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Elevating Calcium in Th2 Cells Activates Multiple Pathways to Induce IL-4 Transcription and mRNA Stabilization

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PMA and ionomycin cause T cell cytokine production. We report that ionomycin alone induces IL-4 and IFN-γ, but not IL-2, from in vivo- and in vitro-generated murine Th2 and Th1 cells. Ionomycin-induced cytokine production requires NFAT, p38, and calmodulin-dependent kinase IV (CaMKIV). Ionomycin induces p38 phosphorylation through a calcium-dependent, cyclosporine A-inhibitable pathway. Knocking down ASK1 inhibits ionomycin-induced p38 phosphorylation and IL-4 production. Ionomycin also activates CaMKIV, which, together with p38, induces AP-1. Cooperation between AP-1 and NFAT leads to Il4 gene transcription. p38 also regulates IL-4 production by mRNA stabilization. TCR stimulation also phosphorylates p38, partially through the calcium-dependent pathway; activated p38 is required for optimal IL-4 and IFN-γ.


Optimal activation of T cells requires engagement of the TCR-CD3 complex and of costimulatory molecules whose ligands are expressed on APCs (1). The binding of Ag/MHC complexes to the TCR elevates intracellular Ca²⁺ concentration ([Ca²⁺]i) (3) and activates protein kinase Cθ (PKCθ). The combination of a calcium ionophore (e.g., ionomycin) and phorbol ester tumor promoters (e.g., PMA), which bypass TCR signals, results in T cell activation (2) and production of a series of cytokines, including IL-2, IL-4, and IFN-γ.

Elevation of [Ca²⁺]i is an essential event following TCR stimulation (3). Imaging studies reveal that TCR stimulation causes sustained elevation in [Ca²⁺]i, over a period of 1–2 h. Such sustained signaling allows dephosphorylated NFAT, a key transcriptional regulator of cytokine genes, to be maintained in the nucleus (4). The cooperative action of NFAT and AP-1 is generally thought to be necessary for full cytokine transcription (5).

PKCθ is a signal regulator of several T cell activation pathways (6). The MAP kinases ERK, JNK, and p38 are among the targets of PKCθ. The ERK and JNK pathways are involved in IL-2 production, predominantly through activation of AP-1 (7). However, relatively little is known about the role of p38 MAPK in cytokine induction.

Four p38 MAPK isoforms have been characterized: p38α, p38β, p38γ, and p38δ. CD4 T cells predominantly express p38α and p38δ (8). p38 can be activated by multiple stressors, such as UV radiation, heat shock, and osmotic shock, as well as by proinflammatory cytokines, such as TNF-α and IL-1. Ligation of the TCR also activates p38. TCR stimulation triggers small GTP-binding proteins such as Ras, Rac-1, and Cdc42, leading to the activation of MAPK cascades (7, 8). p38 is dually phosphorylated at Thr180 and Tyr182 in a highly conserved motif in the p38 activation loop. Salvador and colleagues reported an alternative p38 activation pathway in T cells, in which ZAP70 phosphorylates p38α on Tyr323, leading to autophosphorylation of the Thr and Tyr in the activation loop and to “self-activation” (9). TCR stimulation thus results in induction of both the classic MAPK cascade and this alternative p38 inhibitor-sensitive self-activation pathway; the relative contributions of the two pathways and their physiological functions remain to be determined.

The importance of p38 activity to cytokine production is still controversial. Herein we report that ionomycin alone induces IL-4 and IFN-γ production from in vivo- and in vitro-generated Th2 and Th1 cells, in contrast to IL-2, for which both PMA and ionomycin are essential for production. The production of IL-4 requires elevation of [Ca²⁺]i, NFAT dephosphorylation, and activation of p38 and CaMKIV. Phospho-p38 generated through elevation of [Ca²⁺]i plays a crucial role in ionomycin-induced IL-4 production by promoting Il4 gene transcription and stabilizing its mRNA. Furthermore, we show that phospho-p38 is essential for optimal IL-4 production induced through TCR stimulation.

Materials and Methods

Mice and cell culture

Cells from C57BL/6 mice infected with Schistosoma mansoni cercariae for 8 wk and splenocytes from C57BL/6 mice infected with Toxoplasma gondii for 10 days were kindly provided by Dr. Dragana Jankovic of the National Institute of Allergy and Infectious Diseases (10, 11). C57BL/6 mice were inoculated with three-stage Heligmosomoides polygyrus per os, and spleens were isolated 14 days later, as previously described (12).

CD4 T cells were purified from SC.C7 transgenic Rag2−/− mice by negative selection. Cells were cultured under Th1 and Th2 conditions as described before (13). CD4 T cells were purified from H/4Gfp heterozygous mice by negative selection. The cells were primed with APC, anti-CD3 (3 μg/ml), and anti-CD28 (3 μg/ml) under Th2 conditions.

