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Defective IL-12 production in mitogen-activated protein (MAP) kinase kinase 3 (*Mkk3*)-deficient mice

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The p38 mitogen-activated protein kinase (MAPK) pathway, like the c-Jun N-terminal kinase (JNK) MAPK pathway, is activated in response to cellular stress and inflammation and is involved in many fundamental biological processes. To study the role of the p38 MAPK pathway *in vivo*, we have used homologous recombination in mice to inactivate the *Mkk3* gene, one of the two specific MAPK kinases (MAPKKs) that activate p38 MAPK. *Mkk3*$^{-/-}$ mice were viable and fertile; however, they were defective in interleukin-12 (IL-12) production by macrophages and dendritic cells. Interferon-γ production following immunization with protein antigens and *in vitro* differentiation of naive T cells is greatly reduced, suggesting an impaired type I cytokine immune response. The effect of the p38 MAPK pathway on IL-12 expression is at least partly transcriptional, since inhibition of this pathway blocks IL-12 p40 promoter activity in macrophage cell lines and IL-12 p40 mRNA is reduced in MKK3-deficient mice. We conclude that the p38 MAP kinase, activated through MKK3, is required for the production of inflammatory cytokines by both antigen-presenting cells and CD4^{+} T cells.

Keywords: interleukin-12/inflammation/ lipopolysaccharide (LPS)/p38 MAPK/MKK3

Introduction

The mitogen-activated protein kinase (MAPK) pathway transduces a variety of extracellular signals through a cascade of protein phosphorylation. There are at least three genetically distinct MAPK pathways in mammals including the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs) and the p38 MAPK. These kinases are activated by phosphorylation on both threonine and tyrosine residues in a regulatory TXY loop present in all MAP kinases. This phosphorylation is performed by distinct upstream dual-specificity MAP kinase kinases (MAPKKs). Activated MAP kinases then phosphorylate their respective substrates on serine or threonine residues (Whitmarsh and Davis, 1996; Minden and Karin, 1997; Ip and Davis, 1998).

The physiological function of the ERK pathway is to respond mainly to mitogens and growth factors, such as epidermal growth factor and platelet-derived growth factor, and regulate cell proliferation and differentiation. The JNK and p38 MAPK pathways are referred to as stress-activated MAP kinase (SAPK) pathways since they are both activated by environmental perturbation (e.g. osmotic changes, heat shock) and by inflammatory cytokines including tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1). The physiological functions of the JNK and p38 MAPK pathways might therefore be overlapping (Ip and Davis, 1998). Biochemical and genetic studies have revealed the roles of the JNK pathway as regulators of apoptosis (Xia *et al*., 1995; Dickens *et al*., 1997; Yang *et al*., 1997b), development (Sluss *et al*., 1996; Yang *et al*., 1997a), cell transformation (Dickens *et al*., 1997), T cell activation and differentiation (Su *et al*., 1994; Dong *et al*., 1998; Yang *et al*., 1998a) and cytokine production (Swantek *et al*., 1997). Similarly, the p38 MAPK pathway has been proposed to function in the regulation of cytokine production (Lee *et al*., 1994; Bayaert *et al*., 1996; Rincon *et al*., 1998), B cell and T cell proliferation and differentiation (Crawley *et al*., 1997; Craxton *et al*., 1998; Rincon *et al*., 1998; Sugawara *et al*., 1998), the innate immune response (Han *et al*., 1998), cell cycle control (Molnar *et al*., 1997; Takenaka *et al*., 1998) and apoptosis (Xia *et al*., 1995; Huang *et al*., 1997; Juo *et al*., 1997; Wang *et al*., 1998).

The p38 MAPK, also known as CSBP and RK, was first identified by several independent groups using different strategies: it was shown to be a major tyrosine-phosphorylated 38 kDa protein induced by lipopolysaccharide (LPS) in murine macrophage cell lines (p38; Han *et al*., 1994); to be the target for a group of anti-inflammatory drugs which inhibit IL-1 and TNF-α synthesis in human monocytes (CSBP; Lee *et al*., 1994); and an IL-1-induced protein kinase that activates the protein kinase MAPKAP kinase 2 (RK; Freshney *et al*., 1994; Rouse *et al*., 1994). The p38 MAPK is similar to the yeast HOG1 MAP kinase which is involved in osmolarity regulation (Herskowitz, 1995). Two p38 MAPK isoforms were identified in *Drosophila* that appear to regulate immunity gene expression (Han *et al*., 1998). There are four mammalian isoforms of p38 MAPK: p38α; p38β; p38γ; and p38δ (Freshney *et al*., 1994; Han *et al*., 1994; Lee *et al*., 1994; Rouse *et al*., 1994; Jiang *et al*., 1996, 1997; Li *et al*., 1996; Mertens *et al*., 1996; Cuenda *et al*., 1997; Goedert *et al*., 1997; Stein *et al*., 1997; Wang *et al*., 1997; Enslen *et al*., 1998). The *in vitro* substrates of p38 include the transcription factors ATF-2 (Raingeaud *et al*., 1995, 1996), CHOP/GADD153 (Wang and Ron, 1996), Elk-1

(Raingeaud *et al*., 1996; Whitmarsh *et al*., 1997), MEF-2C (Han *et al*., 1997a) and SAP-1 (Whitmarsh *et al*., 1997), and protein kinases including MAPKAP kinase 2 and 3 (Freshney *et al*., 1994; Rouse *et al*., 1994; Ludwig *et al*., 1996; McLaughlin *et al*., 1996), Mnk1 and 2 (Fukunaga and Hunter, 1997; Waskiewicz *et al*., 1997), Msk1 (Deak *et al*., 1998), PRAK (New *et al*., 1998) and RSK-B (Pierrat *et al*., 1998). However, whether the above are physiologically relevant substrates is unclear.

The availability of p38-specific inhibitory drugs has facilitated rapid progress in the study of the role of p38 MAPK pathways in a variety of biological systems (Lee *et al*., 1994; Lee and Young, 1996); however, since these p38 inhibitors are pyridinyl imidazole derivatives that bind to the ATP-binding groove within the p38 MAPK, the specificity of this inhibition *in vivo* remains to be established (Cohen, 1997; Wilson *et al*., 1997; Young *et al*., 1997). It was reported that SB 203580, one of the widely used p38 MAPK inhibitors, could also inhibit JNK2 activity, albeit with lower potency (Whitmarsh *et al*., 1997; Clerk and Sugden, 1998). p38γ and p38δ are not inhibited by these drugs (Cuenda *et al*., 1997; Goedert *et al*., 1997; Jiang *et al*., 1997; Wang *et al*., 1997). Limitations of p38 chemical inhibitors suggest that alternative methods to study the p38 MAPK pathway would be desirable.

The specific upstream MAPK kinases for p38 MAPK are MKK3 and MKK6 (Derijard *et al*., 1995; Cuenda *et al*., 1996; Han *et al*., 1996, 1997b; Moriguchi *et al*., 1996; Raingeaud *et al*., 1996; Stein *et al*., 1996), although MKK4, an upstream kinase for JNKs, has also been implicated in the activation of the p38 MAPK pathway (Derijard *et al*., 1995; Lin *et al*., 1995; Ganiatsas *et al*., 1998). Both MKK3 and MKK6 are activated upon phosphorylation on serine and threonine residues within subdomain VIII by upstream MAPKK kinases. There are many MAPKK kinases (MAPKKKs) that can activate MKK3 and MKK6 *in vitro*, but the physiologically relevant activators that mediate the effects of specific stimuli remain to be determined (Fanger *et al*., 1997). To study the role of the p38 MAPK pathway *in vivo*, and to study the relative contribution of MKK3 and MKK6 in the activation of p38 MAPK, we have generated mice with a germline mutation of the *Mkk3* gene. Here, we report that MKK3-deficient mice were viable and fertile. Nevertheless, these mice have defective p38 MAPK activation and defects in the production of IL-12 and interferon-γ (IFN-γ), resulting in an impaired Th1 $CD4^+$ immune response. These results suggest that MKK3 is the *in vivo* upstream kinase for p38 MAPK in response to certain specific stimuli.

