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IL-2 Activation of NK Cells: Involvement of MKK1/2/ERK But Not p38 Kinase Pathway

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IL-2 stimulates extracellular signal-regulated protein kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) in various immune cell populations. The functional roles that these kinases play are still unclear. In this study, we examined whether MAPK (MKK)/ERK and p38 MAPK pathways are necessary for IL-2 to activate NK cells. Using freshly isolated human NK cells, we established that an intact MKK/ERK pathway is necessary for IL-2 to activate NK cells to express at least four known biological responses: LAK generation, IFN-γ secretion, and CD25 and CD69 expression. IL-2 induced ERK activation within 5 min. Treatment of NK cells with a specific inhibitor of MKK1/2, PD98059, during the IL-2 stimulation blocked in a dose-dependent manner each of four sequelae, with inhibition of lymphokine-activated killing induction being least sensitive to MKK/ERK pathway blockade. Activation of p38 MAPK by IL-2 was not detected in NK cells. In contrast to what was observed by others in T lymphocytes, SB203850, a specific inhibitor of p38 MAPK, did not inhibit IL-2-activated NK functions. This data indicate that p38 MAPK activation was not required for IL-2 to activate NK cells for the four functions examined. These results reveal selective signaling differences between NK cells and T lymphocytes; in NK cells, the MKK/ERK pathway and not p38 MAPK plays a critical positive regulatory role during activation by IL-2.

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3 Abbreviations used in this paper: ERK, extracellular signal-regulated protein kinase; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MKK, mitogen-activated protein kinase kinase; JAK, Janus kinase; LAK, lymphokine-activated killing; MBP, myelin basic protein; ATF-2, activating transcription factor 2.
did not stimulate ERK phosphorylation in CD3+CD8−CD18+ bright T cells in a previous study (15), we demonstrate that IL-2 activated MKK/ERK activity in freshly isolated NK cells. To determine the functional role of the MKK/ERK pathway, we utilized a highly specific inhibitor of the MKK/ERK pathway, PD98059, which binds to the inactive forms of MKK1/2 and prevents their activation by upstream regulators (23). PD98059 is quite specific for ERK; it does not inhibit 18 other serine/threonine kinases in vitro and p38 and Jun N-terminal kinase MAPK in vivo (23). We examined four sequelae of IL-2-induced NK activation, including enhanced cytolytic activity, IFN-γ secretion, and up-regulation of activation markers CD25 and CD69, and determined that all depended on intact MKK/ERK function. Unlike what was observed in CD3+CD8−CD18+ T cells, IL-2 did not initiate p38 activity in NK cells. In addition, when p38 kinase activity was blocked during IL-2 stimulation by SB203580, the same p38 kinase inhibitor used in a previous T lymphocyte study (15), NK activation was not inhibited. These data suggest that MKK/ERK pathway, not p38 kinase, exerts a positive regulatory signal during IL-2 activation of NK cells, which is different from that previously reported in the IL-2 activation of T lymphocytes.

**Materials and Methods**

**NK cell and T cell isolation, cell culture, and activation**

NK cells were isolated as described previously (24). Briefly, the PBMC were isolated from the venous blood of healthy donors using Histopaque (density = 1.077, Sigma, St. Louis, MO). The PBMC were purified using a negative selection, magnetic cell separation system (Miltenyi Biotec, Auburn, CA), according to the manufacturer’s protocol to obtain a highly enriched population of NK cells. Negative selection was employed to prevent NK stimulation by Ab binding. After isolation, NK cells were rested overnight in Aim V Media (Life Technologies, Grand Island, NY) at 37°C before proceeding with the experiments. Purity of the resultant cell population was measured using flow cytometry by labeling the cells with fluorochrome-conjugated anti-CD3, -CD14, -CD19, and -CD56 Abs (Becton Dickinson, Franklin Lakes, NJ) as described previously (24). The resultant population of a typical experiment was above 85% CD56+ <5% CD3−<1% CD14+ and CD19+. To separate NK cells from T cells from a single donor, macrophages were depleted by adherence for 1.5 h at 37°C/7% CO2. NK cells were isolated from the resultant PBLs using anti-CD56 Ab-conjugated beads (Miltenyi Biotec) per the manufacturer’s instructions. The leftover population was designated as total T cell population.