Intracellular staining

In vitro differentiated Th1 and Th2 cells were rechallenged under different conditions for 4 h, in the presence of monensin, to check cytokine production. The conditions used to rechallenge the cells were: APC + peptide,
cognate cytochrome C peptide (1 μM)-loaded APC, anti-CD3/CD28, plate-bound (PB) anti-CD3 (3 μg/ml) and anti-CD28 (3 μg/ml); P=I (10 ng/ml) and ionomycin (1 μM), and ionomycin (1 μM) alone.

Harvested cells were fixed with 4% paraformaldehyde, washed with 0.1% BSA-containing PBS, and stored at 4°C. For staining, cells were incubated with permeabilization buffer (PBS supplemented with 0.1% BSA/0.1% Triton X-100) and various Abs for 20 min. All Abs were purchased from BD Pharmingen.

To test p38 phosphorylation in PB anti-CD3 and/or anti-CD28-stimulated Th2 cells, cells were put in Ab-coated 6-well plates and were spun at 1600 rpm for 20 s to allow cells to attach to the bottom. Paraformaldehyde (4% final concentration) was added to cell culture to fix the cells immediately after the cells had been cultured in the plates for the indicated periods.

For phospho-p38 staining, fixed cells were incubated with a rabbit monoclonal phospho-p38 Ab (1/25) (336 ng/ml) (Cell Signaling Technology) or a nonspecific rabbit isotype control Ab (336 ng/ml) (Imgenex) for 30 min at room temperature. After extensive washing, cells were stained with a FITC-conjugated donkey anti-rabbit Ab (1/200) (Jackson ImmunoResearch Laboratories).

Small hairpin (sh)RNA lentiviral infection

p38α, ASK1, CaMKII, and CaMKIV shRNA constructs were purchased from Sigma-Aldrich. All the plasmids were purified with EndoFree plasmid purification kit (Qiagen). 293FT “packaging” cells were transfected with shRNA plasmids or nonsilencing control shRNA plasmids, together with the packaging plasmid pCMVΔ8.9 and the envelope plasmid pHCMV-G using the Fugene 6 transfection reagent (Roche Diagnostics) according to the manufacturer’s protocol. Packaging plasmid pCMVΔ8.9 and envelope plasmid pHCMV-G were kindly provided by Dr. N. Hacohen at Harvard Medical School. Lentivirus-containing supernatant was collected twice, at 48 and 72 h after transfection, respectively.

To “knockdown” p38, Th2 cells were cultured for a second round under Th2 conditions. Two days later, the lentivirus, collected as described above, and polybrene (5 μg/ml) (Sigma-Aldrich) were added and the cells were centrifuged at 2500 rpm for 90 min at room temperature. The supernatant was removed immediately and fresh medium was added to the culture, which continued under Th2 conditions. Forty-eight hours after infection, cells were placed in medium containing IL-2 and puromycin (5 μg/ml) (Sigma-Aldrich). One week later, puromycin-resistant cells were purified by cell sorting.

Retroviral infection

GFP-Cre viral supernatant was prepared as described before (14). Splenic CD4 T cells were purified from mice homozygous for a “floxed” TAK1 gene (15) and cultured under Th2 conditions as described above. Two days after a third round of Th2 priming, cells were incubated with a GFP-Cre retrovirus-containing supernatant. Two days after infection, cells were placed in IL-2-containing medium. Twenty-four to 48 h later, cells were stimulated to check cytokine production or p38 phosphorylation in response to ionomycin.

RNA purification and quantitative PCR (qPCR)

Total RNA was isolated using RNeasy Mini kits (Qiagen) (treated with RNase-free DNase I); first-strand cDNA was prepared using SuperScript III (Invitrogen). All PCR was performed on a 7900HT sequence detection systems (Applied Biosystems). The TaqMan universal PCR SuperMix and all the primer and probe sets were purchased from Applied Biosystems. To test Gfp mRNA level, SYBR Green technology was utilized with the primers as reported before (13). To exclude the possible false signal introduced by binding of SYBR Green to likely primer dimer, the PCR conditions were set as 95°C for 15 s, 60°C for 1 min, and 78°C for 30 s for 40 cycles, and the increase in fluorescence was detected only at the 78°C incubation step.

Immunoblotting

Stimulated cells were lysed in 1× SDS sample buffer (0.05 M Tris-HCl (pH 6.8), 1% SDS (w/v), 10% glycerol (w/v), bromophenol blue 1.009% (w/v)) freshly supplemented with the following inhibitors: 1 mM PMSF, 10 μg/ml aprotinin, leupeptin, pepstatin, and 1X phosphate inhibitor cocktail 1 and cocktail 2 (Sigma-Aldrich). Cell lysates were sonicated for 10 s to shear DNA.

Proteins were separated on 12% pre-made tricine-glycine gels (Invitrogen) and then transferred onto nitrocellulose membranes (Millipore) by the Trans-Blot SD semidyey electrophoretic transfer cell (Bio-Rad). Membranes were probed with phospho-p38 MAPK Ab (Cell Signaling Technology) (1/1000), followed by a HRP-labeled anti-rabbit Ab (Pierce) (1/1000). The signal was visualized with ECL. Membranes were stripped by Restore Western blot stripping buffer (Pierce) and then reprobed with p38 MAPK Ab (Cell Signaling Technology) (1/1000).

