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*J Immunol* 2011; 186:4140-4146; Prepublished online 2 March 2011;
doi: 10.4049/jimmunol.1002614
http://www.jimmunol.org/content/186/7/4140

Supplementary Material

http://www.jimmunol.org/content/suppl/2011/03/02/jimmunol.1002614.DC1

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Translational Control of NKT Cell Cytokine Production by p38 MAPK

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NKT cells are known to rapidly produce a large amount of cytokines upon activation. Although a number of signaling pathways that regulate the development of NKT cells have been identified, the signaling pathways involved in the regulation of NKT cell cytokine production remain unclear. In this study, we show that the p38 MAPK pathway is dispensable for the development of NKT cells. However, NKT cell cytokine production and NKT-mediated liver damage are highly dependent on activation of this pathway. p38 MAPK does not substantially affect cytokine gene expression in NKT cells, but it regulates the synthesis of cytokines through the Mnk–eIF4E pathway. Thus, in addition to gene expression, translational regulation by p38 MAPK could be a novel mechanism that contributes to the overall production of cytokine by NKT cells. The Journal of Immunology, 2011, 186: 4140–4146.

The p38 MAPK signaling pathway is known to regulate the production of a number of different cytokines, including TNF-α, IL-6, and IFN-γ. The members of the p38 MAPK family (p38α, p38β, p38γ, and p38δ) are activated by phosphorylation by the upstream activators MAPK kinases (MKK)3 and MKK6 (11). Regulation of cytokine production by p38 MAPK is primarily mediated by the control of gene transcription through the activation of specific transcription factors (12, 13). Additionally, p38 MAPK can also regulate cytokine mRNA stability indirectly through activation of MAPK-activated protein kinase-2 (14, 15) and cytokine mRNA translation by activation of MAPK-interacting serine/threonine kinases (Mnks) that phosphorylate and activate the translation initiation factor eIF4E (16–18). In this study, we show that the p38 MAPK is not essential for NKT cell development and cytokine gene expression, but together with Mnk contributes to cytokine synthesis in NKT cells. Thus, regulation of preexisting cytokine mRNA translation by p38 MAPK could be a novel mechanism for the rapid cytokine production in NKT cells.

Materials and Methods
Mice
C57BL/6J and BALB/cByJ mice were obtained from The Jackson Laboratory. Dominant-negative (dn) p38α transgenic mice have been described (19). The dn p38 mice, which were in B10.BR background, were crossed with C57BL/6 mice and the F1 progeny were used for the study. MKK3−/−MKK6−/− mice (20) were generated by serial intercrossing MKK3−/− (21, 22) and MKK6−/− mice (23). p38α conditional knockout (p38 cKO) mice were generated by crossing the homozygous floxed p38α mice (p38αfloxed) (24) with T cell-specific Lck-Cre transgenic mice [B6.Cg-Tg(Lck-cre)1Cwi N9] (25). The experimental procedures used in this study were approved by the Institutional Animal Care and Use Committee of the University of Vermont.

Surface and intracellular staining
Characterization of thymus, spleen, and liver NKT cells was performed as described (26). Briefly, cells were stained at 4°C in PBS/2% FCS containing 0.1% sodium azide. NKT cells were identified using anti-TCR and CD1d-tetramer/BS57 (National Institutes of Health Tetramer Facility). For intracellular cytokine staining of NKT cells, freshly isolated splenocytes from α-galactosylceramide (αGalCer)-treated mice (100 ng/g, i.p.) were stained with anti-TCR and CD1d-tetramer/BS57, followed by fixation, permeabilization, and staining for intracellular cytokines. The data

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Received for publication August 4, 2010. Accepted for publication January 25, 2011.

This work was supported by National Institutes of Health Grants AI067897 and RR021905 (to J.E.B.), and AI051454 (to M.R.).

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The online version of this article contains supplemental material.

Abbreviations used in this article: ALT, alanine transaminase; AST, aspartate aminotransferase; cKO, conditional knockout; dn, dominant-negative; αGalCer, α-galactosylceramide; MKK, MAPK kinase; Mnk, MAPK interacting serine/threonine kinase.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1002614

The Journal of Immunology
were collected on a BD LSR II (BD Biosciences) flow cytometer and analyzed using FlowJo software (Tree Star).