Results

Generation of Mkk3$^{-/-}$ mice

Mice carrying a null mutation in the *Mkk3* gene were generated using homologous recombination in embryonic stem (ES) cells by a strategy of positive and negative selection. A targeting vector was constructed and is shown in Figure 1A. Homologous recombination with the endogenous *Mkk3* gene will replace an internal 1.5 kb *Bgl*II–*Eco*RV genomic fragment with a *neo* gene cassette. The deleted region includes exons 8 and 9, which encode amino acids 217–221 of the murine MKK3 protein. This region includes the sequence Ser–Val–Ala–Lys–Thr containing the dual phosphorylation sites (serine and threonine) that are required for MKK3 activation (Derijard *et al*., 1995). The deleted region also encompasses sequences that are highly conserved among all protein kinases (Hanks *et al*., 1988). These observations led us to anticipate that the predicted targeted disruption of the *Mkk3* gene would result in a null allele.

The linearized targeting construct was transfected into W9.5 ES cells. Analysis of 159 independent G418- and gancyclovir-resistant clones by Southern blotting identified 14 positive clones. The frequency of homologous recombinants among the G418- and gancyclovir-resistant clones was 9%. Six targeted ES clones were injected into C57BL/6 blastocysts, and two (clones 49 and 54) resulted in chimeric mice that transmitted the mutated *Mkk3* allele through the germline.

Crosses of the *Mkk3*$^{+/-}$ mice resulted in progeny with the expected Mendelian frequencies. A representative Southern blot using genomic DNA isolated from wild-type mice and from mice that are heterozygous or homozygous for the disrupted *Mkk3* allele is shown in Figure 1B. Northern blot analysis of kidney and liver RNA confirmed that the homozygous *Mkk3*$^{-/-}$ mice did not express detectable levels of *Mkk3* mRNA (Figure 1C), nor was the MKK3 protein detected by Western blot analysis of knockout peritoneal macrophages (Figure 1D). Western blot analysis of other kinases in the SAPK pathway indicated that *Mkk3*$^{-/-}$ mice expressed normal levels of MKK6, MKK4, JNK and p38 MAP kinase (Figure 1D) and, therefore, there were no compensatory changes in the expression of these other kinases as a consequence of the MKK3 deficiency.

Normal development of Mkk3$^{-/-}$ mice

The *Mkk3* knockout mice were viable and fertile, with no detected developmental defects. No gross histological abnormalities of the lymphoid organs were apparent in young mice. Gross histological analyses of hematoxylin- and eosin-stained sections of other non-lymphoid organs, including liver and lung, also revealed no obvious abnormalities (data not shown). The knockout mice have normal numbers of thymocytes and splenocytes. Major cell surface markers of T and B lymphocytes were examined by flow cytometric analysis of cells derived from freshly isolated thymus, spleen and lymph nodes. The wild-type and knockout mice have similar expression of CD3, CD4, CD8, CD25, CD44, CD69, T cell receptor (TCR) α/β and γ/δ, B220, F4/80 and major histocompatibility complex class II antigen $I\text{-}A^b$ (data not shown). The number of bone marrow-derived dendritic cells (DCs), as well as CD11c, $I\text{-}A^b$ and B7-2 surface markers also did not differ between DCs from *Mkk3* wild-type and knockout mice (data not shown). No obvious defects in lymphocyte development were therefore evident in *Mkk3*$^{-/-}$ mice.

Defective p38 MAP kinase activity in Mkk3$^{-/-}$ macrophages

By the use of the chemical inhibitors, the p38 kinase pathway has been implicated in the regulation of the expression of TNF-α, IL-1, IL-6 and granulocyte–macro-

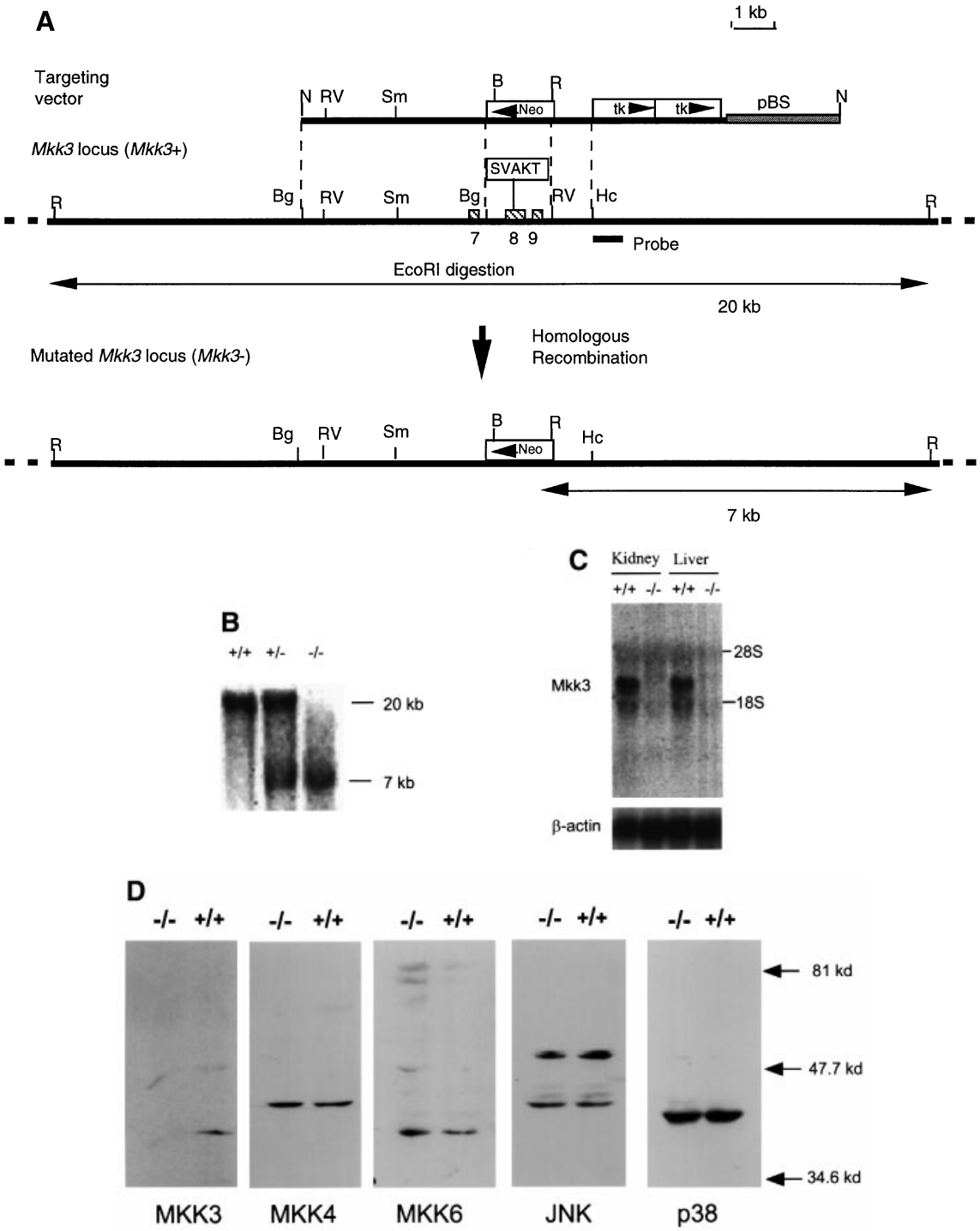


Fig. 1. Disruption of the *Mkk3* gene by homologous recombination. (**A**) Structure of the targeted vector, the *Mkk3* gene and the mutated *Mkk3* gene following homologous recombination. Relevant restriction enzyme sites are indicated. (N, *Not*I; RV, *Eco*RV; Sm, *Sma*I; B, *Bam*HI; R, *Eco*RI; Bg, *Bgl*I; Hc, *Hinc*II). Exons 7, 8 and 9 are depicted as closed boxes. SVAKT is the protein sequence (single letter code) that includes the dual phosphorylation sites that are required for MKK3 activation. The diagnostic probe used for Southern analysis is illustrated. (**B**) Southern blot analysis. Genomic DNA from mouse tails was digested with *Eco*RI, and blots were hybridized with the probe shown in (A). The wild-type allele corresponds to a 20 kb fragment and the mutated allele is a 7 kb fragment. (**C**) Northern blot analysis. Total RNA isolated from kidneys and livers of wild-type and homozygous mice was hybridized with a MKK3 cDNA fragment. The blots were also probed for β-actin mRNA which was used as an internal control. (**D**) Western blot analysis. Protein lysates from peritoneal macrophages of wild-type and homozygous mice were used to examine the expression of MKK3, MKK4, MKK6, JNK and p38 MAP kinase.

