involvement of p38, but not JNK1, in the molecular mechanisms regulating NK cell cytotoxicity. The Journal of Immunology, 2000, 165: 1782–1789.

Natural killer cells exert MHC-nonrestricted cytotoxicity, independently from prior sensitization, against a variety of target cells, including virus-infected, transformed, and IgG Ab-coated cells (reviewed in Ref. 1). While receptors transducing activating signals to trigger spontaneous cytotoxicity are just beginning to be defined (2), the well-characterized low affinity receptor for the Fc fragment of IgG (FcγRIIIA) is responsible for triggering Ab-dependent cell-mediated cytotoxicity (ADCC)3 (reviewed in Ref. 3). Early biochemical events induced upon cross-linking the receptors involved in binding IgG Ab-coated or NK-sensitive target cells include protein tyrosine kinase (PTK) activation and increased intracellular Ca2+ concentration as a consequence of phospholipase C-γ1 and -γ2 activation (4–7). Intermediate molecules in the elicited signaling cascades have been reported to modulate NK cell cytotoxicity. The PTK Syk (8), the adapter protein LAT (linker for activation of T cells) (9), and the Vav-Rac1 pathway (10, 11) play roles in both types of cytotoxicity, which are abolished upon PTK inhibition or activation of protein tyrosine phosphatases (4, 5, 12, 13). Biochemical pathways induced preferentially during ADCC or spontaneous cytotoxicity have also been identified. For example, granule exocytosis-mediated spontaneous cytotoxicity against the prototypic K562 target cells depends on activation of protein kinase C, but not phosphatidylinositol 3-kinase, whereas the reverse is true for ADCC (14).

Mitogen-activated protein kinases (MAPK) transduce signals that regulate cell growth and differentiation (15). They are serine-threonine kinases, the enzymatic activity of which is elicited upon phosphorylation of threonine and tyrosine residues in a Thr-X-Tyr motif in their regulatory domain (16). This is mediated by dual specificity MAPK kinases, which also become activated, following phosphorylation, under the same conditions. The MAPK family includes the extracellular signal-regulated kinases (ERK), the c-Jun N-terminal kinase (JNK)/stress-activated protein kinase, and the p38 MAPK (p38). For most part, ERK are activated by mitogenic factors, while JNK and p38 are activated by stress-inducing agents or proinflammatory cytokines. Although in most cell types each MAPK is activated by specific nonoverlapping kinases, named MEK, activation of both p38 and JNK by a single MEK4 kinase has also been reported (17). In different cell types the different MAPKs may act antagonistically or cooperate with each other to regulate different cell functions (15). Examples for this are the opposite effects of ERK (facilitating) and JNK (protecting) to control B cell receptor-induced apoptosis (18) and the requirement for both kinases in TCR- and CD28-dependent T cell activation and IL-2 production (19).

We and others have shown that ERK-2 activation occurs in NK cells upon target cell binding or FcγRIIIA stimulation, and that cytokine mRNA accumulation, spontaneous cytotoxicity, ADCC, and FcγR-induced degranulation depend at least in part on ERK-2 function (20–23). The possible role of the other MAPK family members is unknown. The observation that p38 plays a role in actin reorganization leading to formation of filamentous actin in endothelial cells upon platelet-derived growth factor (PDGF) (24) and vascular endothelial growth factor (25) stimulation suggests the possibility that the same kinase may be activated to play a
similar role in NK cells upon target cell binding and thus may be involved in regulating granule exocytosis-mediated cytotoxicity. Increased JunB mRNA and emergence of JunB:Fos heterodimers with increased AP-1-activity during spontaneous cytotoxicity have been reported in the human NKL cell line (26). Because ERK, but not JNK, is involved in the regulation of JunB transcription (27, 28), a possible differential role for distinct MAPK in cytotoxicity may be envisaged.

To define the role of non-ERK-2 MAPK in the regulation of NK cell cytotoxicity, we investigated the involvement of p38 and JNK kinases. Our data indicate that both FcyRIIIA triggering and NK cell recognition of nonsensitized target cells generate signals leading to activation of p38, whereas only FcyRIIIA stimulation activates JNK1. Inhibition experiments indicate that only p38 plays a role in ADCC and spontaneous cytotoxicity and exclude a role for JNK1 in ADCC, demonstrating a differential role for p38 and JNK1 in regulating NK cell cytotoxic functions. Similar to ERK, p38 is also involved in the regulation of target cell-induced cytoxin expression.

Materials and Methods

Cells lines and NK cell preparations

The human monocytic THP-1, erythroleukemic K562, T lymphoid Jurkat (clone J32), B lymphoblastoid RPMI-8866 and 721.221 cell lines were maintained in culture in RPMI 1640 medium (Bio-Whittaker, Walkersville, MD) supplemented with 10% heat-inactivated FBS (Sigma, St. Louis, MO) and 100 μg/ml l-glutamine (Life Technologies, Gaithersburg, MD). Homogeneous NK cell preparations were obtained from 10-day cocultures of PBL from healthy individuals with 30-Gy irradiated RPMI-8866 and biotin-labeled mAb 3G8 or B159.5 (both 20 μCi/ml; Sigma) and THP-1, THP-721.221 cells were fixed (30 min on ice) and washed extensively before use. This treatment prevents binding to NK cells or subsequent stimulation of early biochemical events (29). The cell preparations contained >98% CD16^+CD56^−CD3^− and <3% CD3^+ cells, as determined by indirect immunofluorescence (flow cytometry) using a panel of mAbs.