Nuclear extraction and EMSAs

Cells were stimulated with ionomycin for 50 min. Nuclear protein extracts were prepared as previously described (16), and protein concentration was
determined with the BCA kit (Pierce). EMSAs were conducted with the EMSA kit (Promega). The oligonucleotides corresponding to sense and antisense sequences within the Il4 promoter (17) were 5'-biotin labeled (Bio-Synthesis), HPLC purified, and annealed for use as probe. Samples were resolved on a 6% DNA retardation gel (Invitrogen) in 0.5× Trisborate-EDTA buffer. The protein-DNA binding complex was detected by the LightShift chemiluminescent EMSA kit (Pierce). The sequences of the oligonucleotides were: sense, 5'-TAATGTAATACCTTATCTGAGATCTGCTGAC-3' and antisense, 5'-CAAGGGAAATGAGTTTACTA-3'.

**AP-1 ELISA**

An ELISA-based TransAM AP-1 kit, in which oligonucleotide containing AP-1 binding motif has been immobilized to 96-well plate, was used to quantify activated AP-1 components (Active Motif). Five-microgram nuclear extracts prepared as above were used and c-Fos, FosB, Fra-1, Fra-2, c-Jun, JunB, and JunD Abs were added at 1/2000 dilution. The AP-1 binding assay was performed according to the instructions of the manufacturer.

**Results**

**Ionomycin induces IL-4 and IFN-γ, but not IL-2, production from in vivo- and in vitro-differentiated Th cells**

Mesenteric lymph nodes (LNs) were isolated from mice 8 wk after *S. mansoni* infection, which strikingly induces a Th2 response. LN cells were either unstimulated, immediately stimulated with PB anti-CD3/CD28, with a combination of PMA and ionomycin (P+I), or with ionomycin alone. The percentage of CD44brightCD4 T cells from infected mice that produced IL-4 in response to ionomycin was similar to that in response to P+I or to PB anti-CD3/CD28; mean fluorescence intensities (MFI) of the IL-4–expressing cells induced by P+I or by ionomycin alone were also similar (Fig. 1A).

Splenocytes isolated from mice 2 wk after *H. polygyrus* infection, another typical Th2 response inducer, showed only slight differences in the percentage of IL-4–producing cells and in their MFI in response to P+I or to ionomycin. No IL-2 production was observed when cells were stimulated with ionomycin, whereas IL-2 expression was induced in 36% of the cells stimulated with P+I (Fig. 1B).

Ionomycin-induced IL-4 production was also observed in Th2 cells primed in vitro. A similar percentage of cells produced IL-4 when stimulated with peptide/APC, anti-CD3/CD28, P+I, or ionomycin alone (Fig. 1C). The MFI of IL-4–producing cells induced by ionomycin alone was about half that of the IL-4–producing cells induced by PB anti-CD3/CD28 or by P+I.

**FIGURE 2.** Ionomycin and TG induce IL-4 production through a p38-dependent pathway. A, Ionomycin-induced IL-4 production by Th2 cells is highly sensitive to p38 inhibition. Th2 cells were preincubated with DMSO (mock), 10 μM of inhibitors (upper panel), or various concentrations of inhibitors, as indicated (lower panel), of the three distinct MAPK pathways for 30 min and then stimulated. In the lower panel, the degree of IL-4 production by DMSO-treated cells was set as “1.” B, Knocking down p38 inhibits ionomycin–induced IL-4 production. Th2 cells were lentivirally infected with nonsilencing or p38-shRNAs; puromycin-resistant cells were purified and further stimulated under Th2 conditions. These cells were divided into three portions: one portion to check IL-4 production in response to P+I or ionomycin (upper panel), one portion to analyze p38 mRNA by qPCR, and one portion to check p38 protein by immunoblotting (lower panel). C, TG similarly induces IL-4 production through a p38-dependent pathway. Th2 cells were pretreated then stimulated as described. SB203580, 5 μM; SB202190, 5 μM; TG, 10 nM. The experiments were conducted three times.

**FIGURE 3.** p38 is required for optimal IL-4 and IFN-γ production, but is not necessary for IL-2 production. A, Splenocytes isolated from C57BL/6 mice 2 wk after *H. polygyrus* infection were pretreated for 30 min and then stimulated. The cells shown were gated on CD44brightCD4 T cells. B, Splenocytes isolated from C57BL/6 mice 10 days after *T. gondii* infection, and CD4 T cells were purified with CD4 beads by positive selection. The cells shown were gated on CD44brightCD4 T cells. C, Two round-primed Th1 cells were pretreated for 30 min and then stimulated to check IL-2 production. The in vivo experiments were conducted twice and the in vitro experiments multiple times.
FIGURE 4. Calcium is important in p38 phosphorylation mediated by ionomycin and by PB anti-CD3. A, Ionomycin induces p38 phosphorylation. Th2 (left panel) and Th1 (right panel) cells were stimulated for 15 min and then p38 phosphorylation was examined by intracellular staining. C and E, PB anti-CD3 but not anti-CD28 induces p38 phosphorylation. Th2 cells were stimulated with PB Abs for indicated periods and analyzed with intracellular staining (C) and immunoblotting (E). EGTA, CsA, and FK520 abolish p38 phosphorylation induced by ionomycin (B and F) and by anti-CD3 (D and F). Th2 cells were preincubated with indicated inhibitors for 30 min to 1 h and stimulated with ionomycin for 20 min (B and F) or with PB anti-CD3 for 40 min (D–F) to measure phospho-p38. EGTA, 0.8 mM; CsA, 50 ng/ml; FK520, 1 μM; SB203580, 5 μM; BAPTA, 10 μM; BAPTA/AM, 10 μM. The experiments were conducted at least three times.