Purified NK cells were pretreated with 0–50 μM PD98059 or 0–10 μM SB203580. As a negative vehicle control, cells were treated with DMSO at a concentration in a sample collection was 1%. Immediately after stimulation, the NK cells were placed on ice and lysed in 1% Nonidet P-40, 20 mM Tris, 250 mM NaCl, 3 mM each of EDTA and EGTA, orthovanadate, p-nitrophenyl phenolphosphate, β-glycerophosphate, and DTT) supplemented with protease inhibitors (aprotinin, leupeptin) and subjected to fast centrifugation (14,000 rpm × 30 min) at 4°C. The supernatant was collected and frozen at −80°C. Periodically, the IFN-γ concentration in a sample collection was determined with an IFN-γ ELISA kit (Endogen, Woburn, MA) according to the manufacturer’s instructions.

**Results**

**IL-2 activates ERK, but not p38 MAPK, in NK lymphocytes without prior stimulation**

In this study, we collected PBMC from healthy human volunteers and isolated NK cells; the cells were analyzed in experiments without undergoing long-term culture or without any prior in vitro stimulation from cytokines, Ags, or Abs. To determine activation states of ERK and p38 kinase in NK cells, kinase activity in response to increasing concentrations of IL-2 was measured with an in vitro kinase assay using as substrates MBP and ATF-2, respectively. An appreciable increase in ERK activity was observed within 5 min of IL-2 stimulation (Fig. 1A). Above 10 U/ml IL-2, activation peaked between 15 and 30 min and persisted through 30 min. As the concentrations of IL-2 increased, response became more brisk and more intense. However, after a 2-day culture with IL-2, ERK activity in NK cells was comparable to the level in unstimulated cells (data not shown).

A highly selective inhibitor of MKK, PD98059, was utilized to further delineate the importance of MKK molecules during IL-2 activation. PD98059 treatment at concentrations comparable to the range used by other studies blocked MKK stimulation of ERK

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activity in response to IL-2 (Fig. 1A). To verify specificity and potency of the inhibitor, NK cells were treated with increasing concentrations of PD98059 and stimulated with 40 U/ml IL-2 under similar conditions as those in Fig. 1A. The extent to which IL-2-induced ERK activation was inhibited depended on the PD98059 concentration used (Fig. 1B). To further investigate the efficacy of PD98059, Jurkat B64.10 cells that had been transfected with IL-2Rβ chain to confer IL-2 responsiveness were treated with 50 μM PD98059 and stimulated with increasing concentrations of IL-2. At low IL-2 concentrations, ERK activation measured at 15 min of stimulation was completely blocked. However, higher IL-2 concentrations activated additional ERK activity in cells treated at the same PD98059 concentration (data not shown). This finding was consistent with that observed during the initial characterization of PD98059 (23).

In contrast, when p38 activity was assessed in NK cells after IL-2 stimulation, activation of p38 kinase was not detected even when the cells were stimulated with high concentrations of IL-2 (400 U/ml; data not shown). A previous report showed that IL-2

FIGURE 1. Kinetics and dose dependency of IL-2 activation of ERK and dose-dependent inhibition by PD98059. A, Freshly isolated NK cells were activated with 0–80 U/ml IL-2 for 5–30 min. At each IL-2 concentration, one sample (PD) was treated with 50 μM PD98059 for 2 h and activated with IL-2 for 30 min. ERK activity was measured with in vitro kinase reaction using MBP as substrate. B, Freshly isolated NK cells were treated the same as above with 2–50 μM PD98059 or with DMSO (DM) and then activated with 40 U/ml IL-2 for 15 min. ERK activity was measured as above.

