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*J Immunol* 1999; 162:4472-4481; 
http://www.jimmunol.org/content/162/8/4472

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The Functional Synergy Between IL-12 and IL-2 Involves p38 Mitogen-Activated Protein Kinase and Is Associated with the Augmentation of STAT Serine Phosphorylation

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IL-12 and IL-2 can stimulate mitogen- or CD3-activated T cells to proliferate, produce IFN-γ, and kill tumor cells. The magnitude of these functional responses is greatly augmented when T cells are activated by the combination of IL-12 and IL-2. Although peripheral blood T cells are largely unresponsive to these cytokines without prior activation, a small subset of CD8+ T cells (CD8+CD18bright) is strongly activated by the combination of IL-12 and IL-2. In this report we show that the functional synergy between IL-12 and IL-2 in CD8+CD18bright T cells correlates with the activation of the stress kinases, p38 mitogen-activated protein (MAP) kinase and stress-activated protein kinase (SAPK)/Jun N-terminal kinase, but not with the activation of the extracellular signal-regulated kinases. The functional synergy between IL-2 and IL-12 is also associated with a prominent increase in STAT1 and STAT3 serine phosphorylation over that observed with IL-12 or IL-2 alone. By contrast, STAT tyrosine phosphorylation is not augmented over that seen with either cytokine alone. A specific inhibitor of p38 MAP kinase completely inhibits the serine phosphorylation of STAT1 and STAT3 induced by IL-12 and IL-2 and abrogates the functional synergy between IL-12 and IL-2 without affecting STAT tyrosine phosphorylation. This suggests that p38 MAP kinase may play an important role in regulating STAT serine phosphorylation in response to the combination of IL-12 and IL-2. Furthermore, these findings indicate that the optimal activation of T cells by IL-12 and IL-2 may depend on an interaction between the p38 MAP kinase and Janus kinase/STAT signaling pathways. The Journal of Immunology, 1999, 162: 4472–4481.

Interleukin-12 and IL-2 have both overlapping and distinct effects on T cell activation. To respond to either IL-12 or IL-2, T cells must first be activated with a mitogen such as PHA or through ligation of the TCR/CD3 complex (1). One of the primary mechanisms through which T cell activation confers responsiveness to IL-12 or IL-2 is through the up-regulation of the receptor subunits for these cytokines (2–5). Although both cytokines stimulate cytolytic activity (6), proliferation (7, 8), and IFN-γ production (9) by activated T cells, the magnitudes of these functional effects elicited by IL-12 and IL-2 are different. For example, while IL-2 is the more potent stimulator of proliferation and cytolytic activity (7, 10, 11), IL-12 is a stronger inducer of IFN-γ production. In addition, IL-12 differs from IL-2 in its ability to promote the differentiation of CD4+ T cells into Th1 cells (12, 13). The stimulation of T cells with IL-12 and IL-2 together has an additive effect on cytolytic activity and a synergistic effect on proliferation and IFN-γ production (8, 9). While resting T cells are largely unresponsive to IL-12 or IL-2, a small number of resting T cells appear to be capable of producing IFN-γ when stimulated with the combination of IL-12 and IL-2 (9).

The effects of IL-12 and IL-2 on T cell function reflect the similarities and differences in the signaling pathways that are activated in T cells by these cytokines. Among the MAP kinases, IL-2 has been shown to activate extracellular signal-regulated kinase-1 (ERK1) and ERK2 in T cells (14, 15) as well as p38 MAP kinase in a murine T cell line (16), while IL-12 can activate ERK1 in T cells (17). However, it is not known whether ERK or p38 MAP kinase activation plays a role in IL-2- or IL-12-mediated cytolytic activity or IFN-γ production in T cells. One report has suggested that ERK2 activation is necessary but not sufficient for IL-2-induced T cell proliferation (14). Several other studies have implicated p38 MAP kinase in the proliferative response of T cells to IL-2 and IL-7 (16) and in the induction of IL-6 production by TNF-α (18).

IL-12 and IL-2 also activate both distinct and overlapping components of the JAK/STAT signaling pathway in activated T cells. Whereas IL-12 activates JAK2 and Tyk2, IL-2 activates JAK1 and JAK3 (19). STAT1, STAT3, and STAT5 (20, 21) are activated in response to IL-2, while STAT1, STAT3, STAT4, and STAT5 can all be activated in response to IL-12 in T cells (20, 22, 23). The important role of JAK/STAT signaling in mediating the functional effects of IL-12 has been demonstrated by the phenotype of STAT4 knockout mice (24). In mice lacking STAT4, all the major functional effects of IL-12 on T and NK cells are markedly inhibited. The importance of JAK/STAT signaling in lymphocyte IL-2 responsiveness has been shown by the diminished functional response to IL-2 in JAK3 and STAT5 knockout mice (25, 26). In addition to tyrosine phosphorylation, cytokines can induce the

3 Abbreviations used in this paper: MAP, mitogen-activated protein; JAK, Janus kinase; PE, phycoerythrin; ATF-2, activating transcription factor-2; ERK, extracellular signal-regulated kinase; SAPK, stress-activated protein kinase.
serine phosphorylation of STATs as well. The tyrosine phosphorylation of STATs is required for dimerization, nuclear translocation, and DNA binding and is therefore necessary to induce STAT-responsive gene transcription. In addition, STAT serine phosphorylation may be an important modulator of both STAT DNA binding and STAT-mediated transcriptional activity (27, 28). However, the relative importance of STAT tyrosine phosphorylation vs STAT serine phosphorylation in the functional response of T cells to cytokines remains undefined. Several cytokines have been shown to induce STAT serine phosphorylation in addition to tyrosine phosphorylation. IL-2 can induce the serine phosphorylation of STAT5 (29), whereas IL-6 (30) and IFN-γ (27) can stimulate STAT3 and STAT1 serine phosphorylation, respectively. Both IL-12 and IFN-α stimulate STAT4 serine phosphorylation (31).