phage colony-stimulating factor (GM-CSF) (Lee *et al*., 1994; Bayaert *et al*., 1996). Since many of the inflammatory cytokines are produced by macrophages upon activation by LPS, a potent activator of the p38 MAPK pathway, LPS-activated p38 MAPK activity in MKK3-deficient macrophages was studied. There was reduced p38 MAPK activation in *Mkk3*$^{-/-}$ macrophages in comparison with wild-type macrophages (Figure 2A); interestingly, however, the activation of p38 MAPK in response to sorbitol was similar in the wild-type and knockout macrophages

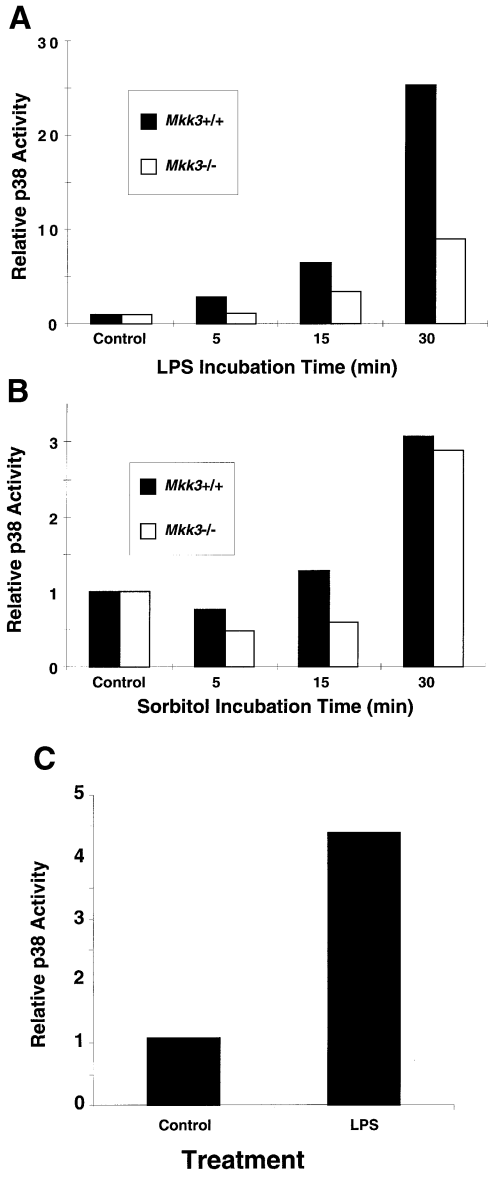


Fig. 2. p38 MAPK activity in MKK3-deficient and wild-type primary macrophages and macrophage cell line. Peritoneal macrophages from MKK3 wild-type and knockout mice were left untreated (control) or were treated with LPS (100 ng/ml) (**A**) or sorbitol (300 mM) (**B**) for 5, 15 and 30 min. The p38 MAPK activity was measured using an immune complex kinase assay with the substrate GST–ATF2. (**C**) RAW264.7 cells were transiently transfected with an MKK3 expression vector. Then, 24 h later, the cells were left untreated or were treated with LPS for 30 min. MKK3 was immunoprecipitated from the cell lysates. Kinase assays were performed using the immunoprecipitates, GST–p38γ, GST–ATF2 and [γ-^{32}P]ATP. The radioactivity incorporated into GST–ATF2 was quantitated after SDS–PAGE by PhosphorImager analysis and is presented as relative p38 MAPK activity. The results shown were obtained in a single experiment and are representative of two separate experiments with similar results.

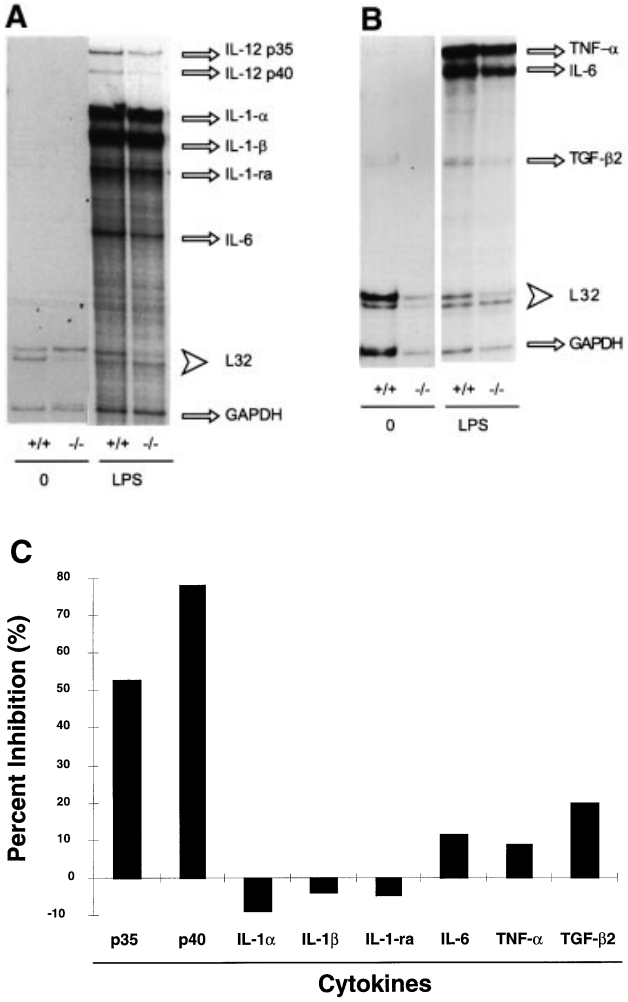


Fig. 3. Inflammatory cytokine mRNA levels induced by LPS are reduced in MKK3-deficient mice. Peritoneal macrophages were left untreated or treated with LPS (100 ng/ml) for 6 h. Total RNA was isolated and an RNase protection assay was performed to examine cytokine mRNA expression using mCK-2b probes (Pharmingen) (**A**) or mCK-3 probes (Pharmingen) (**B**). L32 and GAPDH are housekeeping genes that serve as internal controls. (**C**) The percentage inhibition of cytokine expression in MKK3-deficient macrophages. The intensity of the radioactive band was quantitated by PhosphorImager analysis using GAPDH as an internal control.

(Figure 2B), indicating that LPS-induced p38 MAPK activation was selectively defective in the MKK3-deficient macrophages. On the other hand, JNK activity induced by LPS in MKK3-deficient macrophages was not reduced (data not shown).

In order for MKK3 deficiency to account for the defect in p38 activation in *Mkk3*-deficient macrophages, it is necessary for LPS to be an activator of MKK3. To test this directly, we transfected RAW264.7 cells with an MKK3 expression vector and activated these cells with LPS. MKK3 was then immunoprecipitated and p38 activity was measured (Figure 2C). MKK3-directed p38 activation was stimulated >4-fold by LPS, showing that LPS indeed activates MKK3.