FIGURE 2. Comparison of IL-2 activation of ERK and p38 in NK and T cells. A, Freshly isolated NK cells and T cells from a single donor were activated using 200 U/ml IL-2 or 200 U/ml IL-2 and 100 ng/ml IL-12, respectively, for 15 min. p38 MAPK activity was measured by in vitro kinase reaction using ATF-2 as substrate and probing for phospho-ATF-2 (Thr71) in the Western blot analysis. B, Same two populations of cells from above were activated by 80 U/ml IL-2 for 15 min. ERK activity was measured as described in Fig. 1.
with IL-12-induced phosphorylation of p38 kinase and not ERK in CD18<sup>bright</sup> T lymphocytes (15). We wanted to compare p38 kinase response to IL-2 between NK cells and T cells. Therefore, PBMC from a single healthy volunteer were separated into NK lymphocytes and total T lymphocytes. Then the two cell populations were activated for 15 min with 200 U/ml IL-2 or 200 U/ml IL-2 and 100 ng/ml IL-12, respectively. About 30 × 10<sup>6</sup> T cells and 6 × 10<sup>6</sup> NK cells were used per condition to increase sensitivity of detection. In experiments.

**FIGURE 2.** IL-2 with IL-12-induced p38 kinase activity by hypertonic salt. We treated YT cells (NK cell line) with increasing concentrations of SB203850 to inhibit p38 kinase activity by hypertonic salt. Then we assessed p38 kinase activity as before. SB203850 at 5 and 10 μM concentrations blocked p38 kinase activation to near completion (data not shown).

IL-2 activation of an MKK/ERK pathway is required for NK cells to acquire LAK activity

NK cells possess an intrinsic cytotoxic function toward only a limited number of NK-sensitive targets (e.g., the K562 cell line). Freshly isolated NK cells that were stimulated with as low as 5 U/ml IL-2 for 2 days acquired enhanced cytolytic function (LAK activity) and were able to lyse Raji cells, which were previously resistant. When NK cells were pretreated with PD98059 at concentrations similar to what other studies used, LAK induction was inhibited. As the cells were treated with increasing concentrations of PD98059, IL-2 induction of LAK decreased further; inhibition of >50% was achieved with 30 μM PD98059 at 10 U/ml IL-2 stimulation (Fig. 3A). When higher concentrations of IL-2 (up to 40 U/ml) were used, PD98059 inhibition gradually decreased (Fig. 3C), which correlated with a previous report that PD98059 is a weak inhibitor that is unable to block a strong agonistic signal (23). The inhibitory effect of PD98059 was specific and limited to acquisition of LAK activity. NK cells treated with 50 μM PD98059 (the highest used) still maintained NK activity at levels comparable to those of the control cells with no treatment (Fig. 3B). Treatment with PD98059 also did not affect viability or recovery (data not shown).

**FIGURE 3.** Effect of PD98059 on NK cytolytic activity. A, Inhibition of IL-2-activated LAK activity by PD98059. NK cells were treated with 0-50 μM PD98059 for 2 h and then activated with 10 U/ml IL-2 for 2 days. 51Cr release assay was performed to measure LAK activity using Raji cells as target. B, PD98059 does not inhibit intrinsic NK activity. NK cells were treated with 50 μM PD98059, and NK activity was measured at different concentrations of IL-2 as above using K562 as target. C, Higher dose of IL-2 overcomes PD98059 inhibition. NK cells were treated with 0 or 30 μM PD98059, and LAK activity was measured at different concentrations as in A. Cytotoxicity is given at E:T ratio of 40:1 as the mean of triplicate measurements ± SD. Data shown are representative of three similar experiments.

IL-2-driven IFN-γ secretion and induction of CD25 and CD69 activation markers require intact MKK/ERK pathway

When NK cells are stimulated with IL-2, secondary cytokine secretion is induced. The IFN-γ levels in culture were measured to determine whether PD98059 treatment could block IL-2-induced cytokine secretion. Freshly isolated NK cells cultured with 30–80 U/ml IL-2 for 2–5 days led to IFN-γ secretion. At each IL-2 concentration, blocking of MKK activity in NK cells with PD98059 resulted in less IFN-γ secretion. In a representative experiment, NK cells that were treated with 30 μM or 50 μM PD98059 and stimulated with 80 U/ml IL-2 for 3 days secreted similar levels of IFN-γ as those NK cells that were not stimulated with IL-2 (Fig. 4).