Monoclonal and polyclonal Abs

mAb 3G8 (anti-CD16), B159.5 (anti-CD56), B36.1 (anti-CD5), OKT3 (anti-CD3), and B52.1 (anti-CD14) have been previously described (29); the anti-FLAG M2 mAb was obtained from Sigma. The polyclonal rabbit sera against the wt or dominant negative JNK1 fusion recombinant proteins was obtained from Rockland (Gilbertsville, PA), and polyclonal rabbit sera against Phe185 JNK1 (33) were generated after Bm11H and HindIII digestion, respectively. The blunt-ended cDNA were inserted into the Nhel cloning site of the pcC11 vector and introduced into Vc, WR strain, by homologous recombination, as previously described (34). For infection, NK or Jurkat T cells were incubated with the indicated Vc recombinant (10−20 multiplicity of infection, 37°C, 4 h, 10^7 cell/ml, and 25% 10^7/ml RPMI 1640 medium supplemented with 10% FBS). The cells were used immediately after washing. Expression of the wt or dominant negative JNK1 fusion recombinant proteins was confirmed by Western blot with anti-JNK1 or anti-FLAG Ab, and the expression of the wt JNK1 or JNK2 on the Western immunoblots were performed using a commercial kit following the manufacturer’s recommendations (MPAKAP kinase-2 IP-Kinase Assay Kit, Upstate Biotechnol- ogy, Lake Placid, NY; with a sheep serum reacting with both rabbit and human MAPKAP kinase-2 for immunoprecipitation, and the KKLNRHTLSVA peptide as a substrate).

Vaccinia virus (Vac) recombinant preparations and NK cell infection

To generate JNK1 recombinant Vc, cDNA fragments encoding the p38 activation-domain negative (APF) and dominant negative (APF) p38 kinase (Vac) were cotransfected into NIH 3T3 cells and selected in G418. A total of 10^6 Transfection laboratories (Lexington, KY).

Cell stimulation

Cells were incubated at 37°C for the indicated times with 10^5 cells/ml 37°C for the indicated times with the different stimuli. These were: K562 and 721.221 cells (5.1 NK to target cell ratio), PMA (50 ng/ml) and ionomycin (1 μM, both from Sigma), immune complexes (rabbit IgG-sensitized bovine erythrocytes (EA)), or E (negative control; 0.5% suspension) prepared as previously described (30), and biotin-labeled mAb 3G8 or B159.5 (both 20 μg/ml) with added streptavidin (50 μg/ml; Sigma). In the samples used for Western blotting, K562 and 721.221 cells were fixed (3 × 10^6 cells/ml 1% paraformaldehyde, 30 min on ice) and washed extensively before use. This treatment prevents possible activation of endogenous kinases and has no effect on target cell binding to NK cells or subsequent stimulation of early biochemical events and ERK activation in NK cells (23, 21) (data not shown). When target cells were used, effector/target cell contact was facilitated by centrifugation (600 rpm, 2 min) before incubation. When indicated, the p38 inhibitors SB203580 and SB202190 (Calbiochem, San Diego, CA) were added to the effector cells at the indicated concentrations for 1 h at 37°C before stimulation.

