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Interferon-γ expression by Th1 effector T cells mediated by the p38 MAP kinase signaling pathway

Mercedes Rincón[1,2,3], Hervé Enslen[4], Joël Raingeaud[4,5], Michael Recht[6], Tyler Zapton[1], Michael S-S.Su[7], Laurie A.Penix[6], Roger J.Davis[4] and Richard A.Flavell[2,3,8]

[1]Immunobiology Program, Department of Medicine, Given Medical Building, University of Vermont, Burlington, VT 05405, [2]Section of Immunobiology, [6]Department of Pediatrics, [8]Howard Hughes Medical Institute and Yale University School of Medicine, New Haven, CT 06520, [4]Program in Molecular Medicine, Department of Biochemistry and Molecular Biology, University of Massachusetts Medical School and Howard Hughes Medical Institute, Worcester, MA 01605, [7]Vertex Pharmaceuticals Incorporated, Cambridge, MA 02139-4242, USA and [5]Laboratoire d'Oncogenèse Rétrovirale et Moléculaire, Institut Curie, 91405 Orsay, France

[3]Corresponding authors
e-mail: richard.flavell@qm.yale.edu and mrincon@zoo.uvm.edu

Signal transduction via MAP kinase pathways plays a key role in a variety of cellular responses, including growth factor-induced proliferation, differentiation and cell death. In mammalian cells, p38 MAP kinase can be activated by multiple stimuli, such as pro-inflammatory cytokines and environmental stress. Although p38 MAP kinase is implicated in the control of inflammatory responses, the molecular mechanisms remain unclear. Upon activation, $CD4^+$ T cells differentiate into Th2 cells, which potentiate the humoral immune response or pro-inflammatory Th1 cells. Here, we show that pyridinyl imidazole compounds (specific inhibitors of p38 MAP kinase) block the production of interferon-γ (IFNγ) by Th1 cells without affecting IL-4 production by Th2 cells. These drugs also inhibit transcription driven by the *IFN*γ promoter. In transgenic mice, inhibition of the p38 MAP kinase pathway by the expression of dominant-negative p38 MAP kinase results in selective impairment of Th1 responses. In contrast, activation of the p38 MAP kinase pathway by the expression of constitutively-activated MAP kinase kinase 6 in transgenic mice caused increased production of IFNγ during the differentiation and activation of Th1 cells. Together, these data demonstrate that the p38 MAP kinase is relevant for Th1 cells, not Th2 cells, and that inhibition of p38 MAP kinase represents a possible site of therapeutic intervention in diseases where a predominant Th1 immune response leads to a pathological outcome. Moreover, our study provides an additional mechanism by which the p38 MAP kinase pathway controls inflammatory responses.
Keywords: IFNγ/p38 MAP kinase/T-cell differentiation/Th1 cells/transgenic mice

Introduction

Signal transduction via mitogen-activated protein (MAP) kinases plays a key role in a variety of cellular responses, including growth factor-induced proliferation, differentiation and cell death. Recently, several parallel MAP kinase signal transduction pathways have been defined in mammalian cells (Whitmarsh and Davis, 1996; Ip and Davis, 1998). These pathways include the extracellular signal related kinase (ERK) (Boulton *et al.*, 1990, 1991), c-Jun N-terminal kinases (JNK, also known as SAPK) (Dérijard *et al.*, 1994; Kyriakis, 1994), and p38 MAP kinase (Han *et al.*, 1994; Lee *et al.*, 1994; Rouse *et al.*, 1994). These MAP kinases are activated by phosphorylation on Thr and Tyr by dual-specificity MAP kinase kinases (Dérijard *et al.*, 1994; Raingeaud *et al.*, 1995). The MAP kinase groups are functionally independent and are implicated in different biological processes (Whitmarsh and Davis, 1996; Ip and Davis, 1998).

The *p38* MAP kinase gene was cloned following the purification of a 38 kDa protein that was phosphorylated on tyrosine in response to hyperosmolarity and endotoxic lipopolysaccharide. Independently, human p38 MAP kinase homologs (CSBP1 and CSBP2) were identified as the targets of pyridinyl imidazole compounds, which inhibit the production of pro-inflammatory cytokines [interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα)] in stimulated monocytes (Han *et al.*, 1994; Lee *et al.*, 1994; Rouse *et al.*, 1994). A second gene (*p38*β) that encodes a p38 MAP kinase isoform that is inhibited by pyridinyl imidazole drugs has recently been identified (Jiang *et al.*, 1996; Stein *et al.*, 1996; Enslen *et al.*, 1998).

In mammalian cells, p38 MAP kinase can be activated by multiple stimuli, such as pro-inflammatory cytokines (e.g. IL-1β and TNFα), lipopolysaccharide and physical–chemical changes in the extracellular milieu caused by environmental stress (heat, osmotic stress, UV irradiation) (Freshney *et al.*, 1994; Han *et al.*, 1994; Lee *et al.*, 1994; Rouse *et al.*, 1994; Raingeaud *et al.*, 1995). More recently, it has been reported that a variety of hemopoietic growth factors, such as colony stimulatory factor-1 (CSF-1), granulocytes/macrophages-colony stimulatory factor (GM-CSF) and IL-3 also activate p38 MAP kinases (Foltz *et al.*, 1997).

The p38 MAP kinase is activated by the MAP kinase kinases MKK3, MKK4 and MKK6 (Dérijard *et al.*, 1995; Han *et al.*, 1996; Moriguchi *et al.*, 1996; Raingeaud *et al.*, 1996). These MKKs phosphorylate p38 MAP kinase on Thr and Tyr within the tripeptide motif TGY in kinase sub-domain VIII, thereby increasing enzymatic activity (Raingeaud *et al.*, 1995). The p38 MAP kinase is implicated in the expression of cytokines and the regulation of cell proliferation and death (Lee *et al.*, 1994; Xia *et al.*, 1995). *In vitro* studies demonstrate that the transcription factor ATF-2 is phosphorylated and activated by p38 MAP kinase (Dérijard *et al.*, 1995; Raingeaud *et al.*, 1995, 1996). In addition, p38 MAP kinase activates Elk-1, CHOP, MEF2C and SAP-1 transcription factors (Dérijard

et al., 1995; Raingeaud *et al.*, 1996; Wang and Ron, 1996; Han *et al.*, 1997; Whitmarsh *et al.*, 1997). p38 MAP kinase also phosphorylates and activates the eIF-4E protein kinases Mnk1 and Mnk2 (Fukunaga and Hunter, 1997; Waskiewicz *et al.*, 1997) and the small heat shock protein hsp27 protein kinase MAPKAP kinase-2 (Freshney *et al.*, 1994; Rouse *et al.*, 1994; Mclaughlin *et al.*, 1996).

The p38 MAP kinase is a selective target for pyridinyl imidazole drugs (Lee *et al.*, 1994). These drugs appear to act by inhibiting p38 MAP kinase activity through competition with ATP at the ATP-binding pocket (Tong *et al.*, 1997; Wilson *et al.*, 1997; Young *et al.*, 1997). These compounds are candidate drugs for the treatment of arthritis, bone resorption and endotoxin shock (Griswold *et al.*, 1988; Lee *et al.*, 1993; Reddy *et al.*, 1994; Badger *et al.*, 1996). The SB203580 compound possesses therapeutic activity in collagen-induced arthritis in DBA/LACJ mice, resulting in significant inhibition of paw inflammation and serum amyloid protein levels (Badger *et al.*, 1996). This anti-arthritic activity is associated with reduced production of pro-inflammatory cytokines, such as IL-1β and TNFα, by activated macrophages (Lee *et al.*, 1993; Reddy *et al.*, 1994; Badger *et al.*, 1996; Lee and Young, 1996). However, the role of these inhibitors and the p38 MAP kinase in T-cell mediated responses has not been elucidated.