As NK cells are activated, they express on their cell surface some well-recognized activation markers, CD25 and CD69. Since intact MKK/ERK was necessary for IL-2 to induce LAK generation and IFN-γ secretion, we wanted to analyze whether expression of these activation markers involved the same pathway. NK cells treated with PD98059 were activated with IL-2 for 3–5 days, and the expression of CD25 and CD69 was analyzed using flow cytometry. Resting NK cells from different donors showed variable levels of CD25 and CD69, but in each case, when the cells were stimulated with IL-2, expression of these molecules increased from 2- to almost 12-fold (data not shown). Treatment of NK cells with PD98059 again prevented the up-regulation of CD25 and vate p38 MAPK in NK cells from the same donor treated under similar conditions. When the same two populations from a single donor were used to determine the ERK response, IL-2 activated ERK in NK cells as before whereas it did not induce ERK activation in T lymphocyte (Fig. 2B). Higher basal ERK activity in T cells in Fig. 2B was most likely due to the greater amount of total cellular protein and anti-ERK2 Ab used in T cells than in NK cells. IL-2 with IL-12 also did not activate p38 kinase in NK cells (data not shown).

To determine in vivo effectiveness of the p38 kinase inhibitor, we treated YT cells (NK cell line) with increasing concentrations of SB203850 to inhibit p38 kinase activation by hypertonic salt. Then we assessed p38 kinase activity as before. SB203850 at 5 and 10 μM concentrations blocked p38 kinase activation to near completion (data not shown).
CD69. Increased doses of the inhibitor led to further decreases in CD25 and CD69 expression comparable to the baseline level (Fig. 5).

**SB203580 inhibition of p38 kinase during IL-2 stimulation did not block NK activation**

SB203580, a specific inhibitor of p38 kinase activity, was reported to block IL-2/IL-12-induced activation and proliferation of CD3⁺CD8⁺CD18bright T lymphocytes (15). Although we did not observe any induction of p38 activity by IL-2 in NK cells, p38 kinase activation possibly could have occurred during time periods we did not test or the level of activation could have been below our detection. To confirm that p38 kinase did not have a positive regulatory role during IL-2 activation of NK cells, we examined whether SB203580 could exert a similar inhibitory effect during NK activation by IL-2 as it did during CD3⁺CD8⁺CD18bright T cell activation. NK cells were treated with SB203580 at similar concentrations used to demonstrate p38 kinase requirement during CD8⁺CD18bright T cell activation (15) and then activated with IL-2. The effects of SB203580 on the four activation parameters were examined. When SB203580 (at concentrations demonstrated to inhibit p38 kinase in a previous section of Results) was used during IL-2 activation, levels of LAK activation (Fig. 6A), IFN-γ secretion (Fig. 6B), and CD25 and CD69 expression (Fig. 6C) were not reduced. Treatment of NK cells with SB203580 at concentrations...
as high as 20 μM did not block NK activation by IL-2. In fact, in some of our experiments, augmentation in the level of activation parameters was observed in the SB203580-treated cells.

Discussion

Activation of MAPK family members has been implicated in various immune functions. It has been shown that IL-2 can activate MKK/ERK and p38 in lymphoid cell lines and primary T lymphocytes only after Ag stimulation (14). However, in CD3+CD8+CD18bright T lymphocytes that express functional IL-2R and IL-12R, IL-2 and IL-12 did not induce ERK phosphorylation, but did cause p38 MAPK phosphorylation (15). Our present study demonstrates that IL-2 is able to activate the MKK/ERK pathway within few minutes as part of early signaling events without any prior stimulation in freshly isolated NK lymphocytes and not T lymphocytes. More important, we demonstrate that optimal IL-2 activation of NK lymphocytes is sensitive to a highly specific inhibitor of MKK kinase, PD98059, implicating a positive regulatory function for MKK/ERK pathway during early IL-2 signaling events. Additionally, our study shows that in NK lymphocytes, unlike in T lymphocytes, IL-2 does not send signals through p38 kinase, and p38 kinase activity is not required for NK activation of at least four biological responses.