The tyrosine phosphorylation of STATs induced by IL-12 or IL-2 is mediated by the Janus family kinases that associate with IL-12 or IL-2 receptor subunits and are activated following receptor ligation. However, the serine/threonine kinase(s) responsible for cytokine-induced STAT serine phosphorylation in lymphocytes has not been identified. While epidermal growth factor-induced STAT3 serine phosphorylation in a fibroblast cell line appears to be mediated by ERKs (30), STAT3 serine phosphorylation by IL-6 in HepG2 cells (30) and STAT5 serine phosphorylation by IL-2 in a human T cell line (29) were both found to occur independently of ERK activation. In human chronic lymphocytic leukemia cells, the constitutive serine phosphorylation of STAT1 and STAT3 was found to also occur independently of ERK activation (32). It is not known whether other serine/threonine kinases such as p38 MAP kinase or stress-activated protein kinase (SAPK)/JNK may be capable of mediating cytokine-induced STAT serine phosphorylation in T cells.

Although the JAK/STAT and MAP kinase family signaling pathways appear to play important roles in the functional response of T cells to IL-2 and IL-12, very little is known about the signaling mechanisms that underlie the functional synergy observed between IL-12 and IL-2. One report found that IFN-γ mRNA transcripts were stabilized in lymphocytes stimulated with IL-12 and IL-2 (33), suggesting that this might be responsible for the synergistic increase in IFN-γ production. Although there is evidence that the functional synergy between IL-12 and IFN-γ-inducing factor (IL-18) is partly mediated through the IL-12-induced up-regulation of IL-18R (34), the synergy between IL-12 and IL-2 does not appear to be mediated at the level of cytokine receptor expression. We wanted to determine whether the synergistic activation of T cells by the combination of IL-12 and IL-2 was mediated through the modulation of JAK/STAT and/or MAP kinase family signaling. By examining the activation of these signaling pathways in a subset of peripheral blood T cells that respond poorly to IL-12 alone but demonstrate strong functional responsiveness to the tyrosine 694-phosphorylated form of STAT5 (20, 35, 36). The p38 MAPK-STAT3 Ab recognizes the tyrosine 705-phosphorylated form of STAT3. The phospho-STAT1 and phospho-STAT5 Abs specifically recognize the serine 727-phosphorylated forms of STAT1 and STAT3, respectively (32).

Materials and Methods

Abs and cytokines

The following unconjugated and PE- or FITC-conjugated Abs were obtained from Coulter (Hialeah, FL): negative control, anti-CD4, anti-CD8, anti-CD56 (NK1), and anti-CD20 (B1). Anti-CD18 (8C12, IgM) has been previously described (7) and does not activate resting or PHA-stimulated T cells. In addition, it does not augment the response of resting or PHA-activated T cells to IL-12 or IL-2. 8C12 was used as a 1/400 dilution of mouse ascites. FITC-conjugated goat anti-mouse IgM was purchased from Southern Biotechnology Associates (Birmingham, AL). Unconjugated anti-CD4 (19thy1) and anti-CD56 (N901) Abs were used as dilutions of mouse ascites.

The p38 MAP kinase inhibitor (SB203580) has been described previously and was provided by Dr. John C. Lee (SmithKline Beecham Pharmaceuticals, King of Prussia, PA). SB203580 was dissolved in DMSO (Tocris, Elgol Pharmaceuticals, Buena Park, CA) at a concentration of 10 mM and stored at −20°C. The MEK inhibitor PD98059 was provided by New England Biolabs.

Isolation of PBMC and T cell subsets

Blood samples enriched for white blood cells were obtained from normal volunteer donors undergoing platelet pheresis in the Dana-Farber Cancer Institute Blood Bank. PBMC were isolated from blood samples through density gradient centrifugation using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden).

For the isolation of CD18bright and CD18dim T cell subsets, PBMC were first depleted of monocytes through passage over a nylon wool column. For the preparation of CD8-enriched T cells, monocyte-depleted PBMC were depleted of CD4+ T cells, B cells, and NK cells using Abs to lineage-specific markers (anti-CD4, anti-CD20, and anti-CD56) and magnetic beads coated with anti-mouse Ig Abs (Advanced Magnetics, Cambridge, MA). CD8-enriched T cell preparations were then stained with CD18-FITC and CD8-PE, and PE-positive cells were separated on a FACSort (Coulter EPICS Elite ESP) into CD18bright and CD18dim populations based on CD18FITC fluorescence intensity (CD18bright cells routinely had a CD18FITC mean fluorescence intensity 5–6 times greater than CD18dim cells). Another method for obtaining CD18bright T cells involves the use of PBMC depleted of monocytes alone. These cells were stained with CD18FITC as well as PE-conjugated anti-CD56 and anti-CD20 Abs. PE-negative cells were then sorted based on CD18FITC fluorescence intensity. Through this method, CD18bright T cells were 0% CD56+, 100% CD3+, 95–98% TCR ab+, and >90% CD8+. Cell sorting routinely yielded a maximum of 3–4 × 10^6 CD18bright and CD18dim T cells/experiment.