MAP kinase pathways play a critical role in the activation of T cells during the immune response. Several studies have shown the involvement of the ERK pathway in the positive selection of T cells in the thymus and in T-cell activation (Alberola-Ila *et al.*, 1995; Swan *et al.*, 1995). It has also been shown that JNK is activated during T-cell activation, where this molecule appears to play a role in integrating signals initiated at the T-cell receptor (TcR) complex and the co-stimulatory molecule CD28 (Rincón and Flavell, 1994; Su *et al.*, 1994). In contrast, little is known about the role of p38 MAP kinase. p38 is activated during development in the thymus, suggesting a potentially important role for this MAP kinase in T-lymphocyte development (Sen *et al.*, 1996). More recently, it has been shown that IL-2 and IL-7 activate both JNK and p38 kinases (Crawley *et al.*, 1997).

In response to pathogens, naïve $CD4^+$ T cells differentiate into effector Th1 and Th2 cells. Th1 cells produce mainly IFNγ and TNFβ, which are required for cell-mediated inflammatory reactions. Th2 cells secrete IL-4, IL-5, IL-6, IL-10 and IL-13, which mediate B-cell activation (for review see Paul and Seder, 1994). Development of an inappropriate response can lead to ineffective immunity and may have pathological consequences. One of the most powerful inducers of the differentiation of $CD4^+$ T cells into Th1 or Th2 cells appears to be the local cytokine environment. It is clear that the cytokine IL-12 directs differentiation to a Th1 phenotype, while IL-4 can drive differentiation to a Th2 phenotype. We have recently demonstrated that IL-6 is able to initiate the polarization of naïve $CD4^+$ T cells to effector Th2 cells by inducing the production of endogenous IL-4 (Rincón *et al.*, 1997a). A large number of studies are currently focused on the identification of intracellular signals and transcription factors specific for either Th1 or Th2 (Rincón and Flavell, 1997a).

Since p38 MAP kinase can be activated by different pro-inflammatory cytokines and has been implicated in the expression of specific cytokines, it is possible that this kinase plays a role in the differentiation or activation of Th1 and Th2 cells. Here, we show that p38 MAP kinase is activated upon stimulation in Th1 cells, but not in Th2 cells. Inhibition of the p38 MAP kinase pathway, either by the specific p38 inhibitor SB203580 or by the expression of dominant-negative (dn) p38 in transgenic mice, results in decreased production of IFNγ in Th1 cells. Moreover, the expression of MKK6, a constitutive activator of p38 MAP kinase, caused increased IFNγ expression in transgenic mice. Together, these data demonstrate that the

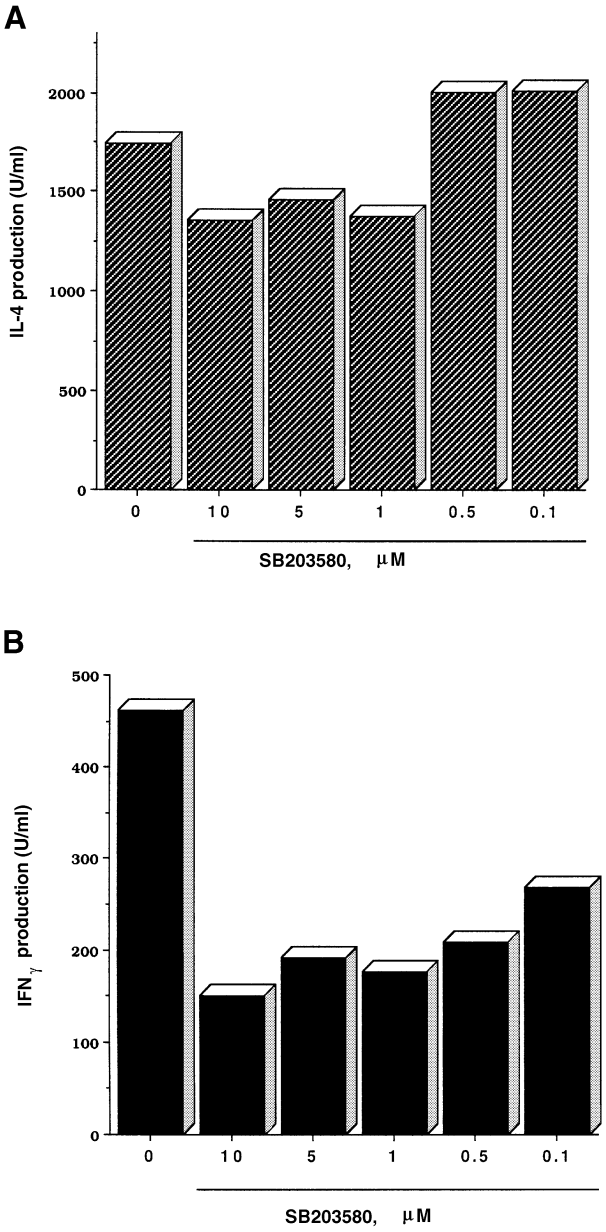


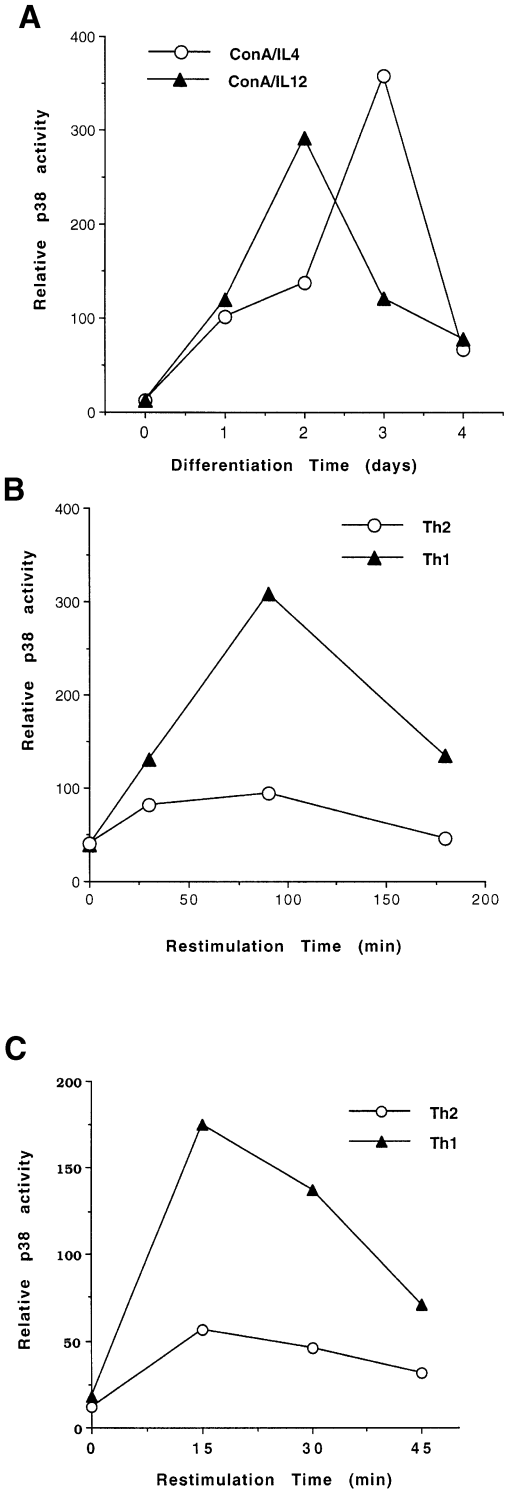
Fig. 1. The p38 MAP kinase inhibitor SB203580 specifically inhibits the production of IFNγ in Th1 cells. $CD4^+$ T cells (10^6 cells/ml) were stimulated with ConA (2.5 μg/ml) plus (**A**) IL-4 (10^3 U/ml) or (**B**) IL-12 (3.5 ng/ml) in the presence of APC. After 4 days the cells were washed and restimulated with ConA alone, in the absence or the presence of different concentrations of the p38 inhibitor SB203580, for 24 h before harvesting the supernatant to analyze IL-4 (A) and IFNγ production (B).

p38 MAP kinase pathway regulates the expression of IFNγ in Th1 cells.