In fresh NK lymphocytes, stimulation of MKK/ERK pathway depended on both the dose of IL-2 and the length of stimulation. ERK activation occurred early as part of IL-2R early signaling events. The level of ERK activity achieved by different concentrations of IL-2 correlated with the level of LAK activity that was induced. PD98059 prevented IL-2 stimulation of ERK activity by blocking MKK activation in a dose-dependent manner.

The functional consequences of IL-2 activation of the MKK/ERK pathway have been elusive. Using truncated IL-2Rβ chain in BAF-3 cells, Hatakeyama et al. (25) have shown that IL-2-induced proliferation is not dependent on MAPK activity. Similar conclusions were made in primary T cells (26). In our present study, we demonstrated that PD98059 used at concentrations comparable to other studies effectively prevented four aspects of IL-2-induced activation processes, implicating a positive regulatory role for the MKK/ERK pathway during activation. We documented a dose-dependent inhibition of LAK induction, inhibition of IFN-γ secretion, and inhibition of increased expression of CD25 and CD69.

During the characterization of PD98059, Alessi et al. (23) measured activities of MKK1/2 and their downstream targets (p42 MAPK and MAPKAP-1αβ) in cells treated with PD98059 and various agonists. The authors found that lower percentage inhibition was consistently observed for targets sequentially down the MKK/ERK pathway. For example, in cells treated with 50 μM PD98059 and activated with epidermal growth factor, 87% of MKK activity was inhibited whereas p42 MAPK and MAPKAP-1αβ were inhibited only by 33 and 13%, respectively (23). For LAK generation in our study, inhibition occurred at higher concentrations of PD98059 than that was apparently needed to partially block ERK activation. Similarly to what was described above, the function of ERK’s downstream targets which were needed for LAK induction were probably not affected by low PD98059 concentration, even though ERK activity was partially blocked. Most likely, more PD98059 was needed to reduce ERK activity below a threshold where activities of ERK’s downstream targets became affected. Thus, once the target’s activity was inhibited, LAK induction was blocked. Alternatively, there may exist other pathway(s) that could compensate for a small loss of ERK activity during IL-2 activation. However, those pathway(s) probably were not able to compensate for the larger losses in ERK activity caused by higher PD98059 concentrations, and, thus, less LAK activity was induced. We also observed that higher IL-2 concentrations could overcome PD98059 blockade of LAK induction. This probably occurred because as mentioned in Results, higher IL-2 concentrations led to additional ERK activity at 15 min of stimulation in the same population of PD98059-treated cells. This increase in ERK activity during the early IL-2R signaling phase most likely led to additional functional activation at a later time.

That PD98059 blocked these four separate functions suggests that ERK activation is an upstream event for all four events, but the relative importance of the MKK/ERK pathway to each activated NK function appears to be different. Induction of MKK/ERK activity appears to be more critical for NK cells to secrete IFN-γ in response to IL-2 because most IL-2-driven IFN-γ secretion was blocked at low concentrations of PD98059, even when a high concentration of IL-2 was used for activation. Conversely, higher concentrations of PD98059 were required to fully block LAK generation. Thus, we propose that LAK induction may rely less on the MKK/ERK pathway; i.e., the downstream effectors of MKK/ERK may contribute less to activate LAK than IFN-γ production or, as mentioned above, there may be one or more alternative pathways that are activated by IL-2 that could compensate for the loss of the MKK/ERK during the process of LAK induction.

