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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 large amounts 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.

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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; GalCer, α-galactosylceramide; Mkk, MAPK kinase; Mnk, MAPK interacting serine/threonine kinase.

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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 (AxXora 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 sorted by staining the splenocytes with PE-conjugated αGalCer-loaded CD1d tetramer and FITC-labeled TCR followed by sorting on a FACS Aria (BD Biosciences). Sorted NKT cells or non-NKT CD4 T cells were stimulated with plate-bound anti-CD3 mAb (5 μg/ml) and stimulated with an anti-CD28 mAb from BD Biosciences, or by multiplex ELISA performed according to the manufacturer’s instructions (Millipore) and read using a Luminox 200 instrument (Millipore).

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. MKK3Δ/Δ-MKK6Δ/Δ mice are not viable (20), but the presence of a single MKK6 allele in MKK3Δ/Δ-MKK6Δ/Δ mice is sufficient to prevent lethality while maintaining impaired activation of p38 MAPK in CD4+ T cells (Supplemental Fig. 1) (30). MKK3Δ/Δ 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 MKK3Δ/Δ mice compared with wild-type mice (Fig. 1A).

To determine whether the reduced serum cytokine levels in MKK3Δ/Δ mice were due to a defect in NKT cell cytokine production, splenocytes were collected 90 min after αGalCer injection in MKK3Δ/Δ 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 MKK3Δ/Δ 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 MKK3Δ/Δ mice were also reduced (Fig. 1C). Furthermore, when the splenocytes from αGalCer-injected wild-type and MKK3Δ/Δ mice were cultured ex vivo for 4 h in the absence of stimulation, both IFN-γ and IL-4 levels were severely diminished in MKK3Δ/Δ splenocytes (Fig. 1D).

To determine whether NKT cell development and/or maturation was altered in MKK3Δ/Δ mice, we examined thymus, spleen, and liver NKT cell numbers through FACS analysis of TCRδ+CD1d-tetramer/PBS57+ leukocytes (Fig. 2A). No difference in the number of NKT cells between MKK3Δ/Δ 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 MKK3Δ/Δ 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 MKK3Δ/Δ 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 MKK3Δ/Δ 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 MKK3Δ/Δ 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 MKK3Δ/Δ mice compared with wild-type mice (Fig. 3A).
analyzed 90 min later. We therefore examined cytokine production in splenocytes from MKK3 cells. We observed no specific defect in NKT cell numbers between p38 cKO 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 T cells were also sorted and activated with anti-CD3 in the presence 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 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 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+ 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 cKO 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.

**FIGURE 1.** Impaired cytokine production in NKT cells from MKK3–/− 6+ mice. MKK3–/− 6+ and wild-type mice were administered αGalCer and analyzed 90 min later. A, Serum cytokine levels were assessed by ELISA. Data represent means ± SD, n = 4 mice per strain, and are representative of three experiments. B, Representative intracellular staining of splenocyte NKT cells stained for IFN-γ and IL-4. Histograms depict the intracellular cytokine staining of gated NKT cells with isotype-matched control mAbs (gray line) or anti-cytokine mAbs (black line). The mean percentage of cytokine positive NKT cells is indicated. C, Median fluorescence intensity (MFI) of NKT cells that were positive for IFN-γ or IL-4. Data represent the mean MFI ± SD, n = 3 mice per strain, and are representative of three experiments. D, Ex vivo analysis of NKT cell cytokine production. Splenocytes from αGalCer-treated mice were placed in culture for 4 h, after which IFN-γ and IL-4 in cell culture supernatant was assessed by ELISA. Data represent the mean ± SD, n = 3 mice per strain, and are representative of two experiments. *p < 0.05. WT, wild-type.
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 it 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 and it correlated with the induction of active p38 MAPK (Fig. 6B). To demonstrate that phosphorylation of eIF4E was indeed dependent on p38 MAPK, we examined phospho-eIF4E in NKT cells activated with anti-CD3 in the presence or absence of SB203580. These data revealed 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<sup>−/−</sup>MKK6<sup>+/-</sup> 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.

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 PKC–NF-xB 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

FIGURE 2. No effect of p38 MAPK inhibition on NKT cell development. NKT cell number and phenotype were compared between age- and sex-matched C57BL/6J and MKK3<sup>−/−</sup>MKK6<sup>+/-</sup> mice. Thymus, spleen, and liver NKT cell numbers were assessed using anti-TCR and CD1d-tetramer/PBS57. A, Representative dot plots depicting percentages of NKT cells (left) and contour plots depicting cell surface expression of CD44 and NK1.1 (thymus) and CD4 and NK1.1 (spleen and liver) on NKT cells (right). The data are representative of two independent experiments. B, Cumulative numbers of NKT cells numbers in thymus, spleen, and liver. The data represent the means ± SD, n = 5 mice per strain, and are representative of two independent experiments. WT, wild-type. C, Cumulative percentage of CD44 and NK1.1-expressing NKT cells in different organs. D, Cumulative percentage of CD4 and NK1.1-expressing NKT cells in spleen (D) and liver (E). The data represent the means ± SD, n = 5 mice per strain, and are representative of two independent experiments.
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