Defective IL-12 production in Mkk3$^{-/-}$ antigen-presenting cells

To test whether the expression of inflammatory cytokines was affected in MKK3-deficient mice, LPS-stimulated macrophage RNA was isolated and subjected to an RNase protection assay (RPA) by using a panel of inflammatory cytokine probe sets. IL-6, TNF-α, IL-1α and IL-1β mRNAs accumulated upon LPS stimulation in the wild-type and the knockout macrophages to a similar degree (Figure 3A and B), indicating that MKK3 deficiency did not affect the expression of these four cytokine mRNAs. Both IL-12 p40 and p35 mRNAs were up-regulated in

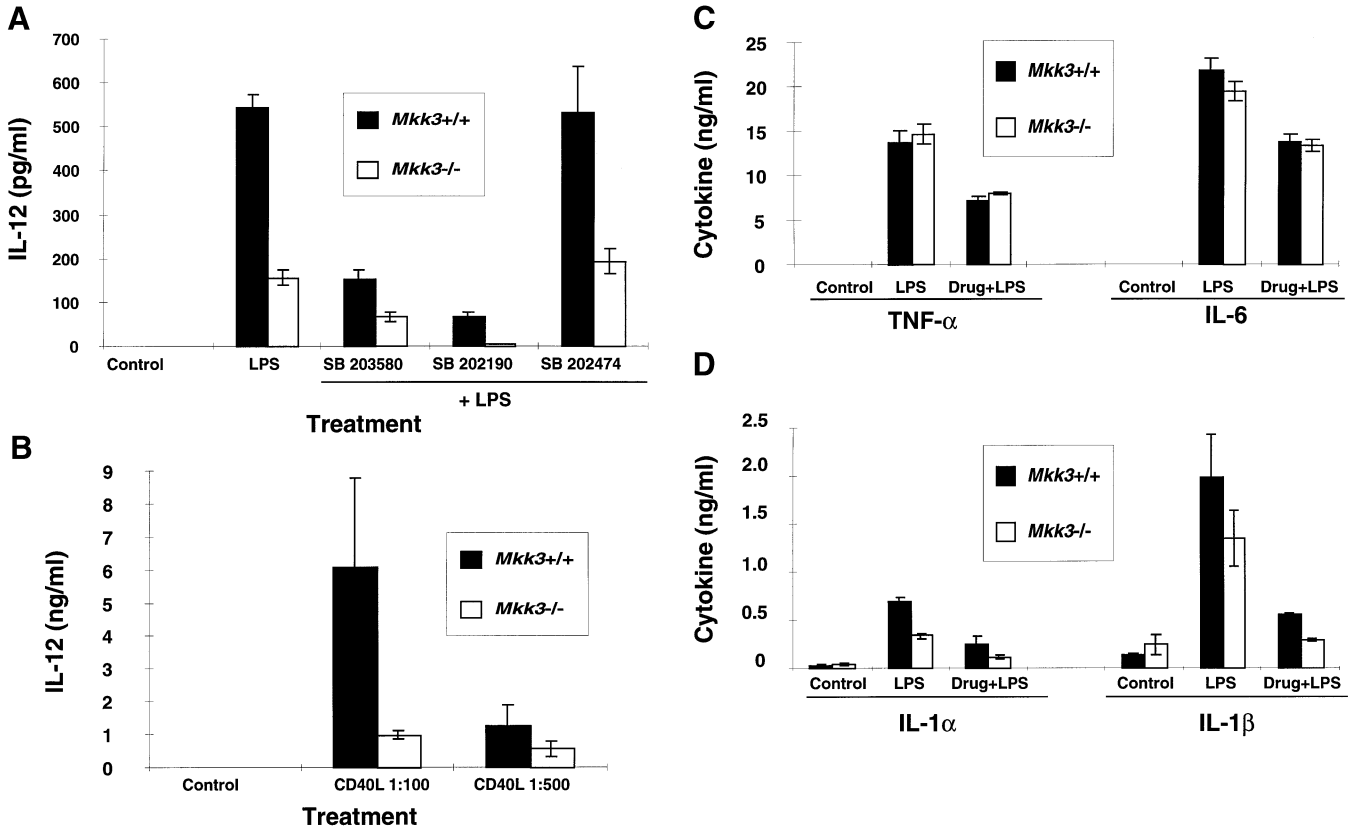


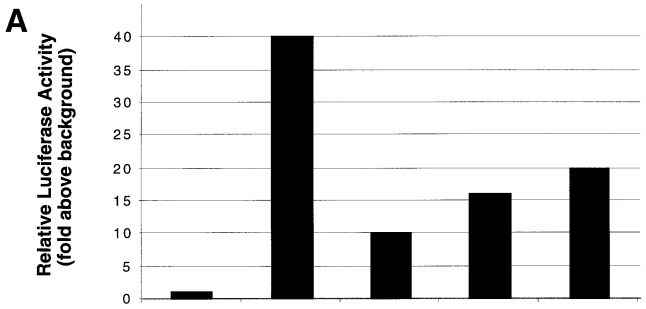
Fig. 4. Inflammatory cytokine production induced by LPS is reduced in MKK3-deficient mice. (**A**) IL-12 production in MKK3 macrophages. Peritoneal macrophages were left untreated, treated with LPS (100 ng/ml) for 20 h or pre-treated with 10 μM SB 203580, SB 202190 or SB 202474 for 2 h before LPS (100 ng/ml) was added for an additional 20 h. Supernatants were collected and IL-12 production was measured by ELISA. The mean and standard error are shown. The results shown in this experiment were representative of six separate experiments with similar results. (**B**) IL-12 production by CD40–CD40L engagement in bone marrow-derived DCs. Bone marrow-derived DCs were left untreated or were treated with different concentrations of membrane-bound CD40L (1:100 and 1:500) for 48 h. Supernatants were collected and IL-12 production was measured by ELISA. The mean and standard error are shown. The results shown in this experiment were representative of two separate experiments with similar results. (**C**) IL-6 and TNF-α production in MKK3 macrophages. Peritoneal macrophages were left untreated or were treated with LPS (100 ng/ml) for 20 h or pre-treated with SB 202190 (10 μM) for 2 h before LPS (100 ng/ml) was added for an additional 20 h. Supernatants were collected and IL-6 and TNF-α production were measured by ELISA. The mean and standard error are shown. The results shown in this experiment were representative of three separate experiments with similar results. (**D**) IL-1α and IL-1β production in MKK3 macrophages. Peritoneal macrophages were left untreated or were treated with LPS (100 ng/ml) for 4 h or pre-treated with SB 202190 (10 μM) for 2 h before LPS (100 ng/ml) was added for an additional 4 h. ATP (5 mM) was added to the culture for 30 min to induce the release of IL-1 into the supernatant. The supernatants were collected and IL-1 (α and β) production was measured by ELISA. The mean and standard error are shown. The results shown were obtained in a single experiment and are representative of three separate experiments with similar results.

wild-type macrophages but, unexpectedly, the level of p40 mRNA was barely detectable and p35 mRNA expression was also greatly reduced in the *Mkk3*$^{-/-}$ macrophages (Figure 3A). There was also a small decrease of TGF-β2 mRNA in the knockout macrophages (20% reduction) (Figure 3B and C). The inhibition of cytokine expression in knockout versus wild-type mice is shown in Figure 3C.

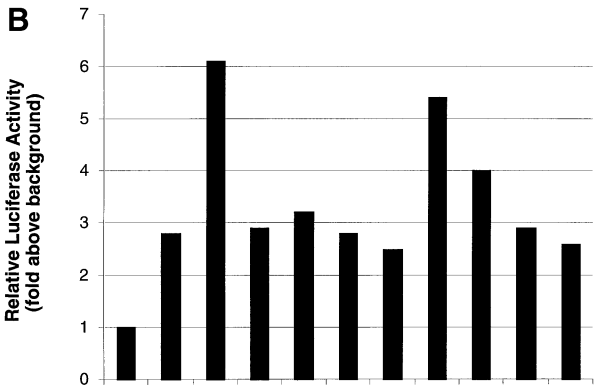
IL-12 is secreted by antigen-presenting cells (APCs), including macrophages and DCs, when microbial pathogens are encountered. To examine the status of IL-12 protein production by APCs in *Mkk3*$^{-/-}$ mice, peritoneal macrophages from both wild-type and knockout mice were activated with LPS, and IL-12 production was measured by enzyme-linked immunosorbent assay (ELISA) after stimulation. There was a marked reduction in IL-12 expressed by the knockout macrophages when compared with the wild-type macrophages (Figure 4A), consistent with the RPA data. This LPS-induced production of IL-12 was also inhibited by SB 203580 and SB 202190, two inhibitors of p38 MAPK, but not by SB 202474, a chemical with similar structure that was used as a negative control. Together, these data indicate that MKK3-directed p38 activation is required for LPS-induced IL-12 production in macrophages.