One or more of many downstream effectors of the MKK/ERK pathway is likely to be required for NK activation. One highly plausible candidate is the AP-1 transcription activator, previously demonstrated to be regulated in many cell types by ERK activity (1). In a previous study, IL-2 in cultured NK cells induced c-fos, jun-B, and egr-1 members of the immediate/early activation gene and induced AP-1-mediated transcription (27). At least three AP-1-binding elements, alone or with other transcription factors, exist within 300 bp proximal to the transcriptional start site of the IFN-γ gene (28, 29). In a study involving CD4+ T cells, the AP-1 element (-196) was important for IL-12 and IL-18 activation of the IFN-γ gene (30). The promoter of CD69 activation Ag contains an AP-1 binding that activates gene transcription (31), whereas the promoter of the IL-2Rα chain contains a putative AP-1/NF-AT composite binding element (32). Blocking the MKK/ERK pathway in NK cells most likely diminished or prevented induction of AP-1 transcriptional activity by IL-2 and thus prevented transcription of those genes that are dependent on AP-1 activity.

Our study for the first time links the MKK/ERK pathway to IL-2-induced LAK generation. The identity of the target molecules of MKK/ERK that are necessary to induce LAK activity is harder to determine because the exact nature and identity of the molecules responsible for this are not well understood. Many surface molecules have been implicated in the activation of LAK function. CD69, CD40 ligand, and Nkp44 were shown to be expressed on NK cells only after incubation with IL-2, and the expression of these molecules resulted in enhanced cytotoxicity of certain targets (33–35). In our present study, MKK/ERK activation was necessary for IL-2 regulation of CD69 expression. The MKK/ERK-dependent expression of CD69 and possibly other molecules necessary for the recognition of target molecules and the triggering of cytotoxicity probably ultimately resulted in LAK dependence on the MKK/ERK pathway. It should be noted that the requirement for ERK in NK killing, but...
not in LAK activation, has been reported previously and indi-
cates a separate role for MKK/ERK (36, 37). In our study, NK killing (as demonstrated by K562 lysis) was not affected by PD98059 because NK cells were treated with PD98059 only during the IL-2 activation phase.

Although T cells and NK cells share the same progenitor, they differentiate via different cytokine milieu and develop close, but different, effector functions (38). IL-2 causes many overlapping effects in T and NK lymphocytes, but each cell type has distinct responses. Our data indicate that, unlike T lympho-
cytes, NK lymphocytes do not require p38 kinase activity during IL-2 responsiveness. The differences in MKK/ERK and p38 kinase activity in T vs NK lymphocytes after stimulation via the same IL-2 receptor system suggest that effector components or interactions of effectors differ between the two cell types. For example, IL-2 activates STAT1, STAT3, and STAT5 in both T and NK lymphocytes, but IL-2 also activates STAT4 only in NK lymphocytes (39). With ERK activation occurring within 15–30 min of IL-2 stimulation, activated STAT4 functioning as a transcription factor in NK cells most likely could not directly influence MAPK activity during the early signaling period. However, the presence of different STATs in NK and T cells during the development of the cells could differentially affect transcription and ultimately the protein level of coupling pro-
tiens required to selectively activate either ERK or p38 MAPK even before IL-2 stimulation. Alternatively, the differential coupl-
ing of ERK and p38 MAPK to IL-2R in NK and T cells, respectively, may lie in the finding that IL-2 activates JAK2 preferentially in NK cell lines and not in activated T cells (39). In the growth hormone receptor system, Winston and Hunter (40) demonstrated that activation of ERK depended on JAK2, Raf-1, and RAS. In that study, the growth hormone stimulated ERK only with kinase-active JAK2 present. As in the growth hormone receptor system, IL-2 may activate ERK only in NK cells because JAK2 was activated only in NK cells.

In summary, our study demonstrates that IL-2 receptor sig-
nals are transmitted through the MKK/ERK pathway to induce several functions in NK cells. Curiously, the IL-2 receptor does not utilize the p38 MAPK during activation of NK cells. There are many possible downstream effectors that may be necessary to drive IL-2-induced effects. Now that the MKK/ERK pathway has been linked to IL-2-activated NK cell functions, each of the proteins that are regulated by MKK/ERK (e.g., AP-1) will be examined in future studies for its role during IL-2 activation of NK cells.

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