Western blotting and kinase assays

After stimulation the cells were lysed (10^6 cells/ml lysis buffer: 1% Nonidet P-40, 10 mM HEPES (pH 7.5), 0.15 M NaCl, 10% glycerol, 10 μg/ml each aprotinin and leupeptin, 1 mM PMSF, 1 mM Na_2VO_4, 50 mM NaF, and 1 mM EDTA). Western blotting was performed according to our published protocols (20), and Ab-reactive proteins were detected with HRP-labeled sheep anti-rabbit Ig sera and enhanced chemiluminescence (Amersham, Arlington Heights, IL). Two methods were used to assess kinase activity: 1) expression (detected by Western blotting) of the enzymatically active phosphorylated forms of p38 and JNK, and 2) JNK1 and p38 kinase assays, performed according to the protocols of Hibi and Rose (31, 32), respectively. For these each kinase was immunoprecipitated from 5 × 10^6 NK cell lysate equivalent using the specific Ab (1 μg) and protein A-Sepharose (Pharmacia, Uppsala, Sweden). After four washes with lysis buffer and two with 10 mM HEPES (pH 7.5), 25 mM MgCl_2, 50 mM NaCl supplemented with 1 mM Na_2VO_4, 50 mM sodium fluoride, and 1 mM PMSF, the protein A-Sepharose beads were incubated (30 min, 30°C) with 30 μl of reaction buffer and occasional tapping. The kinase buffer for the JNK1 assays was 20 mM HEPES (pH 7.5), 2 mM MgCl_2, 25 mM β-glycerol phosphate, 20 mM MgCl_2, 0.1 mM Na_2VO_4, 20 mM ATP, 10 μCi [γ-32P]ATP (sp. act., 4000 Ci/mmol; ICN, Costa Mesa, CA), GST-ATF2 (aa 1–96; Santa Cruz Biotechnology) and GST-c-Jun (aa 1–223) (33), each 1 μg, were used as interchangeable substrates for JNK because both polypeptides have sequences specifically recognized by this kinase (33). The kinase buffer for the p38 kinase assays was 20 mM HEPES (pH 7.5), 25 mM β-glycerol phosphate, 25 mM MgCl_2, 2 mM DTT, 0.1 mM Na_2VO_4, 20 mM ATP, 10 μCi [γ-32P]ATP. The substrate was GST-ATF2, as described above. After the reaction the kinases were eluted from the beads by heating and were analyzed in 10% SDS-PAGE (reducing conditions). Western blots were performed to verify that equal amounts of pre-cipitated MAPK were loaded per sample, and in vitro phosphorylation of the kinase substrates was detected after exposure of the filters to X-AR films (Eastman Kodak, Rochester, NY). MAPKAP kinase-2 assays were performed using a commercial kit following the manufacturer’s recommenda-tions (MAPKAP kinase-2 IP-Kinase Assay Kit, Upstate Biotechnol- ogy, Lake Placid, NY; with a sheep serum reacting with both rabbit and human MAPKAP kinase-2 for immunoprecipitation, and the KKLNRHTLSVA peptide as a substrate).

Vaccinia virus (Vac) recombinant preparations and NK cell infection

To generate JNK1 recombinant Vc, cDNA fragments encoding the p38 activation-domain negative (APF) and dominant negative (APF) p38 kinase (Vac) were cotransfected into NIH 3T3 cells and selected in G418. A total of 10^6 Transfection laboratories (Lexington, KY).

Sodium benzoxylcarbonyl-l-lysine thiol benzyl ester (BLT)-esterase release assay

This was performed as described by Visonneau et al. (35) using as stimuli plastic-immobilized mAb 3G8, B159.5 as a negative control, and PMA (50 ng/ml) and ionomycin (1 μM; both from Sigma) as the positive control. Cell-free supernatants were collected after 4-h incubation at 37°C. The percentage of released BLT esterase activity was calculated for each sample according to the formula (S/S_C) x 100, where S is OD in the supernatant, and C is that in the corresponding cell lysate.

Cytotoxicity assays

K562, THP-1, and 721.221 cells, as indicated, were used as the target in 3-h 51Cr release assays (30). For redirected ADCC, mAb 3G8 or B159.5 as the control (both supernatants, 1/4 predetermined optimal concentration) was present throughout the assay with THP-1 cells. These were not lysed in the presence of the control B159.5 mAb (not shown). A constant number of target cells (5 × 10^6 well, as indicated) and serial dilutions of effector cells were used in triplicate. Spontaneous release from any target cell used was <10%; lytic units (36) were calculated at 40% cytotoxicity.

F-actin detection

After stimulation, NK cells were labeled with N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phallacidin (Molecular Probes, Eugene, OR) and analyzed by...
flow cytometry as described by Salmon et al. (37). Briefly, 3.5 \times 10^6 cells/sample were fixed (10 min, 20°C) in 3.7% formaldehyde in PBS (Sigma); permeabilized, and labeled (20 min, 4°C) in PBS containing 0.5% saponin (Sigma), 0.2% FBS, 0.005% Tween-20, 0.01% NaN₃, and X-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phallacidin-phallacidin (1 U/sample). Relative F-actin content is expressed as the ratio between the mean fluorescence intensity of NDB staining in stimulated and nonstimulated control NK cells.

Northern blot analysis

A 1-h incubation at 37°C with or without the p38 inhibitor SB202190 (50 \mu M), NK cells (5 \times 10^6/ml) were cultured with the indicated stimuli for 1.5 h, and Northern blot analysis was performed as previously described (38), with slight modifications. Briefly, total RNA was extracted using TRIzol reagent (Life Technologies, Gaithersburg, MD), size fractionated in 1% agarose-formaldehyde gels, transferred to Hybond-nylon membranes (Amersham), and hybridized to cDNA probes specific for human IFN-γ, TNF-α, and TCR β-chain (detecting a nonfunctional, truncated, 1.0-kb mRNA species in NK cells) for normalization. cDNA probes were labeled with [α-32P]dCTP (spec. act., 3000 Ci/mmol; ICN) by nick translation (Roche, Indianapolis, IN) (20, 21). Hybridization was detected and quantitated using a PhosphorImager (PhosphorImager SI, Molecular Dynamics, Sunnyvale, CA) with proprietary software (ImageQuant).