DCs, another major APC type, are a key source of IL-12 in response to stimulation with CD40L in the presence of low amounts of LPS (Cella *et al*., 1996; Koch *et al*., 1996; Kato *et al*., 1997; Grewal and Flavell, 1998; Snijders *et al*., 1998). We therefore also measured IL-12 production by DCs. IL-12 production was highly induced by CD40L in wild-type DCs in a dose-dependent manner; however, the induction of IL-12 production by CD40–CD40L engagement was markedly reduced in the MKK3-deficient DCs (Figure 4B). These results indicated that there is a general impairment of IL-12 production by the APCs of the MKK3-deficient mice.

The amount of protein secretion of IL-6 and TNF-α in knockout macrophages was comparable with that of the wild-type macrophages upon LPS stimulation (Figure 4C). Together with the RPA data which showed little difference



	1	2	3	4	5
p40Luc	+	+	+	+	+
LPS		+	+	+	+
SB202190			+		
MKK3Ala				+	
p38AGF					+



	1	2	3	4	5	6	7	8	9	10	11
p40Luc	+	+	+	+	+	+	+	+	+	+	+
MKK3Glu		+	+	+	+	+					
MKK6Glu							+	+	+	+	+
p38α			+					+			
p38β				+					+		
p38γ					+					+	
p38δ						+					+

Fig. 5. IL-12 p40 promoter activity is regulated by the p38 MAPK pathway in RAW264.7 cells. RAW264.7 cells were transiently transfected with the IL-12 p40Luc reporter either together with or without MKK3Ala or p38AGF (**A**); or together with MKK3Glu or MKK6Glu, with or without p38α, p38β, p38γ and p38δ respectively (**B**). The transfected cells were split into two wells and left untreated or were treated with LPS for 24 h. Some cells were treated with SB 202190 (2 μM) for 1 h before the LPS treatment. The luciferase activity was then measured.

in the amounts of mRNA of these two cytokines between the wild-type and the knockout macrophages, these results suggested that MKK3 is not required for the production of IL-6 and TNF-α mRNA in LPS-stimulated macrophages. In contrast, the secretion of both IL-1α and IL-1β protein was reduced in the knockout macrophages (Figure 4D). This contrasts, however, with the RPA analysis for IL-1α and IL-1β mRNA (Figure 3D), suggesting that MKK3-directed p38 MAPK might be involved in IL-1 translational or post-translational regulation.

Regulation of the IL-12 p40 promoter by the p38 MAPK pathway

To study further the molecular mechanisms underlying the regulation of IL-12 by the MKK3-directed p38 MAPK pathway, we studied IL-12 p40 reporter gene expression in macrophages. Since it is very difficult to transfect primary macrophages, we chose instead to use the murine macrophage cell line RAW264.7 in these co-transfection experiments. An IL-12 p40 reporter construct, which contains the –350 to +55 region of the IL-12 p40 promoter driving a firefly luciferase gene (Plevy *et al*., 1997), was transiently transfected into RAW264.7 cells. Luciferase activity was strongly induced by LPS and was dependent on the p38 MAPK pathway since this induction was blocked by SB 202190 (Figure 5A), but not by the control drug SB 202474 (data not shown). This LPS-induced IL-12 p40 promoter-driven luciferase expression was also suppressed when a dominant-negative expression construct of MKK3 or p38 MAPK was co-transfected (Figure 5A) thus the p38 MAPK pathway is required for LPS-induced IL-12 p40 reporter expression, acting at least in part at the transcriptional level. Since the p40 promoter we used contains the 5′-untranslated region (5′-UTR), we cannot yet exclude the possibility that p38 MAPK may also regulate IL-12 p40 promoter post-transcriptionally by acting through the 5′-UTR.

To examine which isoform of p38 contributes to the regulation of IL-12 expression, activated MKK3 and MKK6, MKK3Glu and MKK6Glu respectively, were co-transfected with different p38 isoforms and their ability to activate the p40 promoter was studied. It appeared that MKK3Glu activated the IL-12 p40 promoter through p38α, but less well through p38β, p38γ and p38δ, while MKK6Glu primarily acted through p38α and p38β (Figure 5B). These data indicated that p38α is directly involved in the regulation of IL-12 p40 expression.

Impaired type I cytokine immune response in Mkk3$^{-/-}$ mice

The p38 MAPK pathway is activated upon TCR ligation and T cell activation (Sen *et al*., 1996; Salmon *et al*., 1997; Rincon *et al*., 1998). We have shown recently that p38 MAPK mediates IFN-γ expression in T helper 1 (Th1) effector cells (Rincon *et al*., 1998). IL-12 is a critical inflammatory cytokine linking the innate and adaptive immune response. It induces the production of IFN-γ and is therefore a critical mediator of the proinflammatory antigen-specific Th1, cytotoxic T lymphocyte (CTL) and natural killer (NK) cell cytotoxic response (Wolf *et al*., 1991; Gately *et al*., 1992; Hsieh *et al*., 1993; Abbas *et al*., 1996; Rincon and Flavell, 1997; O'Garra, 1998). To determine if IFN-γ production was impaired in *Mkk3*$^{-/-}$ mice, we differentiated naive T cells into Th1 cells *in vitro* using a well-established protocol (Rincon and Flavell, 1997; Zheng and Flavell, 1997; Flavell *et al*., 1998; Rincon *et al*., 1998). Sorted CD44^{low}CD45RB^{high} naive CD4^{+} T cells were cultured with APCs in the presence

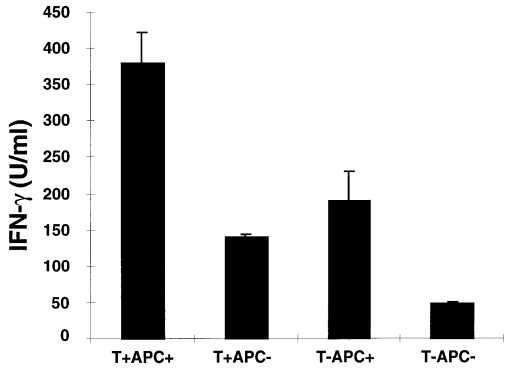


Fig. 6. IFN-γ production after *in vitro* differentiation of naive $CD4^+$ T cells. Sorted $CD44^{low}CD45RB^{high}$ naive $CD4^+$ cells from wild-type (T+) and MKK3-deficient mice (T–) were cultured with either the wild-type APCs (APC+) or knockout APCs (APC–) in the presence of Con A, IL-2 and anti-IL-4 antibody for 4 days. The cells were then washed extensively and restimulated with Con A for another day. IFN-γ production in the supernatants was measured by ELISA. The mean and standard error are shown. The results shown in this experiment were representative of three separate experiments with similar outcome.

of concanavalin A (Con A), IL-2 and anti-IL-4 antibody for 4 days. After extensive washing, the cells were replated and cultured for another day in the presence of Con A. IFN-γ production was then measured. Wild-type T cells incubated with wild-type APCs produce large amounts of IFN-γ, whereas knockout T cells incubated with knockout APCs produced little IFN-γ (Figure 6), indicating that IFN-γ production is impaired in *Mkk3*$^{-/-}$ mice. To test whether this defect resulted from an intrinsic deficiency in the property of Th1 $CD4^+$ T cells with reduced p38 activity that was described previously (Rincon *et al*., 1998), or also from an APC deficiency, as described here resulting from impaired IL-12 production, wild-type naive $CD4^+$ T cells were incubated with knockout APCs and, similarly, knockout naive $CD4^+$ cells were incubated with wild-type APCs under the same conditions. In both cases, IFN-γ production was greatly reduced compared with that of the wild-type T cells and APCs, but was still substantially higher than that of knockout T cells plus knockout APCs (Figure 6). This result suggested that both knockout T cells and knockout APCs were intrinsically defective, and that both contribute to the diminished production of IFN-γ in the knockout mice.