Most in vitro-primed Th1 cells produced IFN-γ (Fig. 1C). Based on the percentage of IFN-γ-producing cells and MFI of these cells, the amount of IFN-γ induced by ionomycin was ~21.5% of that induced by P+I. Ionomycin-induced IFN-γ production by in vivo-generated Th1 cells was also observed, which will be discussed in detail subsequently.

Neither in vitro-generated Th1 or Th2 cells produced IL-2 when stimulated with ionomycin (Fig. 1D).

Inhibiting p38 blocks ionomycin-induced IL-4 production
Ionomycin has been shown to activate calcineurin, which in turn dephosphorylates NFAT, causing the latter to enter the nucleus and promote gene transcription. Since AP-1, a target of MAPKs, is generally thought to cooperate with NFAT to induce gene transcription, we asked whether ionomycin activated a MAPK pathway. We found that 1 μM of the p38 inhibitor SB203580 almost completely abolished IL-4 production, whereas little inhibition was observed in cells pretreated with 1–10 μM of a JNK inhibitor or 1–3 μM of a MEK inhibitor (Fig. 2A). MEK is upstream of the ERK; thus, the inhibitor blocks ERK activation. Also, 10 μM of the MEK inhibitor partially inhibited ionomycin-induced IL-4 production. However, it is possible that chemical toxicity accounted for this decrease since we could detect no phosphorylation of ERK1/2 in the cells stimulated by ionomycin alone (data not shown).

The p38 inhibitor diminished IL-4 production induced by P+I by 50% (Fig. 2A). MEK inhibitor pretreatment also decreased P+I-induced IL-4 production, presumably due to diminished activation of AP-1. The striking inhibition of ionomycin-induced IL-4 production by the p38 inhibitor compared with the partial inhibition of P+I-induced IL-4 production suggests that P+I activates multiple pathways. By contrast, ionomycin appears to selectively activate the p38 pathway.

Knocking down p38 inhibits ionomycin-induced IL-4 production
p38 was knocked down by introducing p38 shRNAs into Th2 cells. The shRNA lentiviruses contain a puromycin resistance gene, allowing for selection of cells that express the viral-encoded sequences. Puromycin-resistant cells were purified and challenged with P+I or ionomycin. Cells infected with either of the two different p38 shRNA lentiviruses expressed less p38 mRNA and protein and produced less IL-4 than did cells infected with the non-silencing control shRNA (Fig. 2B). p38 shRNA construct-2, which caused more efficient diminution of p38 mRNA and protein, resulted in greater inhibition of IL-4 production. p38 shRNA construct-2 reduced the response to P+I by 75%, suggesting that the importance of p38 in IL-4 production in response to P+I was underestimated by experiments relying on the p38 inhibitor.

Thapsigargin (TG) induces IL-4 production through a p38-dependent pathway
We also tested TG for its capacity to stimulate IL-4 production. TG is an endoplasmic reticulum calcium-ATPase inhibitor that induces calcium depletion from endoplasmic reticulum stores and, in turn, causes extracellular calcium influx through Ca\(^{2+}\) release-activated Ca\(^{2+}\) channels. TG caused 24% of Th2 cells to produce IL-4, which was almost completely abolished by pretreatment with p38 inhibitors (Fig. 2C). This strongly supports the concept that elevating [Ca\(^{2+}\)]\(_{i}\) activates p38, which, together with NFAT, plays a critical role in IL-4 production.
p38 is required for optimal TCR-stimulated effector cytokine production

A p38 inhibitor caused a 50% reduction in PB anti-CD3/CD28- or P+I-stimulated IL-4 production by CD44^{hi}CD4 T cells from H. polygyrus infected-mice; the response to ionomycin alone was reduced by 80% (Fig. 3A). The p38 inhibitor similarly blocked the IL-4 response to in vitro-primed Th2 cells stimulated with peptide-loaded APC or PB anti-CD3/CD28 (data not shown).

p38 inhibition resulted in 50% diminution of PB anti-CD3/CD28-stimulated IFN-γ production by CD44^{hi}CD4 T cells from T. gondii-infected mice (Fig. 3B). Similarly, p38 inhibition reduced IFN-γ production by in vitro-primed Th1 cells in response to peptide-loaded APC or PB anti-CD3/CD28 (data not shown).

p38 is dispensable for IL-2 production

The p38 inhibitor failed to diminish TCR- or P+I-mediated IL-2 production by in vitro-primed Th1 cells (Fig. 3C). A similar failure of inhibition of IL-2 production by in vivo-generated Th1 cells and in vitro-primed Th2 cells in response to TCR-mediated stimuli was observed (data not shown). Thus, p38 is required for optimal IL-4 and IFN-γ production, but it is not necessary for IL-2 production.