T cell stimulation and preparation of whole cell lysates for Western blots

Sorted T cells were washed and stimulated for 15 min at 37°C in a total volume of 800 μl under the following conditions: medium alone (RPMI 1640 and 2.5% FCS), 100 μM IL-12, 100 μM IL-2, or IL-12 plus IL-2. Approximately 0.75–1.0 × 10^6 sorted T cells were used per condition. For experiments involving the p38 MAP kinase inhibitor, cells were incubated in medium with either 1–10 μM SB203580 or DMSO vehicle for 1 h at 37°C before addition of cytokine. For experiments involving the MEK inhibitor PD98059, cells were incubated in medium alone or 50 μM PD98059 for 1 h at 37°C before addition of cytokine. For experiments with PMA (Sigma, St. Louis, MO), CD8-enriched T cells were stimulated with 100 ng/ml PMA for 15 min. After stimulation, cells were washed once with ice-cold PBS and then lysed on ice for 20 min in lysis buffer containing 1% Nonidet P-40, 50 mM Tris (pH 8.0), 150 mM NaCl, 2 mM EDTA, 1 mM aprotinin, 100 μg/ml PMSF, 1 mM sodium orthovanadate, and 1 mM NaF. Whole cell lysates were mixed with an equal volume of 2% reducing sample buffer and boiled, and proteins were resolved on a 10% polyacrylamide gel.

For Western blots, proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH) by electroblotting, and membranes were then blocked for 30 min in Tris-buffered saline containing 0.1% Tween-20 (Bio-Rad, Hercules, CA) and either 5% BSA (U.S. Biochemical Corp., Cleveland, OH) or 5% nonfat dried milk. Membranes were
incubated with dilutions of the indicated Abs for 1 h at room temperature, washed with Tris-buffered saline/Tween-20, incubated with either horseradish peroxidase-conjugated rabbit anti-mouse or goat anti-rabbit Abs (diluted 1:10,000) for 1 h, washed again, and developed using enhanced chemiluminescence (Amersham, Aylesbury, U.K.). When reprobed, membranes were first stripped by incubating in a solution containing 2% SDS, 100 mM 2-ME, and 62.5 mM Tris-HCl (pH 6.7) for 30 min at 65°C.

**Proliferation assays and measurement of IFN-γ production**

Sorted T cells were incubated in 96-well U-bottom plates at 3 × 10^5 cells/well with medium alone or the indicated concentration of cytokines at 37°C. For experiments involving the p38 MAP kinase inhibitor, 1–10 μM SB203580 or DMSO vehicle was added at the same time as the cytokines. Proliferation assays were performed as previously described and consisted of a 96-h incubation, with 1 μCi [3H]thymidine (DuPont-New England Nuclear, Boston, MA) added 8 h before harvesting. For IFN-γ assays, supernatants were harvested after a 72-h incubation, and the IFN-γ concentration was assayed using an IFN-γ ELISA (Endogen, Cambridge, MA).

**Cytotoxicity assays**

Sorted CD8^+ CD18^bright T cells were first incubated overnight in 96-well, U-bottom plates with either medium alone or the indicated cytokines at 37°C before the addition of target cells. For experiments involving the p38 MAP kinase inhibitor, 1–10 μM SB203580 was added at the same time as the cytokines. COLO or OKT3 cells were labeled with ^31^Cr and added to the T cells at a 5:1 E:T cell ratio. T cells were incubated with the labeled target cells for 4 h at 37°C, and the supernatants were harvested. ^31^Cr release was assayed using a gamma counter, and the percent specific cytotoxicity was calculated as previously described (11).

**Results**

*p38 MAP kinase and SAPK/JNK are activated by the combination of IL-12 and IL-2 in CD18^bright T cells*

To identify the signaling pathways that mediate the functional synergy between IL-12 and IL-2 in T cells, we examined a subset of peripheral blood T cells that are unique in their ability to respond to these cytokines. The majority of freshly isolated peripheral blood T cells, in the absence of additional activation, do not respond to IL-12, IL-2, or the combination of IL-12 and IL-2. These T cells have a naive phenotype, which includes the relatively weak response to IL-12, IL-2, or the combination of IL-12 and IL-2 (37). In vitro, these T cells respond weakly to IL-12 or IL-2 alone. However, they are strongly activated by the combination of IL-12 and IL-2 to proliferate and produce IFN-γ. In addition, stimulation of these CD8^+ T cells with IL-12 and IL-2 augments non-MHC-restricted as well as CD3-mediated killing of tumor cells. Recent reports have suggested that small subpopulations of peripheral blood lymphocytes in mice, including Vα14 NKT cells and CD8^+ CD44^high T cells, may mediate the antitumor effect of IL-12 (38) and virus-induced bystander T cell proliferation (39), respectively. In humans, the CD8^+ CD18^bright T cells may play a key role in both innate and acquired immunity to infectious pathogens and tumors by virtue of their dual ability to undergo TCR-independent activation via stimulation with cytokine combinations such as IL-12 and IL-2 or IL-12 and IL-15 and to be activated through the TCR by specific Ags. In addition, these T cells may mediate the antitumor effect of cytokines such as IL-12 and IL-2 that are administered to patients with cancer. As CD8^+ CD18^bright T cells can uniquely respond to the combination of IL-12 and IL-2 without first requiring additional in vitro activation with mitogens or CD3 ligation (37), the signaling pathways triggered by IL-12 and IL-2 can be analyzed without concern for any artifact that might be introduced by prior in vitro manipulation.