**In vivo and in vitro stimulations**

For in vivo stimulation, αGalCer (Axzora Pharmaceuticals; 100 ng/g) or vehicle was administered i.p. After 90 min, serum was collected and analyzed for cytokine levels, and intracellular staining of spleen NKT cells was performed as described above. Additionally, splenocytes were cultured ex vivo in complete medium without any further stimulation for 4 h at 37°C for analysis of cytokine production. For liver toxicity studies, mice were injected with αGalCer and serum samples were collected at different time points and liver samples were collected after 24 h. For in vitro stimulation, splenocytes from untreated mice were stimulated with αGalCer (10 ng/ml). NKT cells were assessed by CD1d tetramer staining.

**Ex vivo stimulation, splenocytes from untreated mice were stimulated with plate-bound anti-CD3 mAb (5 μg/ml). Where indicated, 5 μM SB203580 (Calbiochem) or 5 μM CGP57380 (Sigma-Aldrich) was added to the cultures.**

**For Western blot analysis, spleen NKT cells stained with PE-conjugated αGalCer-loaded CD1d tetramer were enriched using anti-PE microbeads (Miltenyi Biotec). Enriched NKT cells were expanded in vitro as described (27) with anti-CD3 and anti-CD28 mAbs in the presence of mouse IL-2 (10 ng/ml) and IL-12 (1 ng/ml). After 48 h, cells were transferred to complete medium supplemented with mouse IL-7 (5 U/ml). Cells were used on day 8 of culture, at which time they were typically >85% pure, as assessed by CD1d tetramer staining.

**Real-time PCR**

RNA was isolated using the RNeasy Micro kit as per the manufacturer’s instructions (Qiagen). RNA was reverse transcribed to cDNA and used for quantitative RT-PCR with IFN-γ- and IL-4-specific primers and probes (Assay on Demand; Applied Biosystems). Relative mRNA levels were estimated using the comparative threshold (Ct) method with GAPDH as housekeeping gene.

**ELISA**

Cytokines were measured either by standard ELISA using anti-IL-4 and anti-IFN-γ mAbs from BD Biosciences, or by multiplex ELISA performed according to the manufacturer’s instructions (Millipore) and read using a Luminox 200 instrument (Millipore).

**ELISPOT**

IFN-γ–producing NKT cells were detected by ELISPOT assay as described previously (28). In brief, sorted NKT cells from wild-type mice were plated (104 cells/well) in an ELISPOT plate coated with an anti-IFN-γ mAb (5 μg/ml) and stimulated with an anti-CD3 (1 μg/ml) mAb and anti-hamster cross-linking mAb (5 μg/ml). After 4 h, plates were washed, incubated with a biotinylated anti-IFN-γ Ab (Mabtech), followed by incubation with a streptavidin–alkaline phosphatase. The total number of IFN-γ–producing cells was determined using an ELISPOT reader (CTL-Immunospot S5 ELISPOT reader; Cellular Technology).

**Liver enzymes analysis**

Alanine transaminase (ALT) and aspartate aminotransferase (AST) activities in serum were measured using an ALT and AST reagent kit (Pointe Scientific) and the results were read using a Tecan Sapphire microplate reader (Tecan Trading).

**Western blotting**

Immunoblot analysis was performed as described previously (29) by probing with Abs for cleaved caspase-3, caspase-3, phospho-p38, phospho-eIF4E (Cell Signaling Technology) and actin (Santa Cruz Biotechnology).

**Immunohistochemistry**

Livers were fixed in 4% paraformaldehyde, processed, and embedded in paraffin. Sections (5 μm) were stained with H&E.

**Statistics**

Statistical analysis was conducted using Prism (GraphPad Software, La Jolla, CA). A Student t test or two-way ANOVA with Bonferroni’s or Dunnett’s post hoc analysis was used as needed. Results were considered significant when p < 0.05.