Results

Inhibition of IFNγ production by the drug SB203580 in Th1 CD4$^+$ T cells

The p38 MAP kinase is implicated in the regulation of cytokine expression and is also regulated by specific cytokines. To determine the potential role of p38 in the regulation of cytokine production by Th1 and Th2 cell subsets, we first analyzed the effect of the p38 MAP kinase inhibitor SB203580, a pyridinyl imidazole drug (Lee *et al.*, 1994; Cuenda *et al.*, 1995), on the production of IL-4 and IFNγ by Th2 and Th1 CD4$^+$ T cells, respectively. To differentiate CD4$^+$ T cells *in vitro* to Th1 and Th2 cells, we followed the protocol that we have used previously in several studies (Rincón *et al.*, 1997a,b). CD4$^+$ T cells were activated with Concanavalin A (ConA) and antigen presenting cells (APC) in the presence of either IL-4 to mediate differentiation to Th2 cells, or IL-12 to mediate differentiation to Th1 cells (Le Gros *et al.*, 1990; Swain *et al.*, 1990; Hsieh *et al.*, 1993; Seder *et al.*, 1993). After 4 days, the cells were exhaustively washed and equal numbers of cells were re-stimulated (in the absence of APC or exogenous cytokines) with ConA alone or ConA in the presence of different doses of SB203580. After 24 h the supernatant was harvested and analyzed for cytokine production by ELISA. IL-4 production by Th2 cells was not significantly affected even by the highest concentration of the p38 MAP kinase inhibitor (10 μM) (Figure 1A). However, a dose-dependent inhibition of IFNγ production was obtained in activated Th1 cells in the presence of different concentrations of SB203580 (Figure 1B). The presence of SB203580 did not affect the viability of the cells at the time point (24 h) when culture supernatants were harvested to determine cytokine production (data not shown). No APC were present during the activation of Th1 and Th2 effector cells, since they do not require co-stimulatory signals (Croft *et al.*, 1994). Therefore, inhibition of IFNγ production by SB203580 was due to a direct effect on the Th1 cells.



p38 MAP kinase activity is selectively induced in Th1 cells

The inhibition of cytokine production (IFNγ) by SB203580 in Th1 cells suggests that the p38 MAP kinase pathway is differentially regulated in Th1 and Th2 cells. We first analyzed the regulation of endogenous p38 MAP kinase activity during the differentiation of naïve CD4$^+$ T cells into Th1 and Th2 cells. No significant differences were observed between the level of p38 MAP kinase activity induced during differentiation of Th2 cells with ConA plus IL-4 and the level of p38 MAP kinase activity during differentiation of Th1 cells with IL-12 (Figure 2A). The activation of p38 MAP kinase was transient and after 4 days of differentiation only low levels of p38 MAP kinase activity were detected. The extent of p38 MAP

Fig. 2. Endogenous p38 MAP kinase activity is induced in Th1 cells, but not in Th2 cells. (**A**) p38 activity during the differentiation of Th1 and Th2 cells. CD4$^+$ T cells (5×10^5 cells) were stimulated with ConA (2.5 μg/ml) plus IL-4 (10^3 U/ml) or IL-12 (3.5 ng/ml) in the presence of APC, harvested and lysed at different periods of time. Whole extracts were assayed for p38 activity using the substrate GST–ATF2, as described in Materials and methods. The phosphorylated ATF2 was detected after SDS–PAGE by autoradiography and was quantitated by PhosphorImager analysis. (**B** and **C**) p38 activity during the activation of Th1 and Th2 cells. CD4$^+$ T cells were cultured as described in (A) for 4 days, exhaustively washed and restimulated (5×10^5 cells) with ConA (2.5 μg/ml) (B) or with PMA (5 ng/ml) plus ionomycin (250 ng/ml) (C) in the absence of APC. At different time points the cells were harvested and lysed to analyze the p38 activity. The results are representative of three (A) or two (B) independent experiments.

kinase activation was similar in cells stimulated with IL-12 and IL-4. Nevertheless, the kinetics of p38 MAP kinase activation was consistently advanced when the cells were stimulated with IL-12 compared with IL-4.

To examine the regulation of p38 MAP kinase activity during activation of Th1 and Th2 effector cells, $CD4^+$ T cells were cultured in the presence of ConA plus IL-4 or IL-12. After 4 days, the cells were washed, counted and restimulated with ConA for different periods of time. No significant increase in p38 MAP kinase activity was detected during the activation of Th2 effector cells (Figure 2B). In contrast, p38 MAP kinase was rapidly activated in Th1 effector cells upon stimulation. These data show that in effector cells the p38 MAP kinase pathway is specific to Th1-cell activation. To determine whether the deficiency in p38 activation in Th2 cells was a TcR proximal or distal event, we examined p38 activity in response to a TcR-independent stimulus, a combination of phorbol myristate acetate (PMA) plus ionomycin. Like ConA, PMA plus ionomycin strongly induced p38 activity in Th1 cells, while only a minor increase was observed in Th2 cells (Figure 2C). Together these results indicated that the p38 MAP kinase pathway was differentially regulated in Th1 and Th2 cells and suggested that its activation in Th1 cells could be critical for the production of IFNγ in these cells.

p38 MAP kinase is required for IFNγ gene transcription

To determine whether inhibition of IFNγ production in Th1 cells by SB203580 was due to a specific effect on *IFN*γ gene expression, we examined the level of mRNA by competitive reverse transcriptase (RT)–PCR (Reiner *et al.*, 1993). Specific primers for HPRT (positive control) and IFNγ were used to amplify the cDNA obtained from stimulated Th1 cells. The level of IFNγ mRNA detected in Th1 cells upon ConA-activation were decreased (45–60%) when the activation was performed in the presence of different doses of SB203580 (Figure 3A). These results indicated that the blockade of p38 MAP kinase by SB203580 in Th1 cells inhibited *IFN*γ gene expression. We cannot, however, exclude the possibility that IFNγ translation may also be affected by SB203580, as reported for certain cytokine mRNAs (Prichett *et al.*, 1995; Pietersman *et al.*, 1997).

ATF/CREB motifs have been identified in two critical regulatory elements of the *IFN*γ promoter, termed 'proximal' and 'distal' elements (Penix *et al.*, 1993, 1996; Cippitelli *et al.*, 1995; Aune *et al.*, 1996). Since the ATF family of transcription factors are known to be substrates of p38 MAP kinase (Dérijard *et al.*, 1995; Raingeaud *et al.*, 1995, 1996), we considered it probable that at least one of the effects of p38 MAP kinase was directly on the *IFN*γ promoter. To test the direct effect of p38 MAP kinase on *IFN*γ gene transcription, we first analyzed the effect of SB203580 on transcription mediated by the *IFN*γ promoter. Jurkat T cells were transiently transfected with a reporter construct containing the luciferase gene driven by the *IFN*γ promoter (–538 to +64 from transcription start site). Luciferase activity was measured in cells activated with PMA and ionomycin in the absence or presence of different doses of SB203580. A dose-dependent inhibition of the transcription mediated by the *IFN*γ promoter was observed in the presence of the p38 MAP kinase inhibitor SB203580 (Figure 3B), suggesting that activation of p38 MAP kinase is required for *IFN*γ gene transcription.