**Results**

**FcyRIIIA- and target cell-induced p38 and JNK1 kinase activation in NK cells**

To determine whether FcyRIIIA ligation induces p38 and JNK1 MAPK activation, Western blot analysis was performed with anti-active p38 or anti-active JNK Ab on lysates from NK cells nonstimulated or stimulated with immune complexes (EA), E, or PMA and ionomycin as a control (Fig. 1A, left panels). Minimal levels of active p38 and JNK1 were detected in NK cells before stimulation or after 10-min incubation with control E. As with ERK1 and ERK2 (20, 22) (not shown), the levels of active p38 and JNK1 significantly increased to plateau by 5–10 min, started declining within 20 min (not shown), and returned to control levels by 1-h stimulation with EA (first and third panels). All samples expressed similar levels of total p38 and JNK1 (second and fourth panels). Active p38 and JNK1 were also detected upon stimulation with PMA/ionomycin. As expected, p38 and JNK1 immunoprecipitated from EA-stimulated NK cells were able to phosphorylate the exogenous substrates ATF2 and GST-c-Jun, respectively, in in vitro kinase assays (Fig. 1B). The same substrates were only minimally phosphorylated by the kinases immunoprecipitated from E-stimulated cells.

To determine whether binding of NK to sensitive target cells leads to activation of the same kinases, the presence of active p38 and JNK1 was analyzed in the lysates from NK cells stimulated with K562 or 721.221 target cells. Lysates from target and NK cells incubated separately for the same time as the experimental samples and mixed after lysis were used as the negative control; positive controls were lysates from NK cells stimulated with PMA/ionomycin. The levels of active p38 were significantly increased in NK cells after interaction with K562 or 721.221 cells, whereas those of active JNK1 were unchanged (Fig. 1A, right, first and third panels). Comparable levels of p38 and JNK1 were detected in all samples (right, second and fourth panels). No active JNK1 was detected upon longer (1-h) stimulation of NK with the target cells (data not shown), and the immunoprecipitated JNK1 did not phosphorylate recombinant ATF2 used as the substrate in in vitro kinase assay (Fig. 1C).

**Role of p38 in FcyRIIIA-induced granule exocytosis and cytotoxicity**

The pyridinyl imidazoles SB203580 and SB202190 specifically inhibit p38, but not other MAPK activity (39), by occupying p38 ATP-binding sites (40). Low doses of SB203580 (1 \mu M) were sufficient to inhibit by 50% the p38 activity in lysates of NK cells stimulated with PMA/ionomycin (not shown). The ability of SB203580 to inhibit the p38-dependent pathway in NK cells was assessed using as a readout the induced activation of MAPKAP kinase-2 (K-2), a physiological substrate of p38 (40, 41) activated in NK cells upon stimulation with immune complexes or target cells (data not shown). Addition of 13 \mu M SB203580 to intact cells inhibited by 50% the PMA/ionomycin-induced MAPKAP K-2 activity (Fig. 2, inset). Further inhibition was obtained at higher doses. Concentrations of this inhibitor up to 50 \mu M did not affect activation of ERK and JNK MAPK (data not shown). Both FcyRIIIA- and PMA/ionomycin-induced BLT esterase secretion were inhibited in a dose-dependent manner in NK cells preincubated with SB203580 (Fig. 2) or SB202190 (data not shown).

The involvement of p38 in ADCC and spontaneous cytotoxicity was assessed analyzing the effect of its pharmacologic inhibition in 51Cr release assays (Fig. 3). The levels of redirected ADCC against THP-1/3G8 (left panels) and of spontaneous cytotoxicity against K562 and 721.221 target cells (middle and right panels) mediated by NK cells pretreated with SB203580 or SB202190 (top and bottom, respectively) were significantly lower than those mediated by control nontreated cells. Based on calculation of lytic units at 40% cytotoxicity in the two experiments reported, 50 \mu M SB203580 or SB202190 inhibited ADCC by 77 and 84%, respectively, spontaneous cytotoxicity against K562 by 90 and 91%, and spontaneous...
cytotoxicity against 721.221 target cells by 64 and 71%. In the same conditions, cell viability, expression of FcγRIIIA, LFA-1, CD18, and CD11b, and formation of conjugates with any of the target cells used were not affected (not shown).

Role of p38 in actin rearrangement

To determine whether FcγRIIIA ligation mediates induction of actin polymerization and, if so, whether p38, alone or with ERK kinases, regulates it, F-actin content was analyzed in NK cells treated with inhibitors of p38 (SB203580, or SB202190; Table I) and/or of MEK (PD098059; not shown) and stimulated for 5 min with anti-FcγRIIIA mAb (Table I). Similar to the changes reported for granulocytes (37) in similar experimental conditions, the F-actin levels detectable in NK cells were significantly increased upon PMA/ionomycin or CD16, but not CD56, stimulation. The levels of CD16-induced actin polymerization did not change in cells pretreated with any of the inhibitors, alone or in combination (not shown).

Role of JNK in ADCC and spontaneous cytotoxicity

As detected in Western blotting with an Ab recognizing specifically Ser63-phosphorylated c-Jun (Fig. 4), expression of the FLAG-tagged, Vac-encoded, recombinant inactive APF mutant (33), but not of the wt JNK, correlated with lack of c-Jun phosphorylation induced by PMA-ionomycin stimulation in J32 cells and by EA in NK cells. This confirms that JNK1 activity is significantly reduced in the APF-inactive mutant-expressing cells, indicating that overexpression of APF inhibits the enzymatic activity of endogenous JNK on its natural substrate (c-Jun) in intact cells.