To examine further whether Th1 $CD4^+$ T cell responses were defective in the *Mkk3*$^{-/-}$ mice *in vivo*, we studied the recall response to the antigen keyhole limpet hemocyanin (KLH) by measuring IFN-γ secretion by lymphocytes after secondary antigenic challenge *ex vivo*. In contrast to the wild-type littermates which exhibited dose-dependent IFN-γ production in response to KLH, the *Mkk3*$^{-/-}$ mice produced little IFN-γ (Figure 7A). To exclude that this was not due to the unresponsiveness of the MKK3-deficient lymphocytes to KLH antigens, T cell proliferation was examined for both wild-type and knockout lymphocytes treated with KLH; there was no significant difference in thymidine incorporation between the wild-type and the knockout mice (Figure 7B). The ability to secrete IFN-γ in response to antigens was therefore greatly impaired, but not completely absent, in *Mkk3*$^{-/-}$ mice.

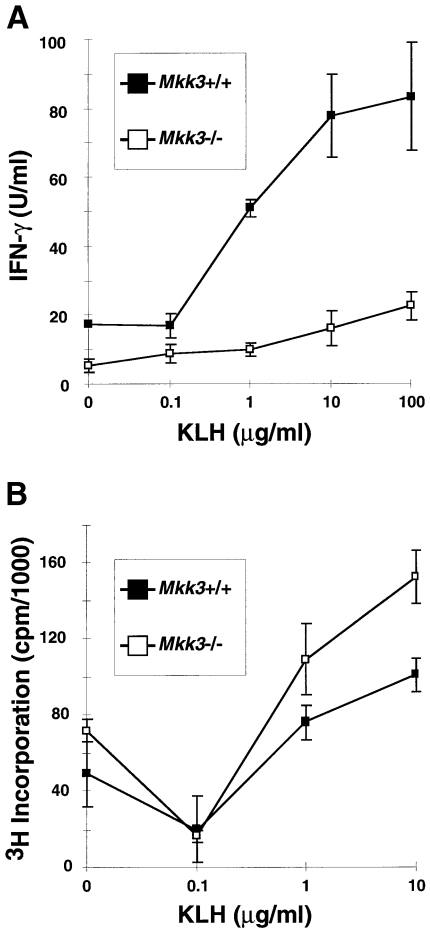


Fig. 7. KLH-induced IFN-γ production is reduced in MKK3-deficient mice. (**A**) IFN-γ production induced by KLH. Mice were immunized with KLH in CFA in the footpads. Nine days later, lymphocytes from the draining lymph nodes in the treated mice were isolated and incubated *in vitro* with different concentrations of KLH for 4 days. The supernatants were collected and ELISA was performed to examine the induction of IFN-γ in the supernatants. The mean and standard error are shown. The results obtained in this experiment were representative of two separate experiments with similar results. (**B**) Proliferation of lymphocytes in response to KLH *in vitro*. Lymphocytes isolated from draining lymph nodes after 9 days of initial challenge with KLH–CFA were incubated *in vitro* with different concentrations of KLH. At day 3, [^{3}H]thymidine was added to the culture media. The proliferation response was measured at day 4 by examining the incorporation of [^{3}H]thymidine. The mean and standard error are shown. The results shown in this experiment were representative of two separate experiments with similar results.

Discussion

We have generated MKK3-deficient mice and shown that they have fundamental defects in the inflammatory response and in the Th1 $CD4^+$ T cell response. Three upstream kinases that activate p38 MAPK have been reported for p38 MAPK: MKK3, MKK4 and MKK6 (Whitmarsh and Davis, 1996). MKK4 activates JNK and p38 MAPK *in vitro* (Derijard *et al*., 1995; Lin *et al*., 1995); however, there is no defect in p38 MAPK activation in MKK4-deficient ES cells although p38 MAPK activation in MKK4-deficient fibroblasts is reduced (Nishina *et al*., 1997; Yang *et al*., 1997a; Ganiatsas *et al*., 1998). At present, MKK3 and MKK6 are the only known specific p38 MAPK activators. By using transient transfection of genes encoding MKK3 or MKK6 and other biochemical

characterization *in vitro*, many reports suggested that MKK6 is the most potent activator of p38 MAPK (Cuenda *et al*., 1996; Moriguchi *et al*., 1996; Raingeaud *et al*., 1996). The relative contribution of MKK3 versus MKK6 *in vivo* is, however, undefined; the generation of MKK3-deficient mice, therefore, provides an opportunity to determine the role of MKK3 versus MKK6 *in vivo*. We found that LPS-activated p38 MAPK activity was reduced, but not absent, in MKK3-deficient macrophages (Figure 4A). On the other hand, p38 MAPK activity induced by sorbitol in MKK3-deficient macrophages is intact; this suggests that MKK3 is required for full activation of p38 MAPK by LPS. The biological significance of this LPS-induced p38 MAP kinase defect in the knockout mice is illustrated by the demonstration that the induction of IL-12 production and IL-12 p40 mRNA expression in macrophages caused by LPS are almost completely blocked in these mice.

Bacterial LPS is one of the most potent activators of cells of the monocytic lineage. LPS forms a complex with the serum protein LPS-binding protein (LBP), the LPS–LBP complex then binds to CD14 on the cell surface to induce a signal within the cell, probably through a Toll-like receptor (Ulevitch and Tobias, 1995; Kirschning *et al*., 1998; Poltorak *et al*., 1998; Yang *et al*., 1998b). LPS has been shown to induce activation of all three MAPK pathways including ERK, JNK and p38, in addition to protein kinase C (PKC), ceramide and PKA; however, the significance of each pathway in connecting LPS to intracellular gene activation is unknown (Sweet and Hume, 1996). It has been shown that the p38 MAPK pathway is involved in the LPS-induced biosynthesis of TNF-α, IL-1, IL-6 and GM-CSF (Lee *et al*., 1994; Bayaert *et al*., 1996) and that the JNK and ERK pathways are also involved in TNF-α production induced by LPS (Swantek *et al*., 1997; Zhang *et al*., 1997). MKK3-deficient mice have enabled us to study the contribution of the MKK3-directed p38 MAPK pathway to the regulation of LPS-induced cytokine production in macrophages. Secretion of IL-6 and TNF-α by peritoneal macrophages in response to LPS stimulation *in vitro* revealed little defect, if any, in the MKK3-deficient mice, suggesting that MKK3 is not required for these processes. The biosynthesis of IL-6 and TNF-α was partially inhibited by the p38 MAPK inhibitor SB 202190 (Figure 4C). Thus, there is an SB 202190-sensitive pathway that contributes to IL-6 and TNF-α production that is not MKK3 dependent; presumably MKK6 may compensate for the MKK3 defect, or be wholly responsible for the induction of these cytokines. On the other hand, IL-1α and IL-1β production (but not mRNA expression) was reduced in MKK3-deficient macrophages (Figure 4D), indicative of a role for the MKK3-directed p38 MAPK pathway in the production of IL-1α and IL-1β that acts at the translational or post-translational level, as suggested previously (Lee *et al*., 1994). The most dramatic effect that we have observed in MKK3-deficient mice, however, was the almost complete absence of IL-12 production caused by LPS in macrophages and by CD40–CD40L interactions in DCs (Figure 4A and B). This was surprising, because IL-12 biosynthesis previously had not been known to be regulated by the p38 MAPK pathway.