**Results**

**Impaired activation of p38 MAPK interferes with in vivo activation of NKT cells**

Unlike conventional CD4 T cells, NKT cells are capable of rapidly producing high levels of a variety of cytokines upon activation. The mechanisms that regulate cytokine production in NKT cells remain unclear. To investigate the role of the p38 MAPK signaling pathway in NKT cell cytokine production we examined mice deficient in the upstream activators of p38 MAPK, MKK3, and MKK6, which regulate all p38 MAPK family members. MKK3−/−/MKK6−/− mice are not viable (20), but the presence of a single MKK6 allele in MKK3−/−/6−/− mice is sufficient to prevent lethality while maintaining impaired activation of p38 MAPK in CD4+ T cells (Supplemental Fig. 1) (30). MKK3−/−/6−/− and wild-type mice were injected with the prototypical CD1d ligand, αGalCer, and 90 min later serum was collected. Analysis of serum cytokine production revealed significantly reduced levels of IFN-γ and IL-4 levels in MKK3−/−/6−/− mice compared with wild-type mice (Fig. 1A).

To determine whether the reduced serum cytokine levels in MKK3−/−/6−/− mice were due to a defect in NKT cell cytokine production, splenocytes were collected 90 min after αGalCer injection in MKK3−/−/6−/− and wild-type mice, and NKT cell cytokine production was examined by intracellular staining. A significant reduction in the frequency of IFN-γ- and IL-4–producing NKT cells was detected in the MKK3−/−/6−/− mice compared with the frequency in wild-type mice (Fig. 1B). Additionally, the intracellular levels of these cytokines (determined by the median fluorescence intensity) in NKT cells in MKK3−/−/6−/− mice were also reduced (Fig. 1C). Furthermore, when the splenocytes from αGalCer–injected wild-type and MKK3−/−/6−/− mice were cultured ex vivo for 4 h in the absence of stimulation, both IFN-γ and IL-4 levels were severely diminished in MKK3−/−/6−/− splenocytes (Fig. 1D).

To determine whether NKT cell development and/or maturation was altered in MKK3−/−/6−/− mice, we examined thymus, spleen, and liver NKT cell numbers through FACS analysis of TCRβ CD1d-tetramer/PBS57 + leukocytes (Fig. 2A). No difference in the number of NKT cells between MKK3−/−/6−/− and wild-type mice was observed in these organs (Fig. 2B). Examination of the developmental stage-specific markers CD44 and NK1.1 revealed no differences in expression on thymic NKT cells between MKK3−/−/6−/− and wild-type mice (Fig. 2C). Similarly, no differences in CD4 and NK1.1 expression on NKT cells in the peripheral organs were observed (Figs. 2D, 2E). Taken together, these results indicate that while in vivo NKT cell cytokine production is defective in MKK3−/−/6−/− mice, p38 MAPK is not essential for NKT cell development.

**Impaired activation of p38 MAPK protects against liver damage induced by αGalCer**

Activation of NKT cells by αGalCer in vivo can lead to TNF-α–dependent liver damage characterized by elevated ALT and AST activities in serum (31, 32). To determine whether the impaired cytokine production by NKT cells in MKK3−/−/6−/− mice affected NKT-mediated liver damage, we examined ALT and AST levels 24 h after injection with αGalCer. Serum ALT and AST levels were markedly increased in αGalCer-stimulated mice as compared with untreated mice (Fig. 3A). However, serum transaminase activities were significantly lower in αGalCer-stimulated MKK3−/−/6−/− mice than in wild-type mice (Fig. 3A). Similarly, a comparison of serum cytokine levels at various times after αGalCer administration revealed significantly lower TNF-α, IFN-γ, and IL-6 in MKK3−/−/6−/− compared with wild-type mice (Fig. 3B).
Because activation of p38 MAPK is systemically compromised in MKK3<sup>−/−</sup> mice, the impaired NKT cell cytokine production in vivo upon αGalCer administration in MKK3<sup>−/−</sup> mice could be due to effects on nonhematopoietic as well as hematopoietic cells. We therefore examined cytokine production in splenocytes from MKK3<sup>−/−</sup> and wild-type mice upon stimulation with αGalCer in vitro. In agreement with the in vivo data, there was a significant reduction in IFN-γ and IL-4 production in MKK3<sup>−/−</sup> splenocytes compared with wild-type splenocytes (Fig. 4A). To rule out the possibility that impaired peripheral NKT cell function was the result of an effect of p38 MAPK during NKT cell development, we also examined the effect of the p38 MAPK-specific inhibitor SB203580 (33, 34) on splenocytes from the wild-type mice. The production of both IFN-γ and IL-4 was dramatically reduced by the p38 MAPK inhibitor (Fig. 4A).