Calcium-dependent p38 phosphorylation

p38 is generally phosphorylated at Thr^{180} and Tyr^{182} in the highly conserved Thr-Gly-Tyr motif of the activation loop. To check the ability of ionomycin to induce p38 phosphorylation, we stained Th2 cells with an Ab that recognizes p38 only when it is dually phosphorylated. Cells stimulated with ionomycin or P+I for 15 min showed an equal increase in staining intensity, involving the entire population of stimulated cells (Fig. 4A). PMA alone had little or no effect on p38 phosphorylation. Ionomycin-induced p38 phosphorylation in Th1 cells was similarly observed (Fig. 4A).

Ionomycin-induced p38 phosphorylation was dependent on calcium. Pretreatment of the cells with extracellular calcium chelator, EGTA, completely abolished p38 phosphorylation (Fig. 4B). Ionomycin-induced p38 phosphorylation was also inhibited by cyclosporine A (CsA) or ascomycin (FK520), a chemical analog of tacrolimus (FK506), suggesting a linkage between calcineurin and the p38 pathway. Ionomycin-induced p38 activation was not affected by p38 inhibition, consistent with the concept that p38 autophosphorylation is mediated through ZAP70, which is bypassed by ionomycin.

In contrast to the rapid induction of phospho-p38 in cells stimulated with ionomycin, only a portion of cells showed p38 phosphorylation in the first 15 min after stimulation with anti-CD3 or anti-CD3/CD28 (Fig. 4C). The proportion of phospho-p38-expressing cells increased with time, reaching essentially 100% by 90 min. Cells stimulated with PB anti-CD28 alone showed no p38 activation, and anti-CD3/CD28-stimulated cells showed no greater staining with anti-phospho-p38 at any time point than did cells treated with anti-CD3 alone, suggesting that CD28 does not play a role in mediating p38 activation.

To determine whether calcium plays an important role in anti-CD3-stimulated p38 activation, cells were pretreated BAPTA, an extracellular calcium chelator, and BAPTA/AM, an intracellular calcium chelator. Each dramatically decreased p38 phosphorylation in response to anti-CD3 stimulation (Fig. 4D), implying that calcium also plays a critical role in TCR-stimulated p38 activation. Anti-CD3-induced p38 phosphorylation was also inhibited by CsA and FK520. By contrast, anti-CD3-induced ERK phosphorylation was not inhibited by calcium chelators or by CsA (data not shown). Pretreatment with the p38 inhibitor resulted in partial decrease in anti-CD3-dependent p38 phosphorylation, which may represent the previously reported p38 autophosphorylation pathway.

p38 phosphorylation was also examined by immunoblotting. It was minimal at 15 min, but easily detectable at 30 min after anti-CD3 stimulation, peaked at 45–60 min, and decreased at 90 min (Fig. 4E). p38 phosphorylation was not observed in cells stimulated by anti-CD28 at any time.

Immunoblotting was also performed to confirm calcium-mediated p38 phosphorylation (Fig. 4F). Phosphorylation was observed in cells stimulated by ionomycin for 20 min and abolished in stimulated cells pretreated with EGTA, CsA, or FK520. More p38 phosphorylation was observed in cells stimulated by anti-CD3 for...
ASK1 is the upstream kinase of p38

To understand the upstream signaling pathway that leads to [Ca^{2+}]\(^{-}\)-dependent p38 activation, we tested the phosphorylation of upstream MAP kinases. Immunoblotting showed that p38 phosphorylation of MKK3/6 (map2k3/6) and TGF-\(\beta\)-activated kinase 1 (TAK1, map3k7), which was diminished in the presence of CsA (Fig. 5A). However, retrovirally introducing GFP-Cre into Th2 cells derived from TAK1-floxed mice (15) resulted in only a subtle decrease in IL-4 production in response to P+I or ionomycin alone (Fig. 5B). TAK1-deficient (GFP\(^{-}\)) cells demonstrated the same degree of ionomycin-induced p38 phosphorylation as did controls (GFP\(^{+}\), TAK1\(^{+/+}\)) cells (Fig. 5B). Thus, despite its ionomycin-induced phosphorylation, TAK1 is not responsible for p38 phosphorylation in ionomycin-treated cells.