IL-12 is a heterodimeric cytokine that consists of p35 and p40 subunits. It plays a central role in driving naive $CD4^+$ T cells into differentiated Th1 cells by inducing the production of IFN-γ, an important effector in both adaptive cellular immunity and innate immunity (Gately *et al*., 1998; Trinchieri, 1998). Among its many immunomodulatory effects in both innate and adaptive immunity, IFN-γ up-regulates major histocompatibility complex (MHC) class I and II antigen expression, stimulates specific $CD8^+$ T cell-mediated cytotoxic immunity through recognition of specific MHC class I and antigen complexes, and enhances innate immunity by activation of macrophages and NK cells (Boehm *et al*., 1997). IL-12, therefore, serves as a bridge connecting innate immunity to adaptive immunity (Trinchieri, 1995). IL-12 is secreted mainly by APCs upon innate immune recognition of pathogen-associated molecular patterns including LPS (Medzhitov and Janeway, 1997). However, the signaling pathway leading to IL-12 production hitherto was undefined (O'Garra, 1998). The p38 MAPK pathway is induced by LPS and peptidoglycan, the major molecular recognition pattern among Gram-negative and Gram-positive bacteria, respectively (Han *et al*., 1994; Dziarski *et al*., 1996). This suggested that the p38 MAPK pathway is activated during the innate immune recognition process. It is particularly satisfying, therefore, that one of the downstream targets of the p38 MAPK pathway is IL-12, which, upon induction by microbial products via the p38 MAPK pathway, would initiate antigen-specific adaptive immunity. We observed a compromised type I immune response to protein antigen (KLH) in complete Freund's adjuvant (CFA), an adjuvant which favors Th1 responses through the production of IL-12 (Forsthuber *et al*., 1996) (Figure 7A). IFN-γ production by differentiated $CD4^+$ T cells was also greatly reduced in MKK3-deficient mice, which had intrinsic defects in both T cells and APCs; both defects contributed to this impairment (Figure 6). The APC defect is probably caused by the impairment of IL-12 production, since IL-12 p40-deficient mice, IL-12 receptor β1-deficient mice and mice deficient for Stat 4, a key signaling molecule that is required in order to respond to IL-12, also exhibit defective type I cytokine immune responses (Kaplan *et al*., 1996; Magram *et al*., 1996; Thierfelder *et al*., 1996; Wu *et al*., 1997). Taken together, our results suggest that MKK3 plays a determining role in driving the downstream p38 MAPK to regulate IL-12 production in APCs.

Analysis of mRNA demonstrated that IL-12 p40 gene expression was almost absent in the MKK3-deficient mice (Figure 3A and C); the IL-12 p35 mRNA level was also reduced (Figure 3A and C). Likewise, p40 promoter-driven reporter expression was blocked by SB 202190 and dominant-negative expression constructs for MKK3 and p38 MAPK (Figure 5A). These results indicated that p38 MAPK regulates IL-12 p40 transcriptionally, at least in part. In comparison with the IL-12 p35 promoter, the IL-12 p40 promoter and the transcription factors that bind to it are better characterized. C/EBP and NF-κB family members together with an ets-2-related factor have been shown to bind to their corresponding sites in the p40 promoter and regulate the expression of this gene (Murphy *et al*., 1995; Ma *et al*., 1996, 1997; Plevy *et al*., 1997; Gri *et al*., 1998). By the use of gene disruptions in mice, IRF-1 and ICSBP (another member of the IRF-1 family) have been shown to be required for IL-12 p40 expression (Giese *et al*., 1997; Lohoff *et al*., 1997; Scharton-Kersten

et al., 1997; Taki *et al*., 1997). Since many of the identified substrates for p38 MAPK are transcription factors and, furthermore, since the p38 MAPK pathway can contribute to NF-κB mediated-transactivation (Bayaert *et al*., 1996; Berghe *et al*., 1998), it is tempting to speculate that the p38 MAPK pathway may regulate IL-12 p40 transcription by regulating the activity of either IRF-1 family members, C/EBP, NF-κB or the ets-2-related factor, directly or indirectly. Further work will be required to test this hypothesis. It is also possible that the inhibition of IL-12 production in MKK3-deficient mice is mediated by IL-10, IL-4 or TGF-β, cytokines that suppress the production of IL-12 (Skeen *et al*., 1996). We considered this unlikely since we observed a reduction rather than an increase of TGF-β2 production in MKK3-deficient macrophages using a sensitive RPA (Figure 3B and C), and since the production of IL-4 and IL-10 mRNA in response to antigen was similar in wild-type and knockout T cells (data not shown). It has been shown that MKK3 selectively activates p38α whereas MKK6 activates both p38α and p38β (Enslen *et al*., 1998). We found that LPS-induced IL-12 production is inhibited by SB 203580 and SB 202190 (Figure 4A). Since p38α and p38β are inhibited by these drugs, the LPS-induced, MKK3-directed IL-12 production is, therefore, probably mediated by the activation of p38α MAPK, at least in part, since p38α, but not p38β, γ or δ, potentiated the transactivation of IL-12 promoter mediated by constitutive active MKK3 (Figure 5B).

Finally, we wish to point out an interesting parallel between the role of the p38 MAPK pathway in inflammatory APCs, such as macrophages and DCs, and its role in T cells. The production of IL-12 in APCs and the production of IFN-γ in T cells all require the p38 MAP kinase pathway, shown by us here and elsewhere (Rincon *et al*., 1998). The ancient p38 MAPK pathway appears to have been used repeatedly during evolution for a variety of stress responses, ranging from osmotic stress in single-cell organisms such as yeast (Herskowitz, 1995), to regulation of antimicrobial peptide expression in insects (Han *et al*., 1998), to the production of inflammatory cytokines by fibroblasts (Wysk *et al*., in press), macrophages and DCs in the innate immune response, and finally in the proinflammatory Th1 adaptive immune response (Rincon *et al*., 1998; this study) developed in higher vertebrates. The conservation of function is unlikely to be fortuitous and may be repeated for other signaling pathways.

Materials and methods

Reagents

LPS (*Escherichia coli* strain 0127:B8), Con A, G418, sorbitol and KLH were purchased from Sigma Chemical Co. (St Louis, MO). CFA was purchased from Life Technologies, Inc. (Gaithersburg, MD). Gancyclovir was purchased from Syntex (Palo Alto, CA). The p38 inhibitors SB 203580 and SB 202190 and the control chemical SB 202474 were purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA). Brewer thioglycollate was purchased from Difco (Detroit, MI). The GM-CSF-producing hybridoma was kindly provided by Dr David Gray (Royal Postgraduate Hospital, London). The membrane-bound CD40L was kindly provided by Dr Marilyn Kehry (Boehringer-Ingelheim, Danbury, CT). Human recombinant IL-2 was kindly provided by Biogen Inc. Murine IL-12 was kindly provided by Genetics Institute. Biotin-labeled CD44, and fluorescein isothiocyanate (FITC)-labeled CD45RB antibodies, and phycoerythrin (PE)-labeled streptavidin were purchased from Pharmingen (San Diego, CA).

Plasmids

The IL-12 p40 promoter reporter plasmid, kindly provided by Dr Steve Smale (UCLA), contains the –350 to +50 region of the p40 promoter driving a firefly luciferase gene (Plevy *et al*., 1997). The PRL-SV40 *Renilla* luciferase plasmid was purchased from Promega (Madison, WI). MKK3Ala and p38AGF, dominant-negative constructs for MKK3 and p38 respectively, MKK3Glu and MKK6Glu, constitutive active constructs of MKK3 and MKK6 respectively, and p38α, p38β, p38γ and p38δ expression constructs were described previously (Raingeaud *et al*., 1996; Enslen *et al*., 1998). All plasmid DNAs were isolated by using endotoxin-free Maxi-prep kit (Qiagen Inc., Valencia, CA).

Generation of *Mkk3*$^{-/-}$ ES cells

Mkk3 genomic DNA clones were isolated from a λ FixII phage library prepared from mouse strain 129/Sv (Stratagene, La Jolla, CA). The PGKneo cassette and herpes simplex virus (HSV)-thymidine kinase gene vector pBSNTK2 were constructed as described (Yang *et al*., 1997a). A 4.4 kb *Bgl*II *Mkk3* genomic fragment was excised with *Not*I and *Spe*I and ligated into the *Not*I–*Xba*I sites of pBSNTK2. Finally, a 920 bp *Eco*RV–*Hin*cII fragment from the 3′ end of the *Mkk3* genomic DNA was inserted into the vector at the *Xho*I site using *Xho*I linkers. The resulting targeted vector construct was linearized with *Not*I and electroporated into W9.5 ES cells. Genomic DNA from transfectants resistant to G418 (200 μg/ml) and gancyclovir (2 μM) was characterized by Southern blot analysis.

Characterization of *Mkk3*$^{-/-}$ mice

For Southern blot analysis, genomic DNA was isolated from mouse tails, digested with *Eco*RI and hybridized with a 240 bp probe located just outside the 3′ arm of the knockout vector which recognizes a 20 kb *Mkk3* wild-type DNA fragment and a 7 kb *Mkk3* mutant DNA fragment.