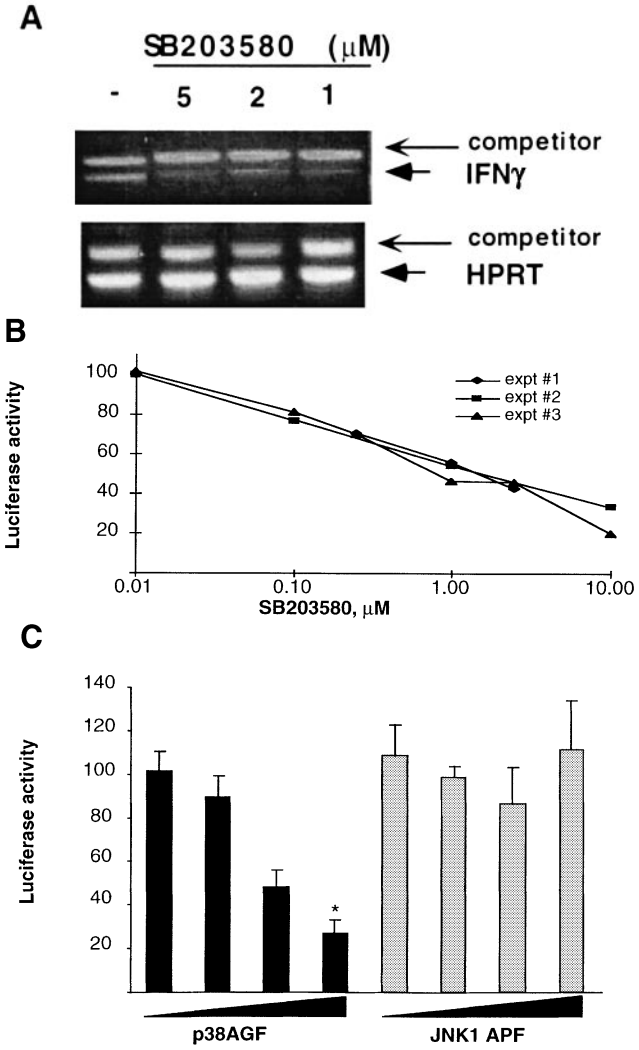


Fig. 3. p38 MAP kinase pathway is required for transcription of *IFN*γ gene. (**A**) $CD4^+$ T cells (10^6 cells/ml) were stimulated with ConA (2.5 μg/ml) plus IL-12 (3.5 ng/ml) in the presence of APC. After 4 days, the cells were washed and restimulated with ConA alone in the absence or the presence of different concentrations of the p38 inhibitor SB203580 (μM). After 24 h, the cells were harvested and total RNA was extracted and analyzed by competitive RT–PCR as described in Materials and methods. Top panel presents the expression of IFNγ transcripts (small arrow), lower panel presents the expression of HPRT (small arrow); long arrow shows the competitor DNA. (**B**) Jurkat T cells transfected with *IFN*γ promoter luciferase reporter plasmid were activated with PMA (25 ng/ml) and ionomycin (1.5 μM) in the presence of different concentrations of the p38 MAP kinase inhibitor SB203580. Luciferase activity was assayed 18 h later. Results are expressed as percentage of DMSO control. (**C**) *IFN*γ promoter luciferase reporter plasmid (15 μg) was co-transfected with 0.5, 1.0, 2.5 or 5.0 μg of pcDNA3–dn p38 MAP kinase expression plasmid or pcDNA3–dn JNK1 kinase expression plasmid. The total amount (5 μg) of expression vector was kept constant by addition of empty pcDNA3. The transfected cells were then stimulated with ionomycin plus PMA, as described in (A). Results are expressed relative to transcriptional activity observed with empty expression vector alone. Statistical analysis by two-tailed Student's *t* test revealed significant differences between the 5 μg of control vector and 5 μg of dn p38 MAP kinase ($P = 0.007$). Results are the mean ± SEM of three experiments.

To examine further the involvement of the p38 MAP

kinase pathway in transcription of the *IFN*γ gene, we investigated the effect of expression of dn p38 MAP kinase. This construct was generated by replacing the activating phosphorylation sites Thr^{180} and Tyr^{182} with Ala and Phe, respectively (Raingeaud *et al.*, 1995). This inactive form of p38 MAP kinase binds endogenous substrates, thereby inhibiting signaling by the endogenous p38 MAP kinase pathway. Expression of this dn p38 MAP kinase by transient transfection in Jurkat T cells did not affect cell viability (data not shown) and did not significantly modify transcription mediated by a constitutive promoter, such as the Rous sarcoma virus (RSV) long terminal repeat (data not shown). However, overexpression of dn p38 MAP kinase inhibited transcription driven by the *IFN*γ promoter in activated Jurkat cells in a dose-dependent manner (Figure 3C). In contrast, a dominant-negative form of JNK (Dérijard *et al.*, 1994) did not affect *IFN*γ promoter transcriptional activity (Figure 3C), indicating the specific involvement of p38 MAP kinase in the regulation of *IFN*γ gene transcription.

Normal T-cell proliferation in dn p38 transgenic mice

To examine the role of p38 MAP kinase in Th1 responses, we generated transgenic mice expressing dn p38 MAP kinase. The cDNA encoding dn p38 was linked to the distal *lck* promoter (Figure 4A) which, though weaker than the proximal *lck* promoter used extensively by Perlmutter and colleagues (Alberola-Ila *et al.*, 1995; Swan *et al.*, 1995), expresses in both peripheral T cells and thymocytes (Wildin *et al.*, 1991). Three different founder mice (lines #18, #25 and #27) were obtained and back-crossed to generate stable lines. Expression of the transgene was examined in both negative and positive littermates by RT–PCR (Figure 4B). Positive littermates from all the lines expressed the dn p38 transgene. High levels of dn p38 expression were observed in T cells and in the thymus and lower levels in the spleen; no expression was observed in B cells (Figure 4C).

The developing thymocytes of dn p38 transgenic mice appeared normal. The ratio of double negative $CD4^-CD8^-$, double positive $CD4^+CD8^+$, and single positive $CD4^+CD8^-$ and $CD8^+CD4^-$ thymocytes was similar in both transgene positive and negative littermates from line #18 (Figure 5A) and the other lines (data not shown). Development and homeostasis of lymphocytes in the periphery also appeared to occur normally, since similar ratios of $CD4^+$ and $CD8^+$ T cells were observed in lymph nodes of dn p38 and wild-type mice (Figure 5A). To test the effect of dn p38 on T-cell activation, splenocytes from positive and negative littermates from all the lines were activated with either ConA or PMA plus ionomycin. No significant differences were observed in the proliferative response (Figure 5B). Moreover, proliferation of purified $CD4^+$ T cells in response to ConA or anti-CD3 monoclonal antibody (mAb) in the presence of APC, was similar for cells obtained from dn p38 and wild-type mice (Figure 5C). Thus, the ability of T cells to produce IL-2 (data not shown) and enter the cell cycle appears to be normal in the dn p38 animals.

Activation of Th1 cells is impaired in the dn p38 transgenic mice

To analyze the activation of Th1 cells in the dn p38 transgenic mice, $CD4^+$ T cells from negative control and dn p38 transgenic littermates were activated with ConA and APC in the presence of either IL-4 (Th2) or IL-12 (Th1) for 4 days. The cells were then exhaustively washed and equal numbers were re-stimulated with ConA alone for 24 h before harvesting the supernatants to analyze cytokine production. In correlation with the results obtained with the p38 MAP kinase inhibitor drug SB203580, the production of IFNγ was significantly inhibited by Th1 cells from dn p38 transgenic mice from line #18 (Figure 6A). In contrast, no significant differences were observed in the production of IL-4 and IL-5 in Th2 cells (Figure 6A). Similarly, the production of IL-10, another Th2 type cytokine, was unaffected (data not shown). A slight increase in IL-4 production was also observed in Th1 cell populations from the dn p38 mice, probably as a consequence of the decrease of IFNγ production, since IFNγ can suppress IL4 production. The reduction of IFNγ production in Th1 cells from dn p38 transgenic mice was consistent in several independent experiments performed using two different transgenic mouse lines (Figure 6B). This effect was caused by the specific overexpression of the dn p38 transgene, since no modifications were observed in Th1 cells from transgenic mice overexpressing dn JNK1 (our unpublished data).

Increased p38 MAP kinase activity was observed during

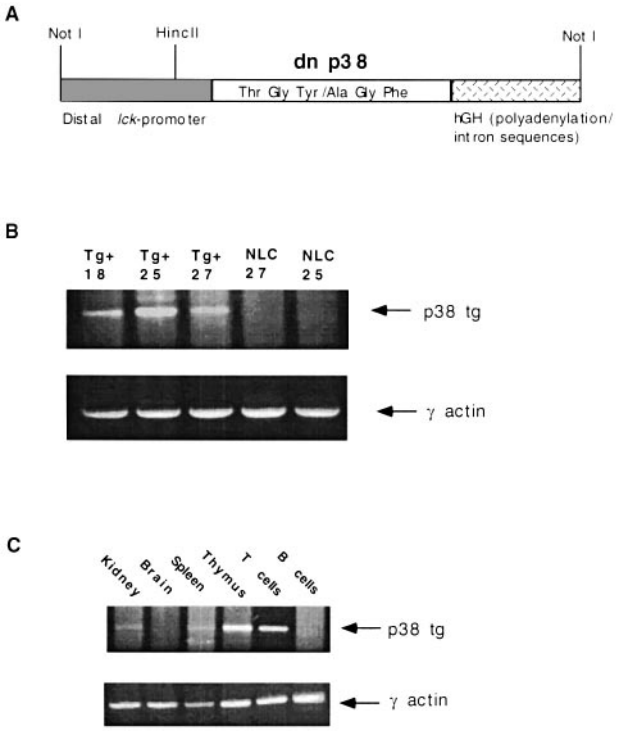


Fig. 4. Generation of dn p38 transgenic mice. (**A**) Schematic representation of the dn p38 transgene. The dn p38 MAP kinase cDNA in which Thr^{180} and Tyr^{182} were replaced by Ala and Phe, respectively, was subcloned downstream of the distal *lck* promoter and upstream of the human growth hormone (hGH) polyadenylation signals and intron sequences. This DNA fragment was used to generate the dn p38 transgenic mice. (**B**) Expression of dn p38 transgene (p38 tg) and endogenous γ-actin in positive littermates (Tg+) or negative littermate controls (NLC) from the stable transgenic lines #18, 25 and 27, analyzed by RT–PCR as described in Materials and methods. (**C**) Tissue distribution of the dn p38 transgene. RT–PCR of cytoplasmic RNA from brain, kidney, thymus, spleen, purified B cells and purified T cells from positive littermates (line #18).