To explore whether signaling events induced by IL-12, IL-2, or the combination of IL-12 and IL-2 differed between CD18^bright and CD18^dim T cells, we first examined the pattern of protein tyrosine phosphorylation in response to these cytokines. CD8^+ CD18^bright and CD8^+ CD18^dim T cells were isolated separately through FACS and stimulated with medium alone, IL-12, IL-2, or IL-12 plus IL-2. Cells were lysed, and a Western blot was performed on whole cell lysates using a phosphotyrosine-specific Ab. In CD18^bright T cells, although the tyrosine phosphorylation of several proteins in the 60–130 kDa range was observed in response to IL-2 alone (Fig. 1A, lane 3), there was no further increase in the tyrosine phosphorylation of those proteins in response to IL-12 and IL-2 (Fig. 1A, lane 4). In contrast, several proteins in the range of 25–42 kDa were strongly tyrosine phosphorylated in response to the combination of IL-12 and IL-2 (Fig. 1B, lane 4) but were only weakly tyrosine phosphorylated or were not phosphorylated at all in response to IL-2 alone (Fig. 1B, lane 3). Protein tyrosine phosphorylation in response to IL-12, IL-2, or IL-12 plus IL-2 was not detected in CD18^dim T cells (data not shown).

The increase in the tyrosine phosphorylation of proteins in the 38–42 kDa range in response to the combination of IL-12 and IL-2 raised the possibility that activation of ERK1/ERK2 (p44/p42) or p38 MAP kinase in CD8^+ CD18^bright T cells was in part mediating the functional synergy between IL-12 and IL-2. To determine whether IL-12 or IL-2 could activate a MAP kinase family member, sorted CD18^bright and CD18^dim T cells were stimulated with medium alone, IL-12, IL-2, or IL-12 plus IL-2. Western blots were performed on whole cell lysates using Abs that recognize the activated, phosphorylated forms of p38 MAP kinase, SAPK/JNK, or ERK1/ERK2. Low level, constitutive activation of p38 MAP kinase was present in CD18^bright and CD18^dim T cells (Fig. 2A, lanes 1 and 5, upper panel). Neither IL-12 nor IL-2 alone consistently activated p38 MAP kinase in CD18^bright cells (Fig. 2A, lanes 2 and 3) or CD18^dim cells (Fig. 2A, lanes 6 and 7). However, the stimulation of CD18^bright T cells with the combination of IL-12 and IL-2 markedly increased the phosphorylation of p38 MAP kinase.
FIGURE 2. p38 MAP kinase and SAPK/JNK are activated by the combination of IL-12 and IL-2, whereas ERK1 and ERK2 are not. A, p38 MAP kinase is activated by IL-12 and IL-2 in CD18bright T cells. Sorted CD18bright or CD18dim T cells were stimulated and lysed as described in Fig. 1. A Western blot was then performed on whole cell lysates using an Ab that recognizes the activated, phosphorylated form of p38 MAP kinase (upper panel). The total amount of p38 MAP kinase protein in each lane was determined by stripping the membrane and reprobing with an Ab that recognizes total p38 MAP kinase regardless of its phosphorylation state (lower panel). Similar results were obtained in four separate experiments using cells from different donors. B, SAPK/JNK is activated by IL-12 and IL-2 in CD18bright T cells. Sorted cells were stimulated and lysed as described in A, and Western blots were performed with Abs specific for the activated, phosphorylated form of SAPK/JNK (upper panel) or total SAPK/JNK (lower panel). Arrows point to the two SAPK/JNK isoforms. Lane 9 serves as a positive control and contains the lysate from CD8+CD18bright and CD8+CD18dim T cells stimulated for 15 min with PMA. Similar results were obtained in four separate experiments using cells from different donors. C, ERK1 and ERK2 are not activated by IL-12 and IL-2 in CD18bright T cells. Sorted cells were stimulated as described in A, and Western blots were performed with Abs specific for the activated, phosphorylated forms of ERK1/ERK2 (upper panel) or total ERK1/ERK2 (lower panel). Similar results were obtained from four separate experiments using cells from different donors.

(Fig. 2A, lane 4), whereas the stimulation of CD18dim T cells with IL-12 and IL-2 did not augment p38 MAP kinase phosphorylation over the basal level (Fig. 2A, lane 8). In addition to the differential effect of the IL-12 and IL-2 combination on p38 MAP kinase activation in CD18bright and CD18dim cells, p38 MAP kinase expression was also much higher in CD18bright T cells compared with CD18dim cells (Fig. 2A, lower panel).

A similar pattern of activation was observed for SAPK/JNK. Whereas low level constitutive activation was seen in CD18bright and CD18dim subsets, strong activation by IL-12 and IL-2 of the p54 and p46 SAPK/JNK isoforms occurred only in CD18bright T cells (Fig. 2B, lane 4). In contrast with p38 MAP kinase, SAPK/JNK expression was similar in CD18bright and CD18dim cells (Fig. 2B, lower panel). Although it has been reported that IL-2 and IL-12 can activate ERK1/ERK2 in mitogen-activated T cells, no activation of ERK1 or ERK2 was detected in CD18bright or CD18dim cells in response to IL-12, IL-2, or IL-12 and IL-2 (Fig. 2C, upper panel). This was not due to the absence of ERK1/ERK2, as similar amounts of ERK1 and ERK2 were present in CD8+CD18bright and CD8+CD18dim T cells (Fig. 2C, lower panel). This was also not due to a defect in the ERK signaling pathway, as the phorbol ester PMA was able to activate ERK2 in these same CD8+ T cells (Fig. 2C, upper panel, lane 9).