Table 1. Actin polymerization upon FcγRIIIA stimulation

| Inhibitor * | Anti-CD56 | Anti-CD16 | PMA/ionomycin |
|------------|-----------|-----------|---------------|
| None       | 1.1 ± 0.1*| 1.4 ± 0.1*| 1.5 ± 0.2*    |
| SB203580   | 1.0 ± 0.1 | 1.5 ± 0.3*| 1.4 ± 0.2*    |
| SB202190   | 0.9 ± 0.2 | 1.3 ± 0.2*| 1.4 ± 0.3*    |

* NK cells were incubated (3.5 × 10⁶/ml, 1 h, 37°C) in medium without (none) or with the indicated inhibitors, 50 μM.

1785The Journal of Immunology

FIGURE 2. Effect of p38 inhibition on FcγRIIIA-induced granule exocytosis. NK cells were incubated (1 h, 37°C) in medium with the indicated concentrations of SB203580. The anti-CD16 mAb 3G8, anti-CD56 mAb B159.5, and PMA/ionomycin, as indicated, were used as stimuli in a 4-h BLT esterase release assay (see Materials and Methods). This experiment is representative of three performed with similar results. Insert. After incubation (40 min, 37°C) in medium with the indicated concentrations of SB203580, NK cells were incubated (10 min, 37°C) without (−) or with (+) PMA/ionomycin, and a kinase assay was performed on the MAPKAP K-2 immunoprecipitated from the cell lysates using KKLRRLSVA peptide as a substrate (see Materials and Methods). This experiment is representative of two performed with similar results.

FIGURE 3. Effect of p38 inhibition on FcγRIIIA-dependent and spontaneous cytotoxicity. NK cells from two separate donors were incubated (1 h, 37°C) without (■) or with (▲ and ●) the indicated concentrations of SB203580 (top) or SB202190 (bottom). FcγRIIIA-redirected lysis (THP-1 target cells in the presence of anti-CD16 mAb 3G8, THP-1/3G8) and spontaneous cytotoxicity against K562 and 721.221 cells were tested in a 3-h ⁵¹Cr release assay. Target cells were 5 × 10⁴/well. The x-axis shows the E:T cell ratio; the y-axis shows the percent specific ⁵¹Cr release. Each experiment is representative of two performed with similar results.
ADCC and spontaneous cytotoxicity were tested in NK cells expressing wt JNK1 kinase or its inactive mutant (APF; Fig. 5). Expression of the exogenous proteins was confirmed by Western blot using an anti-JNK1 or an anti-FLAG Ab (Fig. 5 A, left panels). An ∼46-kDa band corresponding to endogenous JNK1 was detected in all lysates analyzed, while a slower migrating band, corresponding to FLAG-tagged APF or wt JNK1, was detected only in the lysates from cells infected with the wt or the mutant JNK1 Vac recombinant. Similar levels of wt and APF JNK1 were expressed in the Vac-infected cells. To confirm that overexpression of the kinase inactive form of JNK1 results in inhibition of endogenous JNK1 activation, the expression of active JNK1 was analyzed by Western blot in NK cells noninfected or infected with recombinant Vac encoding APF or wt JNK1 and stimulated with EA. In cell expressing the kinase inactive or the wt form of JNK1 the levels of active JNK1 were, respectively, significantly lower or higher than those in noninfected cells (Fig. 5 A, right panel). Additionally, CD16-directed and spontaneous cytotoxicity were not inhibited in APF-JNK1-expressing NK cells or in NK cells infected with wt (Fig. 5 B) or empty Vac (not shown). Similar results were obtained using the human NK cell line NKL infected with the JNK1 recombinant viruses (not shown).

Role of p38 in FcγRIIIA- and target cell-induced cytokine mRNA accumulation

To determine whether p38 plays a role in regulating cytokine expression induced by FcγRIIIA ligation or target cell binding, we analyzed the effects of the p38 inhibitor SB202190 on IFN-γ (Fig. 6).
Discus 

We report that phosphorylation and activation of the p38 MAPK occur in human primary NK cells upon FcγRIIIA ligation and target cell binding, and that, like ERK-2 (21, 23), it plays a role in cytokotoxicity and cytokine expression, whereas JNK, activated exclusively upon FcγRIIIA ligation, does not play a role in either ADCC or spontaneous cytokotoxicity. These observations serve to establish that all MAPK family members can be activated upon ligand binding to FcγRIIIA and, among the MAPK family, identify p38 and ERK as key molecules in the biochemical pathway(s) that regulates activation of two of the NK cell functions, namely cytokotoxicity and cytokine production. All MAPK family members have been previously shown to be activated in murine macrophages (32) upon FcγR cross-linking. However, the identity of the FcγR type responsible for this effect was not definitively established. Using NK cells, we extend those data to report that FcγRIIIA is, in itself, capable of this effect. Upon ligand binding, other immune receptors structurally similar to FcγRIIIA, e.g., the B cell Ag receptor, also transduce signals resulting in activation of the same three kinases (42).