ASK1 (map3k5), another member of the MKKK family, activates both the JNK and p38 pathways through Map2k3/4/6. Of three ASK1 shRNAs introduced into Th2 cells, two caused diminution in both ASK1 and in ionomycin-induced p38 phosphorylation and IL-4 production. Construct 2 diminished Ask1 mRNA by 70%, almost completely inhibited ionomycin-induced p38 phosphorylation, and diminished ionomycin-induced IL-4 production by \(-60\%\). Construct 1 was less effective for all three parameters, and construct 3 and the nonsilencing control were ineffective (Fig. 5C).

p38 stabilizes Il4 mRNA

Many cytokine mRNAs are under tight stability regulation through proteins that bind to adenylate/uridylate (AU)-rich elements (AREs) in their 3' untranslated regions (UTR) (18). Ionomycin could induce IL-4 through message stabilization, new transcription, or both. Th2 cells heterozygous for an Il4 allele in which Gfp has replaced the first exon and a portion of the first intron of Il4 were analyzed to take advantage of the absence of AREs in the Gfp 3' UTR (19) and of their presence in the Il4 3' UTR. The half-life of Il4 mRNA induced by TCR was 27 min in the presence of the p38 inhibitor and 145 min in its absence (Fig. 6A). The half-life of Il4 mRNA induced by ionomycin was 4 min in the presence of the p38 inhibitor and 55 min in its absence (Fig. 6A). The half-life of Il4 mRNA induced by ionomycin was 4 min in the presence of the p38 inhibitor and 55 min in its absence (Fig. 6A). The half-life of Il4 mRNA induced by ionomycin was 4 min in the presence of the p38 inhibitor and 55 min in its absence (Fig. 6A). The half-life of Il4 mRNA induced by ionomycin was 4 min in the presence of the p38 inhibitor and 55 min in its absence (Fig. 6A). The half-life of Il4 mRNA induced by ionomycin was 4 min in the presence of the p38 inhibitor and 55 min in its absence (Fig. 6A). The half-life of Il4 mRNA induced by ionomycin was 4 min in the presence of the p38 inhibitor and 55 min in its absence (Fig. 6A). The half-life of Il4 mRNA induced by ionomycin was 4 min in the presence of the p38 inhibitor and 55 min in its absence (Fig. 6A). The half-life of Il4 mRNA induced by ionomycin was 4 min in the presence of the p38 inhibitor and 55 min in its absence (Fig. 6A). The half-life of Il4 mRNA induced by ionomycin was 4 min in the presence of the p38 inhibitor and 55 min in its absence (Fig. 6A).
CaMKIV and p38 induce AP-1 family gene transcription. A, A CaMK inhibitor markedly diminishes ionomycin-induced IL-4 production. Two round-primed Th2 cells were pretreated for 30 min and then stimulated to check ionomycin-induced IL-4 production. B, CaMKIV but not CaMKII is involved in ionomycin-induced IL-4 production. Th2 cells were lentivirally infected with the indicated shRNA viral supernatant. Puromycin-resistant cells were sorted and then stimulated with ionomycin for 4 h to check IL-4 production, or collected to check Camk2 or Camk4 mRNA level. The mRNA amount in nonsilencing control shRNA infected cells was set as “1.” C, fosB and fosI2, but not fosI1 or junB, mRNA are induced by ionomycin and significantly decreased in the presence of p38 inhibitor and CaMK inhibitor. Th2 cells were pretreated with indicated inhibitors for 30 min and then stimulated with ionomycin for 25 min. mRNA in unstimulated cells was set as “1.” D, Th2 cells were pretreated with indicated inhibitors for 30 min and then stimulated with ionomycin for 50 min. Nuclear extracts were prepared and AP-1 binding was performed by using an ELISA-based AP-1 family transcription factor assay kit. The experiments were conducted at least twice.

anti-CD3/CD28 is more effective than is ionomycin alone in activating p38 and is less completely blocked by the p38 inhibitor.

**p38 plays a role in promoting Il4 transcription**

The absence of an ARE in the Gfp 3'UTR is associated with stability of Gfp mRNA. Thus, the degree of Gfp mRNA induction by ionomycin and its sensitivity to p38 inhibition is a measure of ionomycin-induced transcription and of whether p38 plays a role in such transcription. p38 inhibitor pretreatment caused a 75% inhibition of Gfp mRNA levels and an 80% inhibition of Il4 mRNA (Fig. 6C). Thus, we conclude that p38 plays an important role in Gfp and, presumably, Il4 transcription.

In TCR-induced Il4/Gfp heterozygous Th2 cells, the p38 inhibitor led to a ~50% diminution in Gfp mRNA induction at 4 h but to less inhibition at 2 and 3 h after stimulation. Although cells pretreated with the MEK and JNK inhibitors showed no diminution of Gfp mRNA upon stimulation, pretreatment with all three MAPK pathways inhibitors decreased Gfp mRNA by 75% at all time points (Fig. 6D). This implies that p38 plays an important role in promoting Il4 gene transcription in response to TCR engagement that is underestimated since its absence is compensated for by other MAPKs activated by PB anti-CD3/CD28.

p38 has been reported to have a stimulatory effect on NFAT activation (20). An EMSA using the NFAT binding site in the Il4 promoter as a probe was performed. The capacity of NFAT to bind to the Il4 promoter in ionomycin-stimulated cells was unchanged in the presence of a p38 inhibitor (Fig. 6E). Thus, p38 function in Il4 transcription cannot be accounted for by enhanced activation of NFAT.