p38 MAPK has been reported to affect the CD1d-mediated Ag presentation to NKT cells (35). To rule out a potential indirect effect of p38 MAPK on Ag presentation to NKT cells, we examined splenocyte cytokine production in response to αGalCer in p38α<sup>−/−</sup> mice, in which the p38α gene is disrupted in the T cell lineage (36). This analysis revealed a significant impairment in the production of both IFN-γ and IL-4 in p38α<sup>−/−</sup> mice in comparison with wild-type mice (Fig. 4B). Similar to the MKK3<sup>−/−</sup> mice, we observed no specific defect in NKT cell numbers between p38<sup>−/−</sup> and wild-type mice (Fig. 4C). We also found that the production of IFN-γ was severely compromised in splenocytes from transgenic mice expressing a dn p38 MAPK mutant (Fig. 4D) where endogenous p38 MAPK in T cells is substantially reduced (19). Taken together, these data suggest a role for p38 MAPK in NKT cell cytokine production.

To examine directly the role of p38 MAPK in NKT cells, anti-CD3 mAb was used to stimulate sorted NKT cells in the presence of SB203580 or in medium alone. Simultaneously, NKT-depleted CD4<sup>+</sup> T cells were also sorted and activated with anti-CD3 in the presence or absence of SB203580 for cytokine analysis. The production of both IL-4 and IFN-γ by NKT cells was inhibited by SB203580 (Fig. 5A). In contrast, inhibition of p38 MAPK reduced IFN-γ, but not IL-4, production in non-NKT CD4<sup>+</sup> T cells, as previously described (19) (Fig. 5B). Thus, cytokine production by NKT cells is highly dependent on the activation of p38 MAPK.

Because p38 MAPK regulates IFN-γ gene expression and contributes to IFN-γ promoter activity in CD4<sup>+</sup> T cells (19), we examined whether p38 MAPK affects cytokine gene expression in NKT cells. Sorted NKT cells were activated with anti-CD3 for 24 h in the presence of SB203580 and mRNA levels for IFN-γ and IL-4 were quantified by real-time RT-PCR. Despite the strong effect on NKT cytokine production, inhibition of p38 MAPK had no effect on IFN-γ mRNA levels in NKT cells (Fig. 5C) and caused a <2-fold reduction in IL-4 mRNA levels (Fig. 5C).

Unlike CD4<sup>+</sup> T cells, NKT cells contain preformed cytokine mRNA prior to activation, enabling them to rapidly produce and secrete high levels of cytokines once they encounter Ag (2, 3). We therefore examined the levels of preformed cytokine mRNA in p38 MAPK-deficient NKT cells. No difference in IL-4 or IFN-γ mRNA levels was observed between freshly sorted wild-type and p38<sup>−/−</sup> NKT cells (Fig. 5D). Taken together, these data suggested that p38 MAPK was likely modulating cytokine protein synthesis rather than cytokine gene expression in NKT cells.
p38 MAPK regulates NKT cell cytokine production through regulation of translation

In addition to its effect on cytokine gene transcription and mRNA stability, p38 MAPK can also indirectly regulate cytokine production at a translational level. p38 MAPK phosphorylates the Mnk family members (Mnk1 and Mnk2), which in turn phosphorylate and activate the translational initiation factor eIF4E, which regulates translation of specific mRNAs (16–18). To determine whether inhibition of p38 MAPK could interfere with the early cytokine production that is likely derived from those preformed mRNA, sorted NKT cells were stimulated with anti-CD3 in the absence or presence of SB203580 for 4 h and IFN-γ-producing cells were measured by ELISPOT, the most sensitive cytokine production assay. A substantial reduction in the frequency of IFN-γ-producing cells was found when SB203580 was present (Fig. 6A). We then examined whether eIF4E was phosphorylated upon activation of NKT cells by Western blot analysis using in vitro-expanded NKT cells. Phosphorylation of eIF4E was detected upon activation of NKT cells from MKK3−/−MKK6−/− mice (Fig. 6B). To determine whether eIF4E contributed to NKT cell cytokine production, NKT cells were activated in the presence or absence of the Mnk inhibitor (upstream activator of eIF4E) CGP57380 (37). Inhibition of Mnk caused a strong reduction of both IFN-γ and IL-4 production (Fig. 6F). No effect on NKT cell viability was observed with the concentrations of the compound used (data not shown). Therefore, translational regulation contributes to the overall cytokine production in NKT cells.