We previously reported that FcγRIIIA-dependent ERK-2 activation plays a role in cytokine production, ADCC, and spontaneous cytokotoxicity in NK cells (20, 21). The kinetics of FcγRIIIA-induced activation and inactivation (dephosphorylation) of p38 and JNK in NK cells are very similar, if not identical, to those of ERK, and it is likely that a specific phosphatase(s) induced via FcγRIIIA stimulation controls activation of all MAPK. The most likely candidate for this is the dual specificity protein MKP-1, which dephosphorylates ERK-2, JNK, and p38 MAPK in PMA-stimulated U937 cells (43). Unlike immune complexes, tumor target cells binding to NK cells induce activation of p38, but not JNK1 kinase. One or more activating receptors may be sensitive to fixation, as used here. However, neither ERK (21, 23) nor p38 activation is prevented under these conditions. This indicates that at least one of the target surface molecules triggering spontaneous cytokotoxicity is still capable of responding to fixed cells to induce early biochemical events and activation of at least two MAPK family members, making it unlikely that lack of JNK activation depends on lack of NK cell activation by the fixed target cell. Thus, we favor the hypothesis that, unlike FcγRIIIA, the receptor(s) triggering spontaneous cytokotoxicity transduces signals leading specifically to the activation of ERK and p38, but not JNK, similar to the PDGF receptor (24) or the TCR (19). Whatever the reason for the lack of JNK activation upon target cell binding, our data support a nonredundant role of individual MAPK members in NK cells.

We have used pharmacologic inhibitors to determine the necessary role of p38 in ADCC and spontaneous cytokotoxicity and in cytokine expression, similar to ERK2. Several lines of evidence support that the observed inhibition does not depend on toxicity of the inhibitors or on inhibition of ERK or other biochemical pathways upstream of MAPK. Specifically, 1) cell viability, conjugate formation, and expression of several adhesion molecules involved in spontaneous cytotoxicity or ADCC are not modified in the inhibitor-treated cells; 2) the same treatment does not affect PMA/ ionomycin-induced ERK activation (not shown); and 3) both inhibitors used, which specifically bind the ATP-binding site of the p38 kinase, do not affect the activity of the closely related ERK and JNK or other serine-threonine kinases, such as c-Raf, p90 S6kinase, and P70 S6 kinase (41). The possibility that in our previous report using a MEK inhibitor to prevent ERK activation (21), p38 inhibition was responsible for the almost complete inhibition of both ADCC and spontaneous cytotoxicity can be discounted based on the observation that p38, but not ERK, phosphorylation (and thus activation) is maintained in cells treated with a MEK inhibitor (not shown).

Similar to what we (21) and others (23) previously reported for the ERK pathway, our findings indicate that the inhibition of ADCC following p38 inactivation depends on a regulatory effect of this kinase on NK cell degranulation. Our data suggest that both ERK- and p38-mediated signals, although necessary, are not sufficient alone to activate FcγRIIIA-mediated degranulation and the lytic process. As discussed above, sequential and/or interdependent activation of the two kinases is unlikely, and their roles do not appear to be redundant. Whether the two kinases phosphorylate distinct substrates, or both kinases are needed to phosphorylate a single substrate remains to be determined. Among specific p38 substrates, MAPKAP K-2, activated both upon FcγRIIIA stimulation and target cell binding (data not shown), may represent a biochemical mediator common to the two types of cytokotoxicity.

Direct detection of perforin- and granzyme B-containing intra-cellular granules has indicated the ERK2 dependence of FcγRIIIA-induced granule migration along cytoskeletal structures in NK cells (23). Here we show that, as previously reported for FcγRIIB and FcγRIIA in neutrophils (37), FcγRIIIA stimulation induces actin polymerization in NK cells. Activation of p38 is required for PDGF-induced cell motility responses such as cell migration and actin reorganization (24) and mediates the vascular endothelial growth factor-induced ERK-independent actin reorganization in endothelial cells (25) and the TGF-β1-induced actin polymerization in neutrophils (44). However, we obtained no evidence of a role for p38 (this manuscript), ERK (21), or the two kinases combined (not shown) in target cell-, FcγRIIIA-, or PMA/ ionomycin-induced actin polymerization in NK cells. In cytotoxic T cells degranulation is regulated by the motor protein kinesin, and several kinesin-associated proteins have been identified, the state of phosphorylation of which affects the extent of kinesin motor activity and subsequent granule release (45, 46). Kinesin and/or kinesin-associated proteins might be among the direct or indirect targets of the Ser/Thr kinase phosphorylation cascade induced by p38 or ERK activation during NK cell cytotoxicity.