Having observed that p38 MAP kinase is differentially expressed in CD18bright and CD18dim T cells and is strongly activated by stimulation with IL-12 plus IL-2 in CD18bright cells alone, we next determined whether other proximal and distal components of the p38 MAP kinase pathway were also activated by IL-12 and IL-2. The dual specificity protein kinases MKK3 and MKK6 are phosphorylated in response to cellular stresses or inflammatory cytokines such as TNF-α and IL-1 and, in turn, selectively activate p38 MAP kinase through phosphorylation on tyrosine and threonine residues (40, 41). In CD18bright T cells, there was low level basal activation of MKK3/6, which was not appreciably augmented with stimulation by IL-12 or IL-2 (Fig. 3, lanes 1–3, upper panel). However, stimulation with IL-12 plus IL-2 strongly activated MKK3/6 (Fig. 3, lane 4). This suggested that the synergistic activation of p38 MAP kinase by IL-12 and IL-2 was mediated through MKK3/6. Both p38 MAP kinase and SAPK/JNK activate ATF-2 through the phosphorylation of threonine residues (42, 43). To demonstrate that the phosphorylation of p38 MAP kinase and SAPK/JNK in response to IL-12 plus IL-2 augmented the activity of these serine/threonine kinases, we examined whether ATF-2 phosphorylation was increased by stimulation with IL-12 and IL-2. As was observed with MKK3/6, there was low level basal phosphorylation of ATF-2 in CD18bright T cells (Fig. 3, lane 1, lower panel) which was greatly augmented in response to IL-12 plus
IL-2 (lane 4, lower panel) but not in response to IL-12 or IL-2 alone (lanes 2 and 3, lower panel). This suggests that the activation of p38 MAP kinase and SAPK/JNK in response to IL-2 plus IL-12 is associated with the phosphorylation of a physiologic substrate in these cells.

The serine phosphorylation, but not the tyrosine phosphorylation, of STAT1 and STAT3 is synergistically increased by the combination of IL-12 and IL-2 in CD18^bright T cells

In addition to the activation of components of MAP kinase family pathways, signaling through cytokine receptors in lymphocytes also involves the activation of components of the JAK/STAT pathway. To determine whether the combination of IL-12 and IL-2 could augment STAT activation in CD18^bright T cells in the same manner as p38 MAP kinase and SAPK/JNK activation, we stimulated CD18^bright and CD18^dim T cells with IL-12, IL-2, or IL-12 plus IL-2. The cytokine-mediated tyrosine phosphorylation of STAT1, STAT5, and STAT3 was then analyzed by performing Western blots on whole cell lysates using Abs that specifically recognize the serine-phosphorylated forms of these STATs. No tyrosine phosphorylation of STAT1, STAT5, or STAT3 was observed in response to IL-12 and/or IL-2 in sorted CD18^dim T cells (data not shown). Among CD18^bright T cells, IL-12 and IL-2 each induced the weak tyrosine phosphorylation of STAT1 and STAT3 (Fig. 4, A and B, lanes 2 and 3). IL-2, but not IL-12, induced the relatively strong tyrosine phosphorylation of STAT5 (Fig. 4A, lane 3). Stimulation with the combination of IL-12 and IL-2 did not further increase the tyrosine phosphorylation of these STATs over that which was observed with IL-12 or IL-2 alone (Fig. 4, A and B, lane 4). Thus, the functional synergy between IL-2 and IL-12 is not associated with changes in tyrosine phosphorylation of these STATs.

Given our findings of enhanced p38 MAP kinase and SAPK/JNK activation in response to the combination of IL-12 and IL-2, we examined the effects of IL-12 and IL-2 on the serine phosphorylation of STATs using Abs that specifically recognize the serine-phosphorylated forms of STAT1 and STAT3. Stimulation of sorted CD18^dim T cells with IL-12 and IL-2, alone or together, did not result in the serine phosphorylation of STAT1 or STAT3 (data not shown). The stimulation of CD18^bright T cells with IL-12 or IL-2 weakly increased the serine phosphorylation of both STAT1 and STAT3 over the basal levels (Fig. 5, A and B, upper panels, lanes 2 and 3). However, the combined stimulation with IL-12 plus IL-2 resulted in a striking increase in the levels of STAT1 and STAT3 serine phosphorylation over that observed with either cytokine alone (Fig. 5, A and B, lower panels). This increase in STAT1 and STAT3 serine phosphorylation was not due to an increase in the total amount of STAT1 or STAT3 protein (Fig. 5, A and B, lower panels).

An inhibitor of p38 MAP kinase abrogates the serine phosphorylation of STAT1 and STAT3 in response to IL-12 plus IL-2, while a MEK inhibitor has no effect

Since the augmentation of STAT serine phosphorylation by the combination of IL-12 and IL-2 in CD18^bright T cells was associated with a strong increase in p38 MAP kinase and SAPK/JNK activation by IL-12 and IL-2, we hypothesized that one or both of these serine/threonine kinases might be mediating the serine phosphorylation of STAT1 and STAT3. Although a specific pharmacologic inhibitor of SAPK/JNK is not available, a specific inhibitor of p38 MAP kinase (SB203580) has been described. The p38 MAP kinase has been shown to be activated by IL-2 in a murine T cell line (16) and by TNF-α in L929 cells (18). SB203580 at a concentration of 10 μM has been shown to maximally inhibit IL-2-induced proliferation and TNF-α-induced cytokine production in these cell lines. Used at concentrations ranging from 0.1–1 μM, SB203580 inhibits the IL-2-induced activation of MAP kinase activating protein by p38 MAP kinase without inhibiting the IL-2-induced activation of ATF-2 by SAPK/JNK in a murine T cell line (16). Even at SB203580 concentrations of 10–20 μM, p38 MAP kinase is selectively inhibited in T cells (16) and mouse fibroblasts (44), while ERK and SAPK/JNK activity remain unaffected.