For Northern blot analysis, total RNA was isolated from kidney and liver using the TRIzol reagent (Life Technologies, Inc., Gaithersburg, MD). A murine *Mkk3* cDNA fragment corresponding to *Mkk3* nucleotides 777–1231 was amplified by RT–PCR and was used as a probe.

The polyclonal antibodies used for probing Western blots (MKK3, MKK6, p38 and JNK) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Isolation and culture of peritoneal macrophages

Mice 5–6 weeks old were injected with 3 ml of 3% Brewer thioglycollate i.p. and, 72 h later, peritoneal exudate cells (PECs) were isolated from the peritoneum. The thioglycollate-elicited PECs were cultured at 37°C and 5% CO_2 in 48-well plates at 5×10^5 cells/well in 1 ml/well of high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 2 mM L-glutamine and penicillin/streptomycin. After 2 h, non-adherent cells were removed and the adherent macrophages were subjected to various treatments. Peritoneal macrophages were treated with LPS (100 ng/ml) for different times, the supernatants were collected and immediately analyzed for cytokine production by ELISA. For analysis of IL-1α and β production, the macrophages were treated with LPS for 4 h, ATP (5 mM) was then added into the culture for an additional 30 min to release IL-1 into the media (Hogquist *et al*., 1991). The supernatants were collected for ELISA.

Measurement of MKK3 and p38 MAPK activity

p38 MAP kinase activity in cell lysates was measured using immune complex kinase assays (Raingeaud *et al*., 1995). The activity of MKK3 was measured in transfected cultured macrophages. RAW264.7 cells were grown in DMEM supplemented with 5% FBS, 2 mM L-glutamine and antibiotics (penicillin/streptomycin). Flag-tagged MKK3 was expressed by transfection of 2×10^6 cells using the Superfect reagent (Qiagen Inc.). After transfection (3 h), the cells were divided into two groups and incubated for 24 h. The cells were treated (30 min) without or with LPS (1 μg/ml). Cell lysates were prepared and MKK3 was isolated by immunoprecipitation using the M2 monoclonal antibody to the Flag epitope (Sigma). MKK3 protein kinase activity was measured in the immunoprecipitates in a coupled kinase assay (30 min, 30°C) using 0.5 μg of GST–p38γ, 0.5 μg of GST–ATF2 and 50 μM [γ-^{32}P]ATP. The phosphorylation of ATF2 was quantitated following SDS–PAGE by PhosphorImager analysis (Molecular Dynamics Inc.).

RNase protection assay (RPA)

Peritoneal macrophages were treated with LPS *in vitro* for various times, and total RNA was isolated using TRIzol reagent (Gibco-BRL). RPA was performed using 5 μg of total RNA under conditions suggested by the manufacturer. The RiboQuant RPA kit was purchased from Pharmingen. Probe sets mCK-2b and mCK-3 were used to detect cytokine expression. Normalization was calculated by determination of the ratio of individual cytokine mRNA against GAPDH mRNA on a PhosphorImager screen. The percentage inhibition was determined by the following formula: percentage inhibition (%) = 100×[(+/+ cytokine:GAPDH) – (–/– cytokine:GAPDH)] / (+/+ cytokine:GAPDH).

Isolation and culture of bone marrow-derived dendritic cells

Bone marrow cells were isolated from the femur and tibia of male mice of 6–8 weeks of age as described (Pierre *et al*., 1997). The cells were treated with a combination of depleting antibodies (anti-B220, anti-MHC class II, anti-CD4 and anti-CD8) and complement for 1 h at 37°C (Zheng and Flavell, 1997). The remaining live cells were cultured at 1×10^6 cells/ml in 2 ml/well RPMI supplemented with 5% FCS and GM-CSF (666 U/ml) in a 24-well plate. The culture medium was changed every 2 days to remove non-adherent granulocytes. On day 8, cells in suspension were collected and replated and subjected to various treatments. Bone marrow-derived DCs were treated with membrane-bound CD40L for 48 h, and the supernatants were collected and subjected to ELISA analysis for cytokine production.

Enzyme-linked immunosorbent assay (ELISA)

The ELISA was performed as previously described (Rincon *et al*., 1998). Mouse ELISA paired antibodies for TNF-α, IL-6, IFN-γ and IL-4 were purchased from Pharmingen. A mouse IL-12 ELISA kit was purchased from Biosource International (Camarillo, CA), which detects the mature IL-12 p70 heterodimer. Mouse IL-1α ELISA kit was purchased from Endogen (Cambridge, MA), The mouse IL-1β Duoset™ paired antibodies and mouse IL-1β standard were purchased from Genzyme (Cambridge, MA). The concentration of the coating antibody for TNF-α and IL-6 is 4 μg/ml and the secondary biotinylated antibody is 2 μg/ml. The concentration of the coating antibody used for IFN-γ and IL-4 is 0.625 and 1 μg/ml respectively and of the secondary biotinylated antibody is 2 and 1 μg/ml respectively. The ELISA for IL-12, IL-1α and IL-1β was performed according to the manufacturer's instructions. Mouse IL-6 and TNF-α (R&D Systems, Minneapolis, MN), IFN-γ (Gibco-BRL) and IL-4 (DNAX, Los Angeles, CA) were used as standards. Horseradish peroxidase-conjugated avidin D was purchased from Vector Laboratories (Burlingame, CA). The TMB microwell peroxidase substrate and stop solution were purchased from Kirkegaard and Perry Laboratories (Gaithersburg, MD).

Measurement of IL-12 p40 promoter-driven luciferase activity

The RAW264.7 murine macrophage line was grown in DMEM supplemented with 5% FBS, 2 mM L-glutamine, and penicillin and streptomycin. A total of 2×10^6 cells/well in a 6-well plate were transiently transfected by Superfect (Qiagen Inc.) according to the manufacturer's protocol with 4 μg of IL-12 p40 promoter-driven firefly luciferase reporter plasmid and 0.4 μg of PRL-SV40 *Renilla* luciferase expression vector which was co-transfected to normalize the transfection efficiency. Some cells were also co-transfected with 4 μg of either MKK3Ala, p38AGF, MKK3Glu, MKK6Glu or empty expression vector. At 3 h post-transfection, the cells were harvested and split evenly into two wells. After 24 h, one group of cells was left untreated and the other group was incubated with LPS (1 μg/ml). In some experiments, the transfected cells were pre-treated with SB 202190 (2 μM) for 1 h before LPS treatment. At 24 h after LPS treatment, cell extracts were prepared and luciferase activity was measured by using the Dual-Luciferase system (Promega).

In vitro T cell differentiation

$CD4^+$ T cells were isolated from spleen and lymph nodes of 6-week-old mice by negative selection as described (Zheng and Flavell, 1997; Rincon *et al*., 1998). Naive $CD4^+$ T cells were then isolated by sorting for $CD44^{low}$ $CD45RB^{high}$ cells. APCs were obtained by γ-irradiation and negative selection (Zheng and Flavell, 1997).

A total of 5×10^5 naive $CD4^+$ T cells were incubated with an equal number of APCs in Bruff medium with 5% fetal calf serum (FCS; Life Technologies, Inc.), 2 mM L-glutamine and penicillin/streptomycin in the presence of Con A (2.5 μg/ml), IL-2 (20 U/ml) and anti-IL-4 (Clone 11B11) with or without IL-12 (3.5 ng/ml) for 4 days. The cells were washed exhaustively, counted and re-stimulated at 5×10^5 cells/ml with Con A (2.5 μg/ml) for 20 h. The supernatants were collected and IFN-γ production was measured by ELISA.

Measurement and induction of the KLH recall response

Mice were immunized with 50 μg of KLH in CFA in each of the hind footpads. Draining lymph nodes were isolated 9 days later. The lymphocytes were cultured in 96-well plates (5×10^5 cells/well) in the presence of KLH of different concentrations *in vitro*. One group was incubated with 1 μCi/well of [^{3}H]thymidine at day 3. At day 4, proliferation was assayed by determining the incorporation of [^{3}H]thymidine. The supernatants of other groups were assayed at day 5 by ELISA for IFN-γ production.

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