Discussion

A number of signaling pathways involved in the development, maturation, and/or survival of NKT cells have been identified, including SLAM–SAP–Fyn, calcineurin–NFAT–Egr2, and PKC0–NF-κB pathways (4, 7–9, 38, 39). However, the signaling pathways that are involved in the rapid production of large amounts of cytokines by NKT cells remain unclear. In this study, we show that inhibition of p38 MAPK prevented the phosphorylation of eIF4E (Fig. 6C). We confirmed that inhibition of p38 MAPK also impaired the production of cytokines by in vitro-expanded NKT cells (Fig. 6D). Furthermore, in correlation with a strong reduction of active p38 MAPK, a significant reduction in the level of phospho-eIF4E was found in anti-CD3–stimulated, expanded NKT cells from MKK3−/−MKK6−/− mice (Fig. 6E). To determine whether eIF4E contributed to NKT cell cytokine production, NKT cells were activated in the presence or absence of the Mnk inhibitor (upstream activator of eIF4E) CGP57380 (37). Inhibition of Mnk caused a strong reduction of both IFN-γ and IL-4 production (Fig. 6F). No effect on NKT cell viability was observed with the concentrations of the compound used (data not shown). Therefore, translational regulation contributes to the overall cytokine production in NKT cells.
while p38 MAPK is not critical for NKT cell development, activation of this pathway in NKT cells is essential for the production of IFN-γ and IL-4. These results contrast with the lack of effect of p38 MAPK on IL-4 production by conventional naive CD4 T cells (19). The p38 MAPK pathway has been shown to regulate IFN-γ production in conventional naive CD4 T cells, and this effect is primarily due to the regulation of transcription factors involved in IFN-γ gene expression. In contrast, the data reported in this study suggest that p38 MAPK does not seem to affect cytokine gene expression or mRNA levels in NKT cells. Instead, p38 MAPK appears to be critical for the translational control of NKT cytokine mRNAs. This model is consistent with the presence of preformed cytokine mRNA in NKT cells (2, 3), which would require signals triggered upon recognition of Ag to rapidly initiate cytokine mRNA translation. We propose that the p38 MAPK signaling pathway is involved in this pathway, likely through the activation of the Mnk–eIF-4E pathway. This is a potentially novel mechanism for regulation of cytokine production by p38 MAPK in NKT cells.

The presence of preformed mRNA cytokine transcripts is not unique to NKT cells and indeed appears to be characteristic of some leukocyte subsets that exhibit rapid response kinetics. Mast cells, basophils, eosinophils, and NK cells possess preformed cytokine mRNAs, and all are similar in that translation of cytokines does not occur until after stimulation (40). Interestingly, Mohrs et al. (41) reported that preformed IL-4 is also found in memory, but not...
naive, CD4+ T cells, consistent with the more rapid response kinetics observed in memory cells. As in NKT cells, the regulatory mechanisms that control the onset of cytokine translation in these leukocyte subsets are unclear, allowing us to speculate that p38 MAPK may also be involved in the regulation of cytokine translation in these cells as well.

Activation of the p38 MAPK pathway has previously been implicated in animal models of liver disease (42, 43). It remains unclear, however, in which cells does p38 MAPK activation play an important role in disease pathology. NKT cells have been implicated in a variety of liver pathologies, including a mouse model of autoimmune liver disease triggered by bacterial infection (44) and Con A-induced hepatitis (45), which is dependent on TNFα. Moreover, direct stimulation of liver NKT cells using αGalCer induces liver injury in a TNF-α–dependent manner (31, 32). Our study suggests that the role of p38 MAPK in mediating...
liver injury could be attributed to its activation in NK T cells instead of hepatocytes or macrophages.

Acknowledgments

We thank Colette Charland for flow cytometry and the Vermont Cancer Center DNA facility for real-time PCR analysis. The CD1d tetramer was provided by the National Institutes of Health Tetramer Facility.

Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure S1. Inhibition of p38 MAPK activation in CD4+ T cells from MKK3−/− 6+/− mice. CD4+ T cells isolated from WT or MKK3−/− 6+/− mice were activated with anti-CD3 and western blot analysis was performed with anti-phospho p38, p38 or actin at the indicated times.