To determine whether p38 MAP kinase was mediating the serine phosphorylation of STAT1 and STAT3 in response to IL-12 plus IL-2, sorted CD18^bright T cells were stimulated with IL-12 plus IL-2 in the presence of 10 μM SB203580 or vehicle (DMSO) alone. As shown in Fig. 6A, the combination of IL-12 and IL-2 greatly increased the serine phosphorylation of STAT1 and STAT3 over the low to absent basal serine phosphorylation (lanes 1 and 2, top and middle panels). When cells were incubated with 10 μM SB203580 alone, an increase in the amount of basal STAT1 and STAT3 serine phosphorylation could be detected (Fig. 6A, top and middle panels, lane 5). However, when cells were stimulated with IL-12 plus IL-2 in the presence of 10 μM
SB203580, the serine phosphorylation of STAT1 and STAT3 was completely abolished (lane 6). Similar effects on basal and IL-12 plus IL-2-induced STAT serine phosphorylation were observed with as little as 1 μM SB203580 (data not shown). In contrast, STAT tyrosine phosphorylation in response to IL-12 or IL-2 was not affected by SB203580 (data not shown). This suggested that p38 MAP kinase activity was necessary for STAT1 and STAT3 serine phosphorylation in response to the combination of IL-12 and IL-2.

Since neither ERK1 nor ERK2 was activated by IL-12 and IL-2, it was unlikely that the ERK MAP kinase pathway was mediating the serine phosphorylation of STAT1 and STAT3 in response to IL-12 and IL-2. To further demonstrate this point, CD18 bright T cells were stimulated with IL-12 plus IL-2 in the presence of a MEK inhibitor (PD98059), which prevents the phosphorylation and activation of ERK1/ERK2. As shown in Fig. 6B, the serine phosphorylation of STAT1 in response to IL-12 and IL-2 was unaffected by PD98059, as was the serine phosphorylation of STAT3 (data not shown).

The inhibition of p38 MAP kinase abolishes the functional synergy between IL-12 and IL-2 in CD8^+ CD18^bright T cells

To determine whether the activation of p38 MAP kinase is essential to the functional response of CD8^+ CD18^bright T cells to the combination of IL-12 and IL-2, sorted CD18^bright T cells were stimulated with IL-12 and/or IL-2 in the presence or the absence of SB203580. IFN-γ production was assessed after 72 h, and proliferation was assessed after 96 h. As shown in Fig. 7A, proliferation and IFN-γ production in response to IL-2 alone or IL-12 plus IL-2 was almost completely inhibited by 10 μM SB203580. In addition, non-MHC-restricted cytolytic activity against the COLO cell line induced by IL-12 or IL-2 was reduced by SB203580 (Fig. 7B), and there was a modest 50% reduction in cytotoxicity induced by the combination of IL-12 and IL-2. The inhibition of functional responses to IL-2 or IL-12 plus IL-2 was also observed with 1 μM SB203580 (data not shown). The p38 MAP kinase inhibitor did not inhibit the IL-12- or IL-2-induced augmentation of CD3-mediated cytolytic activity against the OKT3 cell line (Fig. 7B), but did partially inhibit (30% reduction) cytotoxicity induced by the combination of IL-12 and IL-2.
To gain insight into the mechanism underlying the synergistic activation of T cells by the combination of IL-12 and IL-2, we have examined two of the signaling pathways activated by IL-12 and IL-2. Rather than using T cell lines or T cells activated in vitro with mitogens or CD3 cross-linking to analyze the signaling pathways activated by IL-12 and IL-2, we have focused on two subsets of peripheral blood T cells that differ dramatically in their intrinsic ability to respond to the combination of IL-12 and IL-2. This not only allows for the analysis of cytokine-activated signaling pathways and functional responses unperturbed by prior in vitro manipulation, but also adds a component of physiologic relevance to the signaling pathways being studied, as the cytokine-induced activation of CD8\(^+\)CD18\(^{bright}\) T cells may be critical to the host response to both infectious pathogens and tumors (37, 39).

Within both the JAK/STAT and MAP kinase family signaling pathways, we have demonstrated that IL-12 and IL-2 together synergistically increase the activation of signaling proteins in CD8\(^+\)CD18\(^{bright}\) T cells in a manner that correlates with the augmentation of CD8\(^+\)CD18\(^{bright}\) T cell proliferation, IFN-\(\gamma\) production, and cytolytic activity. Specifically, we have shown that T cell stimulation with IL-12 and IL-2 is required to maximally activate the stress-activated MAP kinases, p38 MAP kinase and SAPK/JNK, whereas this same cytokine combination does not activate the ERKs. In addition, we have shown that stimulation with IL-12 and IL-2 is required for optimal STAT1 and STAT3 serine phosphorylation, but does not further augment STAT1 and STAT3 tyrosine phosphorylation over the level observed in response to either cytokine alone. Finally, through the use of a specific inhibitor of p38 MAP kinase, we have found that the ability of IL-12 and IL-2 to augment functional responses and STAT1 and STAT3 serine phosphorylation in CD8\(^+\)CD18\(^{bright}\) T cells is p38 MAP kinase dependent.

While ERK activation in response to IL-2 and IL-12 has been observed in murine cell lines and in mitogen-activated murine and human T cells, the CD8\(^+\)CD18\(^{bright}\) subset of peripheral blood T cells expressing IL-12 and IL-2 receptors did not display activation of ERK1 or ERK2 in response to stimulation with IL-12 and/or IL-2. This was not due to a defect in the ERK signaling pathway, as PMA was able to activate ERK2 in the same CD8\(^+\) T cells. Although the ERKs may play a role in IL-12 and IL-2 responsiveness in T cell lines or in mitogen- or CD3-activated T cells, they do not appear to have a role in the TCR-independent activation of human peripheral blood CD8\(^+\)CD18\(^{bright}\) T cells by the combination of IL-12 and IL-2. In contrast, the activation of the stress kinases by IL-12 and IL-2 correlated well with the functional synergy between these cytokines in CD18\(^{bright}\) T cells. Since the activation of both p38 MAP kinase and SAPK/JNK was greatly augmented in CD18\(^{bright}\) T cells with the addition of IL-12 and IL-2, it is possible that both kinases play a role in the functional responsiveness to IL-12 and IL-2. However, the inhibition of proliferation, IFN-\(\gamma\) production, and, to a lesser extent, non-MHC-restricted cytolytic activity in the presence of the p38 MAP