Our data indicate that both p38 and ERK MAPK activation regulate at least in part the immune complex-induced IFN-γ and TNF-α mRNA accumulation and the target cell- induced IFN-γ mRNA accumulation in human NK cells. These data add to a recent report indicating a role for p38 MAPK in integrin-triggered IL-8 production by human NK cells (47). FcγRIIIA stimulation induces AP-1-dependent transcription of the cytokines tested (48), and both ERK and p38 phosphorylate and regulate the activity of this and other transcription factors (49, 50). Thus, in this case, cytokine regulation may occur at least in part at the transcriptional level. However, post-transcriptional regulation of the expression of...
several cytokines has been reported for p38 (39, 51), and the level(s) at which this kinase regulates FcγRIIIA- and/or target cell induced IFN-γ and TNF-α mRNA remains to be determined. The possibility that JNK1 may also be involved (at least in cytokine expression induced upon FcγRIIIA stimulation) is not excluded by our data and unfortunately cannot be tested at present. No specific inhibitors of this kinase are available, and the use of cells infected with Vae-encoding inactive kinase is inappropriate to study non-inmediate events that require host cell RNA transcription and protein translation, both known to be subverted by the vaccinia virus. Collectively, our data serve to establish that only p38 and ERK, among MAPK, play a role in NK cell cytotoxicity and cytokine production induced upon target cell recognition. They also open the way to future studies to define the mechanism(s) through which p38 regulates NK cell lytic functions, the common or specific MAPK substrates involved, and the possible role of JNK1 specifically in functions other than cytotoxicity.

Acknowledgments
We thank Dr. Danilo Perrotti for helpful discussions, Dr. Roger J. Davis for the vectors encoding wt and APF JNK1, Dr. Michael Karin and Masahiko Hibi for the GST-c-Jun expression vector, and Matthew Loza and Jason McCormick for assistance with flow cytofluorometry.

References
1. Trinchieri, G. 1989. Biology of natural killer cells. Adv. Immunol. 47:187.
2. Moretta, A., S. Sivori, M. Ponte, M. C. Mingari, and L. Moretta. 1998. Stimulatory receptors in NK and T cells. Curr. Top. Microbiol. Immunol. 280:15.
3. Perussia, B. 1998. Fc receptors on natural killer cells. Curr. Top. Microbiol. Immunol. 230:63.
4. Einspahr, K. J., R. T. Abraham, B. A. Binsztad, Y. Uehara, and P. J. Leibson. 1991. Tyrosine phosphorylation provides an early and requisite signal for the activation of natural killer cell cytotoxic function. Proc. Natl. Acad. Sci. USA 88:6279.
5. O’Shea, J. J., D. W. McVicar, D. B. Khuns, and J. R. Ovtadlo. 1992. A role for protein tyrosine kinase activity in natural cytotoxicity as well as antibody-dependent cellular cytotoxicity: effects of herbinychm A. J. Immunol. 148:2497.
6. Azzoni, L., M. Kamoun, T. W. Salcedo, P. Kanakaraj, and B. Perussia. 1992. Stimulation of Fcγ-RIIIA results in phospholipase Cγ-1 tyrosine phosphorylation and p60ph activation. J. Exp. Med. 176:1745.
7. Ting, T. T., L. L. Lanier, R. T. Abraham, and P. J. Leibson. 1992. Fcγ receptor activation induces the tyrosine phosphorylation of both phospholipase C (PLC)-γ1 and PLC-γ2 in natural killer cells. J. Exp. Med. 176:1751.
8. Brumbaugh, K. M., B. A. Binsztad, D. D. Billadeau, R. A. Schoon, C. J. Dick, R. Ten, and P. J. Leibson. 1997. Functional role for Syk tyrosine kinase in natural killer cell-mediated natural cytotoxicity. J. Exp. Med. 186:1965.
9. Jevremovic, D., D. D. Billadeau, R. A. Schoon, C. J. Dick, B. J. Irvin, W. Zhang, L. E. Samelson, R. T. Abraham, and P. J. Leibson. 1999. A role for the adaptor protein LAT in human NK cell-mediated cytotoxicity. J. Immunol. 162:2453.
10. Galdarnini, R., G. Palmeiri, M. Piccoli, L. Frati, and A. Santoni. 1999. Role for the Rac1 exchange factor Vav in the signaling pathways leading to NK cell cytotoxicity. J. Immunol. 162:3148.
11. Billadeau, D., K. M. Brumbaugh, C. J. Dick, R. A. Schoon, X. R. Bustelo, and P. J. Leibson. 1998. The Vav-Rac1 pathway in cytotoxic lymphocytes regulates the generation of cell-mediated killing. J. Exp. Med. 188:549.
12. Burshtyn, D. N., A. M. Scharenberg, N. Wagtmann, S. Rajagopalan, K. Berrada, P. Roncaiolli, L. Bisogno, G. Palmieri, L. Frati, M. G. Cifone, and A. Santoni. 1997. CD16 cross-linking induces both secretory and extracellular signal-regulated kinase (ERK)-dependent cytosolic phospholipase-A2 (PLA2) activity in human natural killer cells: involvement of ERK, but not PLA2a, in CD16-triggered granule exocytosis. J. Immunol. 158:5458.
13. Wei, S., A. M. Gamerro, J. H. Liu, A. A. Daulton, N. I. Valkov, A. C. Trapani, A. C. Larner, M. J. Weber, and J. Y. Djeu. 1998. Control of lytic function by mitogen-activated protein kinase/extracellular regulatory kinase 2 (ERKK2) in a human natural killer cell line: identification of perforin and granzyme B mobilization by functional ERK2. J. Exp. Med. 187:1753.
14. Matsumoto, T., K. Yokote, K. Tamura, M. Takenoto, H. Ueno, Y. Saito, and S. Mori. 1999. Platelet-derived growth factor activates p38 mitogen-activated protein kinase through a Ras-dependent pathway that is important for actin reorganization and cell migration. J. Biol. Chem. 14:19504.
15. Rousseau, S., F. Houle, J. Landry, and J. Huot. 1997. p38 MAP kinase activation by vascular endothelial growth factor mediates actin reorganization and cell migration in human endothelial cells. Oncogene 16:2169.
16. Bernard, A., C. Cambiaggi, S. Guia, F. Bertucci, B. Granjeaud, R. Tagett, C. N’Guyen, B. R. Jordan, and E. Vivier. 1999. Engagement of natural cytotoxicity programs regulates AP-1 expression in the NK human cell line J. Immunol. 162:4062.
17. Coffler, P., M. de Jonge, A. Mettouchi, B. Binetruy, J. Gysdael, and W. Kruijer. 1994. JUNb promoter regulation: Ras mediated transactivation by c-Ets-1 and c-Ets-2. Oncogene 9:911.
18. Kallunki, T., T. Deng, M. Bibi, and M. Karin. 1996. c-Jun can recruit JNK to phosphotyrosine dimerization partners via specific docking interactions. Cell 87:929.
19. Perussia, B., C. Ramoni, I. Anegon, M. C. Cuturi, J. Faust, and G. Trinchieri. 1987. Preferential proliferation of natural killer cells among peripheral blood mononuclear cells cultured with lymphoblastoid cell lines. Nat. Immun. Cell Growth Regul. 6:171.
20. Perussia, B., G. Trinchieri, and J. C. Cerrotti. 1979. Functional studies of Fc receptor-bearing human lymphocytes: effect of treatment with proteolytic en-"
kinase homologue which is stimulated by cellular stresses and interleukin-1. 
FEBS Lett. 364:229.