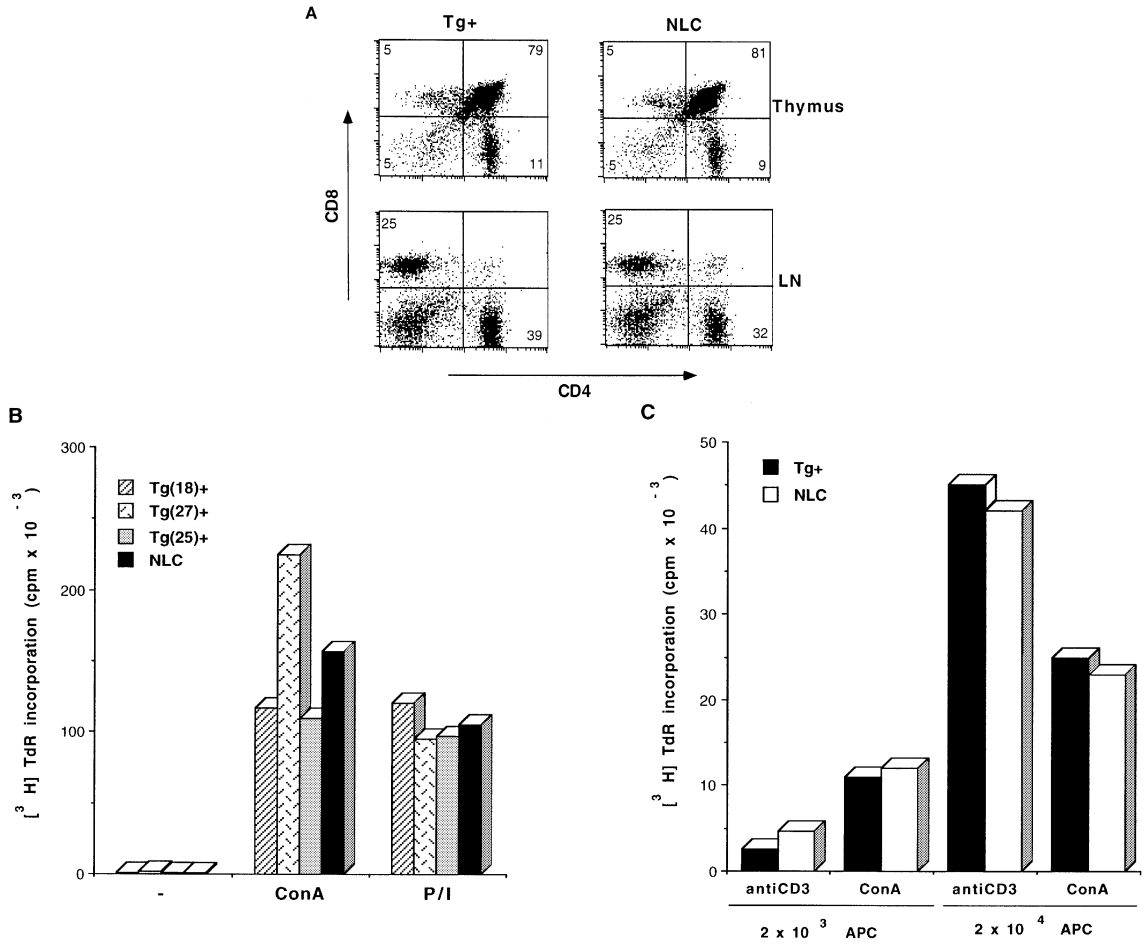


Fig. 5. Normal lymphocyte development and T cell proliferation in dn p38 transgenic mice. (**A**) Thymic and lymph node (LN) populations in dn p38 transgenic mice (Tg+) and negative littermate controls (NLC). Cells were stained with an anti-CD4 mAb and an anti-CD8 mAb, and analyzed by flow-cytometry. Numbers represent the percentage of the population in each quadrant from the total thymocytes. (**B**) Total spleen cells (2×10^5 cells/well) from dn p38 transgenic mice (Tg+) lines 18, 27 and 25, and NLCs were stimulated in the presence of medium (–), PMA (5 ng/ml) plus ionomycin (250 ng/ml) (P/I) or ConA (2.5 μg/ml). Proliferation was examined after 3 days. (**C**) $CD4^+$ T cells were isolated from dn p38 transgenic mice (Tg+) and negative littermates. 5×10^4 cells were stimulated with soluble anti-CD3 mAb (1 μg/ml) or ConA (2.5 μg/ml) in the presence of 2×10^3–2×10^4 mitomycin C-treated syngeneic splenocytes from wild-type mice as the source of APC. Proliferation was assayed. This is representative of four experiments.

activation of Th1 cells, but not Th2 cells. We examined the effect of expression of dn p38 on endogenous p38 MAP kinase activity. Rapid and transient activation of p38 MAP kinase was observed in wild-type Th1 cells (Figure 6C). Significant reduction of endogenous p38 MAP kinase activity was observed in Th1 cells from the dn p38 transgenic mice. The grade of inhibition of the endogenous p38 MAP kinase activity (40–50%) correlated with the inhibition of IFNγ production (50–60%) observed in these mice. As expected, an inhibition of p38 MAP kinase was also detected in Th2 cells from the dn p38 transgenic mice, since the distal *lck* promoter drives equal expression in both Th1 and Th2 cells. However, in correlation with the results obtained in the presence of SB203580, the inhibition of p38 MAP kinase did not affect IL-4 production in Th2 cells (Figure 6B). We also analyzed the regulation of p38 activity in response to a TcR-independent stimulus. The p38 MAP kinase activity induced by PMA plus ionomycin was also inhibited in Th1 cells from dn p38 transgenic mice (Figure 6D).

To determine whether p38 MAP kinase activation was required to trigger Th1 responses by specific antigens, we crossed the dn p38 transgenic mice with cytochrome *c* (Cyt *c*) TcR transgenic mice (Kaye *et al.*, 1989). These mice express the α and β chain of the TcR from a $CD4^+$ T-cell clone that recognizes a peptide of Cyt *c* in the context of MHC class II $I\text{-}E^k$. $CD4^+$ T cells were isolated from both Cyt *c* TcR transgenic mice and dn p38×Cyt *c* TcR double transgenic mice, and differentiated with specific Cyt *c* peptide alone or Cyt *c* peptide in the presence of IL-12. After 4 days, cells were washed and restimulated with peptide alone. Supernatants were harvested after 24 h. Although low levels of cytokines were produced when the cells were differentiated with the peptide in the absence of the driving cytokine, a preferential Th1 response was observed in Cyt *c* TcR transgenic mice. This Th1 response was significantly reduced in dn p38×Cyt *c* TcR double transgenic mice (Figure 6E). Th1 cells differentiated with Cyt *c* peptide in the presence of IL-12 produce higher levels of IFNγ (Figure 6E).