**FIGURE 7.** Inhibition of p38 MAP kinase abrogates T cell proliferation and IFN-\(\gamma\) production induced by IL-12 and IL-2, but only partially blocks cytolytic activity. A. Sorted CD8\(^+\)CD18\(^{bright}\) T cells were incubated with IL-12, IL-2, or IL-12 and IL-2 in 96-h proliferation assays or 72-h IFN-\(\gamma\) assays. Where indicated, 10 \(\mu\)M SB203580 in DMSO or DMSO alone was present along with the indicated cytokines for the entire incubation period. Results are representative of three separate experiments. B. Sorted CD8\(^+\)CD18\(^{bright}\) T cells were incubated overnight with medium alone or the indicated cytokines in the presence or the absence of SB203580. \(^{51}\)Cr-labeled COLO or OKT3 target cells were then added at a 5:1 E:T cell ratio, and 4-h cytotoxicity assays were performed as described in Materials and Methods. Results are representative of two separate experiments.
kinase inhibitor is strong evidence that p38 MAP kinase is necessary for the response of CD8\(^+\)CD18\textsuperscript{bright} T cells to IL-12 and IL-2. The observation that p38 MAP kinase, but not SAPK/JNK or ERK1/ERK2, is highly expressed in CD18\textsuperscript{bright} T cells and is weakly expressed in CD18\textsuperscript{dim} T cells (two peripheral blood T cell subsets that are responsive and unresponsive, respectively, to the IL-12 and IL-2 combination) lends further support to the hypothesis that p38 MAP kinase plays an important role in mediating the functional synergy between IL-12 and IL-2. A recent study examining T cell proliferation in response to IL-2 also found that although p38 MAP kinase and SAPK/JNK are both activated by IL-2, proliferation could be inhibited with a p38 MAP kinase inhibitor (16). Nonetheless, it is still possible that p38 MAP kinase activation is necessary, but not sufficient, for mediating the functional effects of IL-12 and IL-2, and SAPK/JNK activation may play an important role in conjunction with p38 MAP kinase.

A recent report demonstrated that p38 MAP kinase activation is required for mitogen-induced IFN-\(\gamma\) production in CD4\(^+\)Th1 cells (45). In that study 1–10 \(\mu\)M SB203580 had a profound effect on Th1 cell IFN-\(\gamma\) production in response to Con A without affecting IL-4 production by Th2 cells. A dominant negative form of p38 MAP kinase had the same inhibitory effect on IFN-\(\gamma\) production, whereas a dominant negative form of JNK had no effect. These findings support our observation that p38 MAP kinase, rather than SAPK/JNK, is a key regulator of cytokine-induced IFN-\(\gamma\) production in CD8\(^+\)CD18\textsuperscript{bright} T cells. As IL-12 plays a key role in Th1 cell development and, along with IL-2, is a potent stimulator of IFN-\(\gamma\) production by Th1 cells, it is probable that p38 MAP kinase activation is critical to both CD4\(^+\) and CD8\(^+\) T cell IL-12/IL-2 responsiveness. As a parallel to our findings with CD18\textsuperscript{bright} T cells, we have recently observed that IL-12 and IL-2 can also activate p38 MAP kinase in NK cells, a lymphocyte subset that bears a striking immunophenotypic and functional resemblance to CD8\(^+\)CD18\textsuperscript{bright} T cells. Furthermore, the functional response of NK cells to IL-12 and/or IL-2 is inhibited by SB203580 (J. A. G. and D. A. F., unpublished observations), suggesting that the responses of peripheral blood NK cells and CD8\(^+\)CD18\textsuperscript{bright} T cells to Th1 type cytokines are p38 MAP kinase dependent.

Whereas STAT activation in response to IL-12 or IL-2 was not observed in CD18\textsuperscript{dim} T cells weakly expressing IL-12 and IL-2 receptors, the tyrosine and serine phosphorylation of STATs was seen in CD18\textsuperscript{bright} T cells in response to both cytokines. However, the functional synergy between IL-12 and IL-2 correlated not with STAT tyrosine phosphorylation but, rather, with STAT serine phosphorylation. One explanation for this finding is that the small amount of STAT1 and STAT3 tyrosine phosphorylation induced by IL-12 or IL-2 alone in CD8\(^+\)CD18\textsuperscript{bright} T cells is insufficient to mediate gene activation in and of itself. Only when STAT1 and STAT3 contain the additional phosphorylation of serine 727, as occurs following treatment with IL-12 and IL-2, can the necessary gene activation occur that results in the functional activation of T cells. The importance of serine phosphorylation in the function of STATs has been demonstrated at the level of DNA binding and gene transcription. While STAT tyrosine phosphorylation is critical for STAT dimerization, nuclear translocation, and DNA binding, STAT3 serine phosphorylation is necessary for the binding of STAT3-STAT3 homodimers to DNA (28). Serine phosphorylation is also necessary for maximal transcriptional activation by tyrosine-phosphorylated STAT1 and STAT3 (27). A recent report (46) demonstrated that STAT1\(\alpha\)-mediated transcriptional activation by IFN-\(\gamma\) is dependent on the phosphorylation of serine 727, and that this serine phosphorylation is required for the interaction of STAT1\(\alpha\) with MCM5, a member of the minichromosome maintenance (MCM) family involved in DNA replication.