Ionomycin-induced Il4 transcription requires CaMKIV

Although KN-93 failed to prevent ionomycin-induced p38 phosphorylation (Fig. 5D), it strikingly diminished ionomycin-induced IL-4 production (Fig. 7A). Knocking down CaMK proteins by lentiviral shRNAs specific for CaMKIV but not CaMKII diminished ionomycin-induced IL-4 production, implying that CaMKIV is the major CaMK involved in the ionomycin pathway (Fig. 7B). Consistent with the results from the CaMK inhibitor (Fig. 5D), ionomycin-induced p38 phosphorylation remained normal in CaMKIV shRNA-infected cells (data not shown).
CaMKIV contributes to cytokine production by inducing CREB phosphorylation and thereaf ter, transcription of the CREB-dependent immediate-early genes, including fosB, fosL1, and junB (21, 22). Similarly, p38 phosphorylation of CREB and induction of AP-1 transcription has been reported (21). Indeed, fosB and fosL1 mRNA levels were increased >15-fold by ionomycin and significantly decreased in cells pretreated with CaMK inhibitors or p38 inhibitor (Fig. 7C). Fosl1 and junB were only modestly induced by ionomycin. Ionomycin induced increased AP-1 binding activity that was blocked by a p38 inhibitor and by the CaMK inhibitor (Fig. 7D). Thus, p38 and CaMKIV control the amount and activity of AP-1 family transcription factors. This, together with NFAT, explains ionomycin-induced Il4 transcription. Together with ionomycin-induced p38 prolongation of Il4 mRNA half-life, this explains the increase in Il4 mRNA (Fig. 8).

**Discussion**

Full activation of T cells requires signaling through the TCR/CD3 complex and the CD28 costimulatory receptor. P+I are often utilized to bypass TCR stimulation, through the action of PMA on PKCθ and the capacity of ionomycin to elevate [Ca\(^{2+}\)]. Unexpectedly, we found that ex vivo CD44\(^{\text{high}}\)CD4 T cells isolated from either S. mansoni- or H. polygyrus-infected mice produced similar amounts of IL-4 when stimulated with ionomycin alone or with P+I. Both ionomycin and a second Ca\(^{2+}\) elevating agent, TG, induced IL-4 production from in vitro-primed Th2 cells. Ionomycin also induced IFN-γ production by CD44\(^{\text{high}}\)CD4 T cells from T. gondii-infected mice and in vitro-primed Th1 cells. Ionomycin alone has been recently reported to induce IL-21 production in preactivated T cells (23). By contrast, as has been previously reported (24), IL-2 was not produced when these cells were stimulated with ionomycin, although it was produced in response to cognate peptide-loaded APC, anti-CD3/CD28, or P+I.

Ionomycin activates calcineurin, which in turn dephosphorylates NFAT, causing the latter to enter the nucleus and promote gene transcription. Our data demonstrate that blocking p38 activity by inhibitors or diminishing p38 expression by RNA interference strikingly inhibited ionomycin-induced IL-4 production, although the capacity of NFAT to bind to the Il4 promoter remained normal. Thus, ionomycin-induced NFAT translocation alone is not sufficient for IL-4 production; p38 activity is essential. p38 mediates its function in ionomycin-induced IL-4 production through a mechanism other than modification of NFAT binding to Il4 promoter.

We observed that ionomycin induces p38 phosphorylation in Th2 and Th1 cells. Intracellular and extracellular calcium chelators completely abolished p38 phosphorylation induced by ionomycin and greatly decreased phosphorylation in response to PB anti-CD3, indicating that calcium plays an important role in p38 activation even when cells were stimulated through TCR. Ionomycin-induced p38 activation was not affected by the p38 inhibitor SB203580, indicating that this calcium-mediated p38 activation represents a separate pathway from the ZAP70-mediated p38 autopshorylation pathway (9). CsA and FK502 completely abolish ionomycin-induced p38 phosphorylation and partially blocked p38 phosphorylation in response to anti-CD3. These results indicate that calcineurin activation is required for ionomycin-induced p38 phosphorylation and contributes to TCR-induced p38 phosphorylation.

Ca\(^{2+}\) elevation has been reported to activate the MAPK cascade (25–28). Several MKKKs, such as TAK1 and ASK1, can be activated by elevated [Ca\(^{2+}\)], and in turn activate the classical MAPK cascade, leading to p38 phosphorylation (27, 29). The involvement of calcineurin in MAPK activation in Jurkat cells was suggested by the finding that JNK activation by PMA plus A23187 was blocked by CsA (30).

Calcineurin-like molecules have been reported to interact with the MAPK analog. In Caenorhabditis elegans, KIN-29, a Ser/Thr kinase, binds to the calcineurin-like phosphatase Tax6 (31). In Arabidopsis, calcineurin B-like proteins (CBLPs) are reported to bind to CBL-interacting Ser/Thr kinases (AtCIPKs) in a calcium-dependent manner (32). Mammalian kinases corresponding to AtCIPKs include Raf-1. Raf-1 possesses an autoinhibitory site that is generally phosphorylated in quiescent cells. Activation of Raf-1 involves dephosphorylation of this autoinhibitory site (33).