42. Sutherland, C. L., A. W. Heath, S. L. Pelech, P. R. Young, and M. R. Gold. 1996. 
Differential activation of ERK, JNK, and p38 mitogen-activated protein kinases 
by CD40 and B cell antigen receptor. J. Immunol. 157:3381.

43. Franklin, C. C., and A. S. Kraft. 1997. Conditional expression of the mitogen-
activated protein kinase (MAPK) phosphatase MKP-1 preferentially inhibits p38 
MAPK and stress-activated protein kinase in U937. J. Biol. Chem. 272:16917.

44. Hannigan, M., L. Zhan, Y. Ai, and C. K. Huang. 1998. The role of p38 MAP 
kinase in TGF-β1-induced signal transduction in human neutrophils. Biochem. 
Biophys. Res. Commun. 246:55.

45. Burkhardt, J. K., J. M. McIlvain, M. P. Sheetz, and Y. Argon. 1993. Lytic gran-
ules from cytotoxic T cells exhibits kinesin-dependent motility on microtubules 
in vitro. J. Cell Sci. 104:151.

46. McIlvain, J. M., J. K. Burkhardt, S. Hamm-Alvarez, Y. Argon, and M. P. Sheetz. 
1994. Regulation of kinesin activity by phosphorylation of kinesin-associated 
proteins. J. Biol. Chem. 269:19176.

47. Mainiero, F., A. Soriani, R. Strippoli, J. Jacobelli, A. Gismondi, M. Piccoli, 
L. Frati, and A. Santoni. 2000. RAC1/P38 MAPK signaling pathway controls β1 
integrin-induced interleukin-8 production in human natural killer cells. Immuno-
ity12:7.

48. Aramburu, J., L. Azzoni, A. Rao, and B. Perussia. 1995. Activation and expres-
sion of the nuclear factors of activated T cells, NFATp and NFATc in human 
natural killer cells: regulation upon CD16 ligand binding. J. Exp. Med. 182:901.

49. Price, M. A., F. H. Cruzalegui, and R. Treisman. 1996. The p38 and ERK MAP 
kine pathways cooperate to activate ternary complex factors and c-fos trans-
scription in response to UV light. EMBO J. 15:6552.

50. Tan, Y., J. Rouse, A. Zhang, S. Cariati, P. Cohen, and M. J. Comb. 1996. FGF 
and stress regulate CREB and ATF-1 via a pathway involving p38 Map kinase 
and MAPKAP kinase-2. EMBO J. 15:4629.

51. Ramirez, M., N. Fernandez-Troy, M. Buxade, R. P. Casaroli-Marano, D. Benitez, 
C. Perez-Maldonado, and E. Espel. 1999. Wortmannin inhibits translation of tu-
mor necrosis factor-α in superantigen-activated T cells. Int. Immunol. 11:1479.