In addition to STAT1 and STAT3 serine phosphorylation, the serine phosphorylation of STAT5 may also be important in enhancing STAT5-mediated transcription in response to the combination of IL-2 and IL-12. Although we do not have Abs that specifically recognize the serine-phosphorylated form of STAT5, it is known that the tyrosine phosphorylation of STAT5 in response to IL-2 is accompanied by serine phosphorylation (29). It is possible, therefore, that the increase in serine phosphorylation of STAT1 and STAT3 seen after IL-12 and IL-2 treatment in CD8\(^+\)CD18\textsuperscript{bright} T cells may occur in STAT5 as well. Despite the prominent STAT5 tyrosine phosphorylation relative to STAT1 and STAT3 tyrosine phosphorylation seen in response to IL-2 alone in CD18\textsuperscript{bright} T cells, an increase in serine phosphorylation in response to combined stimulation with IL-12 and IL-2 might still be capable of further augmenting STAT5-mediated transcriptional activation.

It is intriguing that STAT serine phosphorylation in response to IL-12 and IL-2 is completely abolished by an inhibitor of p38 MAP kinase, for it raises the possibility that STAT1 and STAT3 serine phosphorylation in CD18\textsuperscript{bright} T cells is either directly or indirectly mediated by p38 MAP kinase. To our knowledge, this is the first evidence in lymphocytes that the JAK/STAT and MAP kinase family signaling pathways may interact at the level of STAT serine phosphorylation, and that this interaction may be necessary to maximize the functional response to Th1-type cytokines. The fact that SB203580 inhibits both STAT1 and STAT3 serine phosphorylation and the functional response to IL-12 and IL-2 without affecting STAT tyrosine phosphorylation further suggests that tyrosine phosphorylation may be necessary, but not sufficient, for STATs to mediate the functional effects of the IL-12/IL-2 combination. It should be noted, however, that if STAT1 and STAT3 serine phosphorylation is occurring via p38 MAP kinase, it is possible that this is not responsible for the functional synergy between IL-12 and IL-2. Instead, the activation of other known substrates of p38 MAP kinase, such as ATF-2, heat shock protein 27, or MAP kinase activating protein kinase-2 (47, 48), may be contributing to that effect. Furthermore, if p38 MAP kinase is responsible for STAT1 and STAT3 serine phosphorylation in response to IL-12 and IL-2, it may not be responsible for cytokine-induced STAT5 serine phosphorylation, as STAT5a and STAT5b lack the -Pro-X-Ser-Pro- MAP kinase phosphorylation motif (49) shared by STAT1 and STAT3 (27, 28). This MAP kinase phosphorylation motif is also shared by STAT4, and as STAT4 is activated by IL-12 and is necessary for lymphocyte IL-12 responsiveness (24), it will be important to determine whether STAT4 serine phosphorylation is also augmented through stimulation with IL-12 and IL-2.

Interestingly, in the presence of SB203580 alone, there is an increase in the basal level of STAT1 and STAT3 serine phosphorylation in unstimulated CD18\textsuperscript{bright} T cells. It is noteworthy that in addition to its ability to inhibit the activity of phosphorylated p38 MAP kinase, SB203580 has been shown to increase the activity of MKK6 (44). While MKK6 is one of the major upstream activators of p38 MAP kinase, it does not activate SAPK/JNK. In unstimulated CD8\(^+\)CD18\textsuperscript{bright} T cells, therefore, the heightened level of STAT serine phosphorylation induced by SB203580 may in part be due to the MKK6-induced activation of an unidentified serine/threonine kinase distinct from p38 MAP kinase that is not inhibited by SB203580. While we observed that SB203580-treated cells were unable to mediate STAT serine phosphorylation in response to IL-12 and IL-2, we also found that stimulation with IL-12 and IL-2 in the absence of p38 MAP kinase activity abrogated the
highened basal levels of serine-phosphorylated STATs. This suggests that concomitant signaling through both IL-12 and IL-2 receptors may activate a STAT serine phosphatase that normally serves to limit the duration of STAT serine phosphorylation following augmentation of the serine kinase activity of p38 MAP kinase by IL-12 and IL-2. When this putative serine phosphatase is activated by IL-12 and IL-2 in the presence of SB203580, the phosphatase activity may predominate in the absence of p38 MAP kinase activity, thereby favoring the loss of STAT serine phosphorylation.

The ability to correlate the functional synergy between IL-12 and IL-2 with synergy observed at the level of signal transduction provides a basis for understanding which signaling pathways mediate select functional responses to these cytokines in lymphocytes. Our findings suggest that T cell proliferation and IFN-γ production induced by IL-12 and IL-2 are regulated through p38 MAP kinase activation, although SAPK/JNK activation may play a role as well. Cytolytic activity induced by these cytokines, especially CD3-activated cytotoxicity, appeared to be less affected by inhibition of p38 MAP kinase activity and may therefore be regulated by a different signaling pathway. Although we have observed a strong association between p38 MAP kinase activation and STAT1 and STAT3 serine phosphorylation, additional studies will be required to prove that STAT1 and STAT3 serine phosphorylation are indeed mediated by p38 MAP kinase. In addition, a detailed analysis of the effect of STAT1 and STAT3 serine phosphorylation on IL-12 and IL-2-induced gene transcription will be necessary to more cogently establish the role of STAT serine phosphorylation in the functional synergy between IL-12 and IL-2.

Acknowledgments

We thank John Daley and Suzan Lazo for their expert assistance with cell sorting and FACS analysis. We thank Genetics Institute for generously providing human IL-12, and SmithKline Beecham for generously providing the p38 MAP kinase inhibitor (SB203580).

References