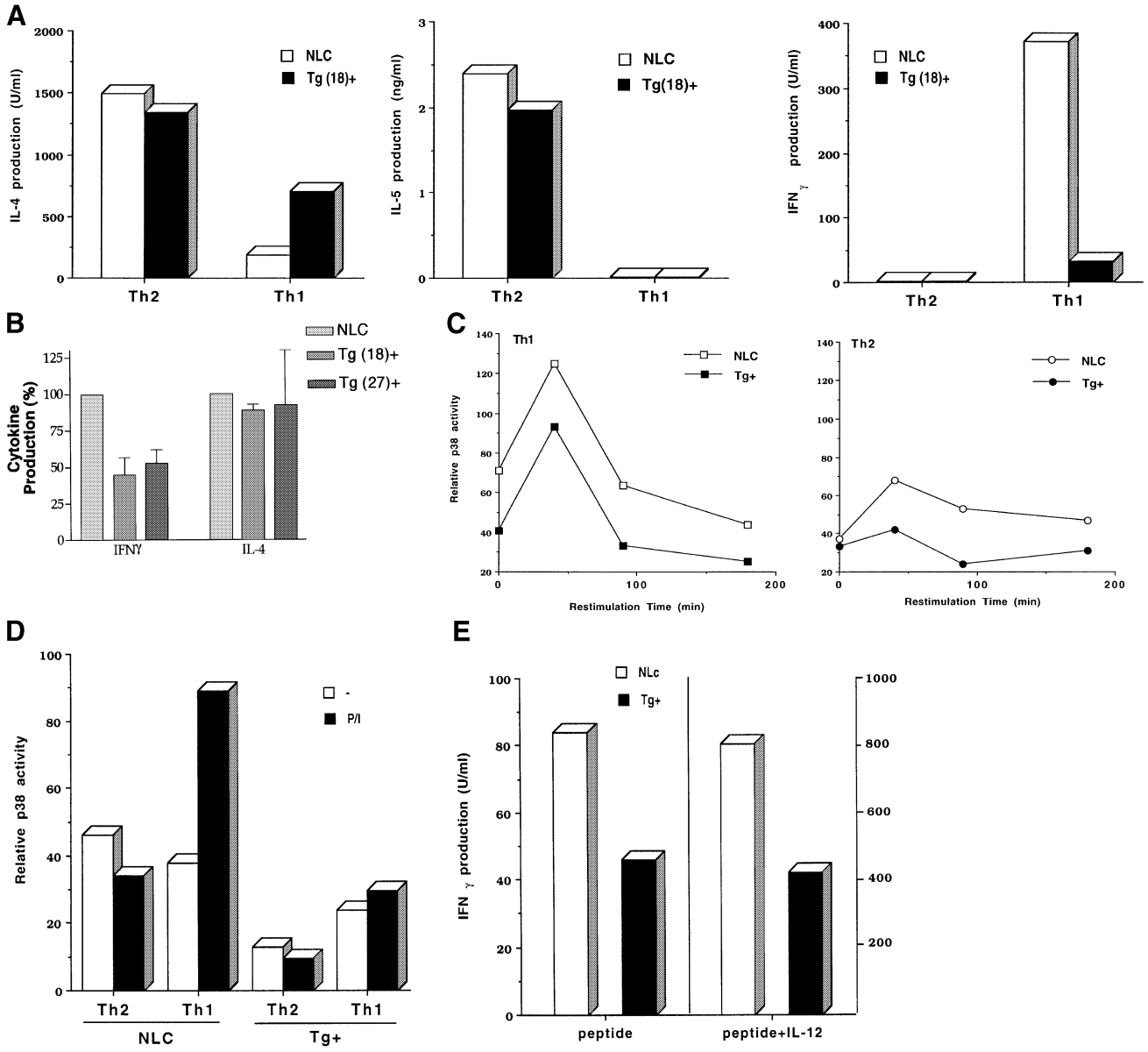


Fig. 6. IFNγ production by Th1 cells is impaired in dn p38 transgenic mice. (**A**) $CD4^+$ T cells were purified from dn p38 transgenic mice (Tg+) (line #18) and negative littermate controls (NLC), and stimulated (10^6 cell/ml) with ConA (2.5 μg/ml) plus IL-4 (10^3 U/ml) (Th2) or IL-12 (3.5 ng/ml) (Th1) in the presence of APC (5×10^5 cells/ml). After 4 days, the cells were exhaustively washed and restimulated with ConA alone (2.5 μg/ml). The supernatants were harvested 24 h later and analyzed for IL-4, IL-5 and IFNγ production by ELISA. (**B**) IFNγ production in Th1 cells and IL-4 production in Th2 cells from negative littermates or transgenic mice from lines #18 and #27. Values represent percentage of cytokine (IFNγ or IL-4) production detected in transgenic (Tg) mice versus NLC mice. The mean and standard deviation from four independent experiments are presented. (**C**) Inhibition of p38 MAP kinase activity induced by ConA in Th1 cells from dn p38 transgenic mice. $CD4^+$ T cells from dn p38 transgenic mice (Tg+) and NLC mice were differentiated into Th1 and Th2 as described in (A). After 4 days, the cells were washed, activated with ConA and lysed at different periods of time. Whole extracts were assayed for p38 activity using the substrate GST–ATF2 as described in Figure 2A. The data are representative of three independent experiments. (**D**) Inhibition of p38 MAP kinase activity induced by PMA plus ionomycin. $CD4^+$ T cells from dn p38 transgenic mice (Tg+) and NLC mice were differentiated into Th1 and Th2 as described in (A). After 4 days, the cells were washed, activated with PMA (5 ng/ml) plus ionomycin (250 ng/ml) (P/I) for 45 min. Whole extracts were assayed for p38 activity using the substrate GST–ATF2. (**E**) $CD4^+$ T cells were purified from Cyt *c* transgenic mice (NLC) or from Cyt *c*×dn p38 double transgenic mice (Tg+) and stimulated (10^6 cell/ml) with Cyt *c* peptide (5 μg/ml) alone or Cyt *c* peptide (5 μg/ml) plus IL-12 (3.5 ng/ml), in the presence of APC. After 4 days, the cells were washed and restimulated with Cyt *c* peptide (5 μg/ml) in the presence of APC. The supernatants were harvested at 24 h and analyzed by ELISA.

Despite the high production of IFNγ, ~50% inhibition was observed in dn p38×Cyt *c* TcR double transgenic mice (Figure 6E).

Together, these data indicate that the inhibition of the p38 MAP kinase pathway in $CD4^+$ T cells causes an impairment of Th1 responses.

Increased IFNγ production in Th1 cells from constitutively-activated MKK6 transgenic mice

To confirm the results obtained with the dn p38 transgenic mice, we generated transgenic mice expressing constitutively-activated MKK6, an upstream activator of p38 MAP kinase (Han *et al.*, 1996; Moriguchi *et al.*, 1996; Raingeaud

et al., 1996). MAP kinase kinases are activated by dual phosphorylation within subdomain VIII. These phosphorylation sites are conserved in MKK6 (Ser^{207} and Thr^{211}). The mechanism of MKK6 activation may therefore be mediated by increased negative charge on these phosphorylation sites. Thus, the replacement of these two residues with Glu, which provides a constitutive negative charge, generates an activated MKK6 [MKK6(Glu)] that is able to phosphorylate p38 MAP kinase in the absence of prior stimulation (Raingeaud *et al.*, 1996). To generate transgenic mice in which p38 MAP kinase is constitutively activated, the cDNA encoding MKK6(Glu) was linked to the distal *lck* promoter (Figure 7A) and microinjected into fertilized eggs. Three transgenic mouse lines were obtained and characterized. To determine the expression of the transgene, we directly examined p38 MAP kinase activity, since expression of MKK6(Glu) should result in an increased level of p38 MAP kinase activity in the absence of stimulation. We first examined p38 MAP kinase activity in total thymocytes isolated from negative and positive littermates from the different transgenic mouse lines. In the three transgenic lines, the level of p38 MAP kinase activity in the transgenic thymocytes was 2- to 4-fold higher than negative control thymocytes (Figure 7B). MKK6 is a specific activator of p38 MAP kinase that activates neither JNK nor ERK (Han *et al.*, 1996; Moriguchi *et al.*, 1996; Raingeaud *et al.*, 1996). In correlation, no significant changes were observed in the level of JNK activity in total thymocytes from MKK6(Glu) transgenic mice compared with wild-type mice (Figure 7B).