Among the kinases upstream of p38 in the classical MAPK pathway, ASK1 has been reported to be activated by calcium signal in primary neurons and synaptosomes (27, 34) and has a well-conserved autoinhibitory site (35), suggesting that it might be a target of calcineurin. It has been reported that ASK1 activity is regulated through this autoinhibitory site (36, 37). In a yeast two-hybrid screen, the calcium-binding subunit B of calcineurin and ASK1 were found to be direct physical partners (38). Furthermore,
in an in vitro phosphatase assay, calcineurin directly dephosphorylated ASK1, resulting in its activation. In cardiomyocytes, the endogenous subunit B of calcineurin could be coimmunoprecipitated with ASK1, suggesting physiological importance of their interaction.

Herein, we showed that knocking down ASK1 resulted in diminished ionomycin-induced p38 phosphorylation and IL-4 production. By contrast, ionomycin-induced p38 phosphorylation and IL-4 production were not impaired in TAK1-deficient cells, although TAK1 was phosphorylated in ionomycin-stimulated cells. We conclude that ASK1 is the major kinase upstream of p38 in the ionomycin-stimulated cells. Inhibiting or knocking down CaMKs did not affect ionomycin-induced p38 phosphorylation. Thus, ionomycin does not activate ASK1 through CaMK. Instead, it is likely that elevated [Ca\(^{2+}\)]\(_i\) activates calcineurin, which in turn dephosphorylates the autoinhibitory site of ASK1 and leads to its activation.

The importance of p38 for effector cytokine production is still controversial. IL-4 production from Th2 cells has been reported to be impaired by a p38 inhibitor (39). However, in another report, p38 phosphorylation was described to be selectively induced by Con A in Th1 cells but not in Th2 cells (40). Our data showed that p38 is essential for both IL-4 and IFN-\(\gamma\) production in response to ionomycin and for optimal production in response to anti-CD3 and anti-CD28. Since Con A, like anti-CD3 and anti-CD28, can induce IL-4 by both p38-dependent and independent pathways, the degree of priming could have had a major impact on the relative use of these pathways and may account for the difference between our results and those reported by Rincon et al. (40). More recently, Berenson and colleagues using p38\(^{-/-}\) and p38\(^{±/-}\)CD4 T cells prepared by RAG2-Gfp knockin into the IL4/Gfp locus, consistent with the absence of AREs in the Gfp 3’UTR. However, the striking increase in Gfp mRNA in ionomycin-stimulated Th2 cells from IL4/Gfp heterozygous donors implies that ionomycin’s effect involves substantial induction of transcription. Furthermore, since Gfp induction is also blocked by the p38 inhibitor, it must be concluded that p38 regulates IL-4 production in response to ionomycin both by a striking induction of transcription as well as by mRNA stabilization.

Inhibiting or knocking down CaMKs greatly diminished ionomycin-induced IL-4 production, although p38 activation in response to ionomycin remained normal. fosl2 and fosb, but not fosl1 and junB, mRNA were induced by ionomycin; p38 and CaMK inhibitors blocked the induction. AP-1 is regulated by phosphorylation as well as by induction (43); p38 has been reported to mediate such phosphorylation (44). Thus, calcium activates at least three pathways that contribute to IL-4 induction: through calcineurin to dephosphorylate NFAT; likely through calcineurin to activate ASK1, leading to p38 activation; through CaM and CaMKIV to induce AP-1 protein synthesis; and activated p38 may induce AP-1 production and phosphorylation (Fig. 8). Cooperation between AP-1 and NFAT leads to Il4 gene transcription, and activated p38 also exerts a role in stabilization of Il4 mRNA half-life.

AP-1 and NFAT are also critical for Il2 transcription; however, IL-2 production was not detected in Th1 or Th2 cells. This can be accounted for by the requirement for additional factors, not induced by ionomycin, for Il2 transcription. Indeed, the CD28 response element (CD28RE) is essential for Il2 transcription, and c-Rel homodimers up-regulated in response to CD28 signaling bind to the Il2 CD28RE (45–47). c-Rel-deficient cells showed a striking defect in IL-2 production (48). When cells are stimulated through TCRs, p65/p50 dimers also interact with CD28RE in the Il2 promoter and act as potent transactivators (49). In resting cells and ionomycin-stimulated CD4T cells, p50/p50 homodimers, potent repressors of Il2 transcription, bind to Il2 promoter (24, 50, 51). Thus, the absence of c-Rel or p65/p50 in ionomycin-stimulated CD4 T cells explains the lack of Il2 transcription.

Do physiologic ligands that lead to robust calcium elevation cause direct effector T cell cytokine production in tissues, or do physiologic ligands that can activate p38 in Th2 or Th1 cells enhance the minimal cytokine production that would have occurred in response to limiting amounts of Ag/APC? Such activation might be particularly important in secondary responses to pathogens to ensure rapid cytokine production even before substantial amounts of Ag are released during infection. Thus, the p38 effect might confer on Th cells a type of innate-immune status. The nature of the ligands that would act to elevate [Ca\(^{2+}\)]\(_i\), in effector cell resident in tissues remains to be determined, but many candidates could be considered, particularly from the G protein-coupled receptor family of molecules, which would include chemokines.

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