**FIGURE 3.** Activation of p38 MAPK is essential for NKT-mediated liver damage. A, Serum transaminase activity (ALT and AST) in wild-type and MKK3−/−-6+/− mice after 24 h treatment with αGalCer. Data represent the means ± SEM, n = 15 mice per condition, from a single experiment. *p < 0.05. B, Serum cytokine levels at different intervals posttreatment with αGalCer were measured by multiplexed ELISA. Data represent the means ± SD, n = 8 mice per time point, from a single experiment. Statistical comparisons were made between wild-type and MKK3−/−-6+/− mice after treatment for 24 h with αGalCer are presented. Original magnification ×20. C, Representative H&E-stained liver sections prepared from wild-type and MKK3−/−-6+/− mice after treatment for 24 h with αGalCer are presented. Original magnification ×20. D, Liver extracts prepared from wild-type and MKK3−/−-6+/− mice after treatment for 24 h with αGalCer were examined by immunoblot analysis for caspase-3 and cleaved caspase-3. WT, wild-type.

**FIGURE 4.** αGalCer-dependent IFN-γ and IL-4 production is p38 MAPK-dependent. A, Splenocytes from wild-type and MKK3−/−-6+/− mice were stimulated in vitro with αGalCer (10 ng/ml) for 48 h and the cytokine production was determined by ELISA. Additionally, splenocytes from wild-type mice were activated with αGalCer in the presence of SB203580 (5 μM) and assayed for IFN-γ and IL-4 production. Data represent the means ± SD and are representative of two experiments. B, Wild-type and p38 cKO splenocytes were activated in vitro with αGalCer as in A and IFN-γ and IL-4 production were determined by ELISA. Data represent the means ± SD, n = 2 mice per strain, and are representative of two experiments. C, Percentage of NKT cells in thymus, spleen, and liver in wild-type and p38−/−. Data represent the means ± SD, n = 3 mice per strain. D, Wild-type and dn p38 transgenic splenocytes were activated in vitro with αGalCer as in A and IFN-γ production was determined by ELISA. Data represent the means ± SD, n = 2 mice per strain, and are representative of two experiments. **p < 0.01, ***p < 0.001. WT, wild-type.
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

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**FIGURE 5.** Activation of p38 MAPK is essential for IFN-γ and IL-4 production by NKT cells. Sorted NKT cells (A) and non-NKT CD4 T cells (B) were stimulated with anti-CD3 mAbs for 24 h in the presence of medium (Med) or SB203580 (SB), and IFN-γ and IL-4 production was determined by ELISA. Data represent the means ± SD and are representative of two experiments. *p < 0.01, ***p < 0.001. C, Sorted NKT cells were stimulated with anti-CD3 mAbs for 24 h in the presence of medium (Med) or SB203580 (SB) and mRNA levels of cytokines were determined quantitative PCR. Data represent the means ± SD. D, Preformed mRNA levels in sorted NKT cells as determined by quantitative PCR. WT, wild-type. Data represent the means ± SD.

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**FIGURE 6.** Gene expression-independent regulation of cytokine production in NKT cells by p38 MAPK. A, Number of IFN-γ-producing cells after 4 h stimulation of sorted NKT cells with anti-CD3 mAb in the presence of medium or SB203580 as determined by ELISPOT. B, Western blot analysis for phospho-p38 MAPK and phospho-eIF4E in expanded NKT cells stimulated with anti-CD3 mAbs for the indicated times. Actin was used as a loading control. C, Western blot analysis for phospho-eIF4E in expanded NKT cells stimulated with anti-CD3 mAbs in the presence of medium or SB203580. Actin was used as a loading control. D, Inhibition of expanded NKT cell line cytokine production by SB203580 after in vitro anti-CD3 stimulation. E, Western blot analysis comparing phospho-p38 MAPK and phospho-eIF4E in expanded NKT cells between wild-type (WT) and MKK3⁻/⁻ MK6⁻/⁻ mice. NKT cells were stimulated with anti-CD3 mAbs for the indicated times. Actin was used as a loading control. F, Sorted NKT cells were stimulated with anti-CD3 mAbs for 24 h in the presence of medium (Med) or 5 μM CGP57380 (CGP) and cytokine production was determined by ELISA. *p < 0.05, ***p < 0.001.
liver injury could be attributed to its activation in NK T cells instead of hepatocytes or macrophages.

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Disclosures

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