The analysis of cell-surface staining showed no major differences in the distribution of thymic subpopulations in MKK6(Glu) transgenic mice. The number and the phenotype of $CD4^+$ T cells from lymph nodes and spleen from these mice were also normal (data not shown). To examine the expression of MKK6(Glu) in peripheral T cells, we first isolated the $CD4^+$ population from spleen and lymph nodes from negative littermate control and MKK6(Glu) transgenic mice. p38 MAP kinase and JNK activities were examined in these cells prior to stimulation. The level of p38 MAP kinase activity was higher in $CD4^+$ T cells from MKK6(Glu) transgenic mice (Figure 7C). No significant increase in JNK activity was observed.

$CD4^+$ T cells from control and MKK6(Glu) transgenic mice were differentiated into Th1 and Th2 cells with ConA in the presence of IL-12 or IL-4, respectively. After 4 days, the cells were washed and equal numbers of cells were restimulated with ConA. Cytokine production was then analyzed. In contrast to the dn p38 transgenic mice, an increase in IFNγ production was observed in Th1 cells from the MKK6(Glu) transgenic mice compared with the negative littermates (Figure 7D). Reduced IL-4 production was also observed in Th2 cells from the MKK6(Glu) transgenic mice (Figure 7D). These data indicate that the MKK6(Glu) transgenic mice exhibit polarization of the effector T cells to the Th1 phenotype with reduced IL-4 secretion and increased IFNγ production. Since the expression of MKK6(Glu) caused constitutive activation of p38 MAP kinase in unstimulated $CD4^+$ T cells, we also examined the production of IFNγ by these cells during their differentiation into Th1 cells. $CD4^+$ T cells were stimulated with ConA and IL-12 and culture supernatants were harvested at several time points during the differentiation. Increased IFNγ production in $CD4^+$ T cells from MKK6(Glu) mice was detected by day 3 (Figure 7E).

Together, the data obtained from experiments using the p38 MAP kinase inhibitor SB203580 and the two transgenic mouse models indicate that that p38 MAP kinase pathway plays an important role in Th1 responses.

Discussion

In response to pathogens, naïve $CD4^+$ T cells differentiate into effector Th1 and Th2 cells. The development of an inappropriate response can lead to ineffective immunity and may lead to pathological consequences. The balance of Th1 and Th2 responses is critical for the generation of physiologically appropriate immune responses and alterations of this balance are associated with several immune disorders, such as diabetes, leishmaniasis, arthritis and experimental encephalomyelitis. A large number of studies are currently focused on the identification of intracellular signals and transcription factors specific for either Th1 or Th2 cells (Rincón and Flavell, 1997a). While several transcription factors have been described to be specifically active in one of these subsets (principally in Th2 cells), little is known about the regulation of the intracellular signals that lead to the activation of these factors in Th1 and Th2 cells. p38 MAP kinase can be activated by pro-inflammatory cytokines and is involved in the expression of specific cytokine genes. In this study, we have used three different experimental approaches (p38 MAP kinase specific inhibitors, dn p38 transgenic mice and constitutively-activated MKK6 transgenic mice) to show that the p38 MAP kinase pathway is critical for the development of Th1 responses.

Pyridinyl imidazole compounds were initially identified as powerful anti-arthritic drugs (Griswold *et al.*, 1988; Lee *et al.*, 1993; Reddy *et al.*, 1994; Badger *et al.*, 1996). These drugs also constitute the most specific inhibitors of p38 MAP kinases that have been identified to date (Lee *et al.*, 1994). However, the anti-arthritic activity of these drugs has been exclusively associated with blocking the production of pro-inflammatory cytokines such as IL-1β, IL-6 and TNFα by macrophages (Lee *et al.*, 1993; Reddy *et al.*, 1994; Badger *et al.*, 1996; Beyaert *et al.*, 1996; Lee and Young, 1996). We have shown here, for the first time, that inhibition of IFNγ production by $CD4^+$ Th1 cells is another mechanism by which the pyridinyl imidazole compounds could modify the course of arthritis. The blockade of Th1-cell activation was not due to an indirect effect of SB203580 on the APC, since no APC were present during the restimulation of Th1 cells. Furthermore, these results were corroborated by the inhibition of IFNγ production observed in the dn p38 transgenic mice where the transgene is expressed only in T cells (distal *lck* promoter) and wild-type APC were used. The description of the role of these drugs in the Th1/Th2 balance is essential due to their potential therapeutic use in the treatment of arthritis. Although the contribution of Th1/Th2 responses in arthritis is not fully established, a predominant Th1 response has been found in several animal models of arthritis, such as collagen-induced and Lyme arthritis, but the implication of a Th1 response is less clear in human rheumatoid arthritis.

