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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. FcyRIIIA-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 FcyRIIIA-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 FcyRIIIA 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.

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We and others have shown that ERK-2 activation occurs in NK cells upon target cell binding or FcyRIIIA 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 FcγRIIIA triggering and NK cell recognition of nonsensitized target cells generates signals leading to activation of p38, whereas only FcγRIIIA 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 cytoxic functions. Similar to ERK, p38 is also involved in the regulation of target cell-induced cytokine 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 (BioWhittaker, 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 721.221 cells were fixed (3 × 10^6 cells/ml 1% paraformaldehyde, 30 min, 4°C) for the indicated times with the cell ratio), PMA (50 ng/ml) and ionomycin (1 μM; both from Sigma), in the samples used for Western blotting, K562 cells were used, effector/target cell contact was facilitated by centrifugation (30 min, 1500 g, 10°C). For redirected ADCC, mAb 3G8 or B159.5 as effector/ target cell was present throughout the assay with THP-1 cells. These were not lysed at examination.

Cell stimulation

Cells were incubated (5 × 10^6/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 NaVO_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 DTT, 20 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) (31), 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 precipitated 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 recommendations (MAPKAP kinase-2 IP-Kinase Assay Kit, Upstate Biotechnology, Lake Placid, NY; with a sheep serum reacting with both rabbit and human MAPKAP kinase-2 for immunoprecipitation, and the KKLNRILSV peptide as a substrate).

Vaccinia virus (Vac) recombinant preparations and NK cell infection

To generate JNK1 recombinant Vac, cDNA fragments encoding the FcγRIIIA-tagged wild-type (AF-P) and dominant negative (AF-Phe185) JNK1 (33) were generated after BamHI and HindIII digestion, respectively. The blunt-ended cDNA were inserted into the Nhel cloning site of the psiC11 vector and introduced into Vac, WR strain, by homologous recombination, as previously described (34). For infection, NK or Jurkat T cells were incubated with the indicated Vac recombinant (10–20 multiplicity of infection, 37°C, 1.5 h, 10^6 cell/ml, and at optimal 4 h in 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 effect of the expression of AF-P JNK1 on c-Jun phosphorylation was determined by Western immunoblotting with anti-phospho-c-Jun Ab. Cell lysates for the latter were in 0.5 M NaCl.

Sodium benzoxyloxy carbonyl-l-lysine thio benzyl ester (BLT)-esterase release assay

This was as described by Vissonneau 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 at 40% cytotoxicity.

Cytotoxicity assays

K562, THP-1, and 721.221 cells, as indicated, were used as the target in 3-h¹¹¹I 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 × 10⁶ 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 10^{-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**

After 1-h incubation at 37°C with or without the p38 inhibitor SB202190 (50 μM), NK cells (5 × 10⁵/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 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 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 μM) 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 μM 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 μM 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 μM 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 I. Actin polymerization upon FcyRIIIA stimulation**

<table>
<thead>
<tr>
<th>Inhibitor*</th>
<th>Anti-CD56</th>
<th>Anti-CD16</th>
<th>PMA/ionomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.1 ± 0.1*</td>
<td>1.4 ± 0.1*</td>
<td>1.5 ± 0.2*</td>
</tr>
<tr>
<td>SB203580</td>
<td>1.0 ± 0.1</td>
<td>1.5 ± 0.3*</td>
<td>1.4 ± 0.2*</td>
</tr>
<tr>
<td>SB202190</td>
<td>0.9 ± 0.2</td>
<td>1.3 ± 0.2*</td>
<td>1.4 ± 0.3*</td>
</tr>
</tbody>
</table>

* NK cells were incubated (3.5 × 10^6/ml, 1 h, 37°C) in medium without (none) or with the indicated inhibitors, 50 μM. Biotin-labeled anti-CD56 (B159.5) and anti-CD16 (3G8) mAb, 20 μg/ml, with added streptavidin, 50 μg/ml; PMA, 5 ng/ml; ionomycin, 1 mM (see Materials and Methods). Relative F-actin content was determined in the cells by flow cytometry after NBD-phallacidin incorporation. Values are the ratio between mean fluorescence intensity in stimulated and nonstimulated cells (mean ± SD, n = 5, 10,000 cells sample analyzed). * p < 0.01 (two-tailed, Student t test), CD16 or PMA/ionomycin vs CD56 stimulation, under any condition; inhibitor-treated vs control nontreated cells, nonsignificant.

**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. Inset, 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 kinase assay was performed on the MAPKAP K-2 immunoprecipitated from the cell lysates using KKLNRTLSVA 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 ^51^Cr release assay. Target cells were 5 × 10^4/well. The x-axis shows the E:T cell ratio; the y-axis shows the percent specific ^51^Cr 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 analysis performed on the lysates using anti-phospho c-Jun Ab (top panel), anti-FLAG (middle panel), and anti-HSP90 (bottom panel) sequentially on the same filters to assay for c-Jun phosphorylation, Vac-encoded protein expression, and total amount of protein loaded per lane. 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. 5A, right panel). Additionally, CD16-redirected and spontaneous cytotoxicity were not inhibited in APF-JNK1-expressing NK cells or in NK cells infected with wt (Fig. 5B) 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).
Discussion

We report that phosphorylation and activation of the p38 MAPK occurs in human primary NK cells upon FcγRIIIA ligation and target cell binding, and that, like ERK-2 (21, 23), it plays a role in cytotoxicity and cytokine expression, whereas JNK, activated exclusively upon FcγRIIIA ligation, does not play a role in either ADCC or spontaneous cytotoxicity. 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 cytotoxicity 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 cytotoxicity 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 cytotoxicity 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 cytotoxicity 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 cytotoxicity 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 S6 kinase, 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 cytotoxicity.

Direct detection of perforin- and granzyme B-containing intracellular 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...
some cytokines has been reported for p38 (39, 51), and the lev-
el(s) at which this kinase regulates FcγRIIA- 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 FcγRIIA 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 VAC-encoding inactive kinase is inappropriate to study non-

immediate events that require host cell RNA transcription and pro-
tein 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.

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