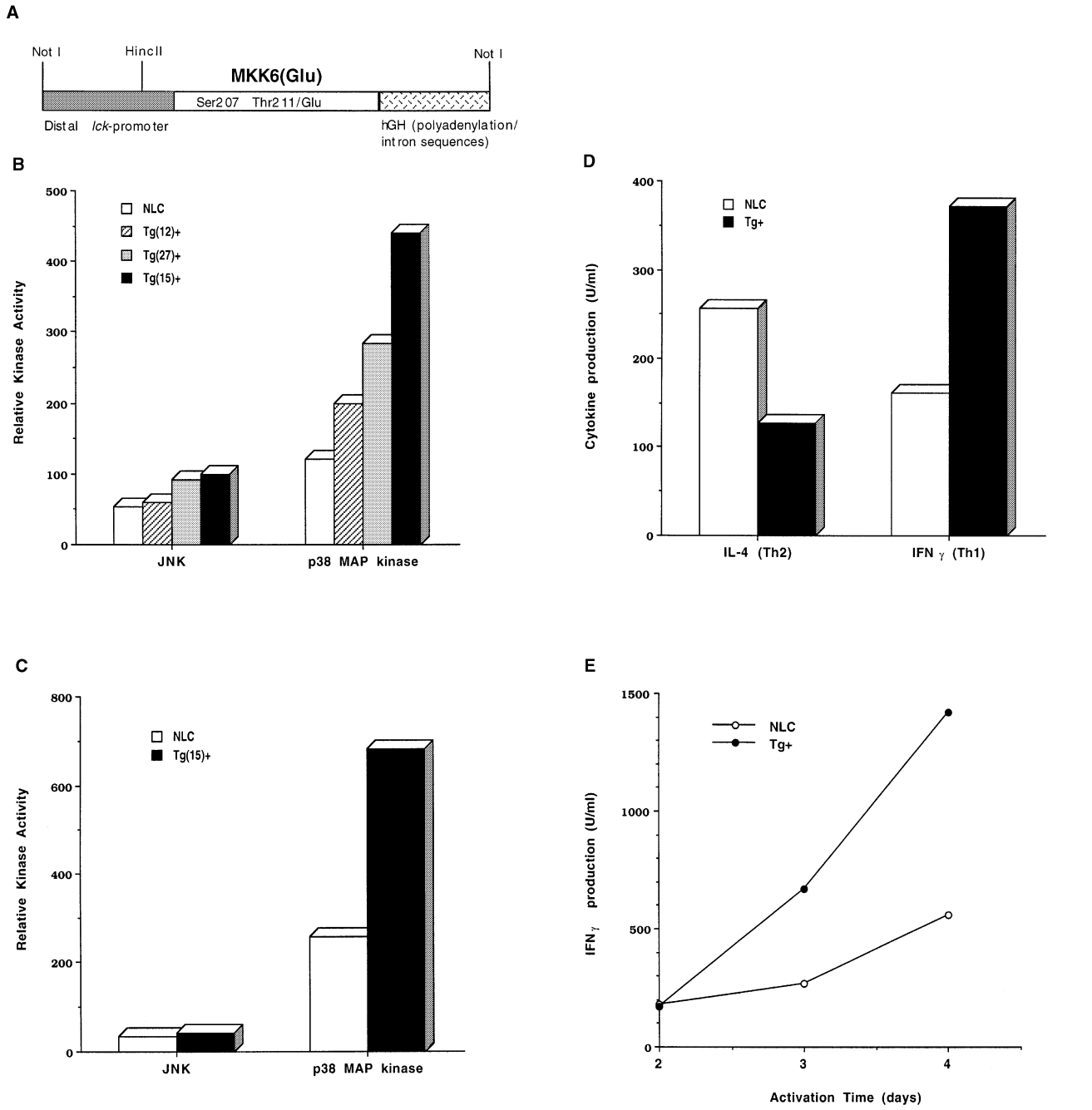


Fig. 7. Generation and analysis of IFNγ production in the MKK6(Glu) transgenic mice. (**A**) Schematic representation of the MKK6(Glu) transgene. The constitutively activated MKK6 cDNA in which Ser^{207} and Thr^{211} were replaced by Glu [MKK6(Glu)] was subcloned downstream of the distal *lck* promoter and upstream of the human growth hormone (hGH) polyadenylation signals and intron sequences. This fragment of DNA was used to generate the MKK6(Glu) transgenic mice. (**B**) Total thymocytes (5×10^5 cells) from MKK6(Glu) transgenic mice (Tg+) from three different lines (#15, #12, #27) or from negative littermate control (NLC) mice were lysed. Whole extracts were assayed for p38 activity or for JNK activity using the substrate GST–ATF2 or GST–c-Jun, respectively. (**C**) Unstimulated $CD4^+$ T cells (5×10^5 cells) were purified from MKK6(Glu) transgenic mice (Tg+) (line #15) and NLC mice and lysed. Whole extracts were assayed for p38 activity or for JNK activity as described in (B). (**D**) $CD4^+$ T cells were purified from MKK6(Glu) transgenic mice (Tg+) (line #15) and NLCs, and stimulated (10^6 cell/ml) with ConA plus IL-4 (Th2) or IL-12 (Th1) in the presence of APC (5×10^5 cells/ml). After 4 days, the cells were exhaustively washed and restimulated with ConA alone. The supernatants were harvested 24 h later and analyzed for IL-4 and IFNγ production. Results represent the production of IL-4 in Th2 cells and IFNγ in Th1 cells. (**E**) $CD4^+$ T cells were purified from MKK6(Glu) transgenic mice (Tg+) and NLCs, and stimulated (10^6 cell/ml) with ConA plus IL-12 in the presence of APC. Supernatants were harvested after 2, 3 or 4 days in culture and analyzed for IFNγ production.

Little is known about the role of p38 MAP kinase in T cells. p38 MAP kinase is constitutively activated in immature thymocytes (Sen *et al.*, 1996), suggesting a role for this pathway in thymic development. We could not find significant alterations in the distribution of thymic subpopulations in dn p38 or MKK6(Glu) transgenic mice at the age that experiments were performed (6–12 weeks). Further experiments are required to determined whether p38 MAP kinase is involved in positive or negative selection of immature thymocytes.

Activation of p38 MAP kinase has been associated with the induction of apoptosis in neuronal cells (Xia *et al.*, 1995; Kawasaki *et al.*, 1997; Kummer *et al.*, 1997). Expression of activated MKK3 in PC12 cells (Xia *et al.*, 1995) or activated MKK6 in Jurkat T cells (Huang *et al.*, 1997) causes increased apoptosis. Conversely, dn MKK3 (Xia *et al.*, 1995) or dn MKK6 (Huang *et al.*, 1997) can inhibit apoptosis. These data strongly support the hypothesis that the p38 MAP kinase pathway contributes to apoptotic responses. Nevertheless, p38 MAP kinase does not appear to be universally required for apoptosis. Thus, it has been shown that p38 MAP kinase activation by Fas in Jurkat T cells is dependent on the activation of caspase proteases (Juo *et al.*, 1997), suggesting a role of p38 MAP kinase downstream of apoptotic events. Similarly, p38 MAP kinase is not required for TNF-induced apoptosis of murine L cells, although this pathway is required for TNF-induced IL-6 gene expression (Beyaert *et al.*, 1996). Furthermore, it is possible that some of the apoptotic effects of p38 MAP kinase activators (e.g. MKK6) are not mediated by p38 MAP kinase (Huang *et al.*, 1997). In our analysis of Th1 cells, we observed increased apoptosis during the differentiation of Th1 cells from MKK6(Glu) transgenic mice compared with wild-type mice, but no significant cell viability differences were detected between Th1 cells from wild-type and dn p38 transgenic mice (data not shown). These data do not support a major role for the p38 MAP kinase pathway in the apoptosis of Th1 cells under the conditions of our experiments. However, our study does implicate the p38 MAP kinase pathway in the regulation of IFNγ production by Th1 cells.

In addition to the activation of p38 MAP kinase by pro-inflammatory cytokines (IL-1 and TNF) and physical–chemical stresses, several growth factors can also stimulate p38 MAP kinase pathway. Hematopoietic growth factors including IL-2, IL-3, IL-7, GM-CSF and Steel locus factor (SLF) induce p38 MAP kinase activity (Crawley *et al.*, 1997; Foltz *et al.*, 1997). Furthermore, IL-2 -stimulated proliferation is inhibited by SB203580 (Crawley *et al.*, 1997). In contrast, IL-4 does not affect p38 MAP kinase activity (Foltz *et al.*, 1997). The potential role of p38 MAP kinase in the IL-12 pathway has not yet been addressed. Our data show no significant effect of the dn p38 transgene on T-cell proliferation. The drug SB203580 did not affect cell recovery during the activation of Th1 and Th2 cells; however, SB203580 did cause partial inhibition of $CD4^+$ T-cell proliferation during differentiation to Th1 and Th2 cells, but it is unclear whether this was a direct effect on the T cells or whether it was indirectly mediated by an effect of SB203580 on the APC.

Pyridinyl imidazole drugs can affect the secretion of several cytokines by interfering with the translation of the specific mRNAs (Prichett *et al.*, 1995; Pietersman *et al.*, 1997). These drugs can also decrease the level of cytokine mRNA, for example IL-6 (Beyaert *et al.*, 1996). Here we demonstrate that the inhibition of IFNγ production by Th1 cells caused by SB203580 is associated with reduced IFNγ mRNA expression. Furthermore, we demonstrate that transcription mediated by the *IFN*γ promoter is inhibited by treatment of cells with SB203580. The expression of dn p38 in $CD4^+$ Th1 cells caused inhibition of IFNγ expression. Moreover, the constitutive activation of p38 MAP kinase caused by expression of MKK6(Glu) in $CD4^+$ Th1 cells resulted in increased production of IFNγ. Together, these results demonstrate the importance of the p38 MAP kinase pathway for the expression of the *IFN*γ gene in Th1 cells. Several substrates for p38 MAP kinase have already been identified, including ATF-2, Elk-1, CHOP, MEF2C and SAP-1 transcription factors (Dérijard *et al.*, 1995; Raingeaud *et al.*, 1995, 1996; Wang and Ron, 1996; Han *et al.*, 1997; Whitmarsh *et al.*, 1997). p38 MAP kinase also phosphorylates and activates the eIF-4E protein kinases Mnk1 and Mnk2 (Fukunaga and Hunter, 1997; Waskiewicz *et al.*, 1997) and the small heat shock protein hsp27 protein kinase MAPKAP kinase-2 (Freshney *et al.*, 1994; Rouse *et al.*, 1994; Mclaughlin *et al.*, 1996). Two regulatory elements have been defined in the *IFN*γ promoter, termed proximal and distal elements (Penix *et al.*, 1993, 1996; Cippitelli *et al.*, 1995; Aune *et al.*, 1996). These regulatory elements bind several nuclear factors. These elements include ATF/CREB motifs present in both the proximal and distal regulatory regions of the *IFN*γ promoter which bind ATF-2 and related bZIP transcription factors. Thus, it is possible that p38 MAP kinase-stimulated *IFN*γ gene transcription is mediated, in part, by regulatory phosphorylation of ATF2 or related proteins by p38 MAP kinase.

Several transcription factors have been described to function selectively in Th2 cells, and to be required for Th2 responses. STAT-6 is induced by IL-4 and the disruption of this gene results in an impairment of Th2 responses (Kaplan *et al.*, 1996; Shimoda *et al.*, 1996; Takeda *et al.*, 1996). GATA-3 expression is maintained in Th2 cells and is eliminated in Th1 cells (Zheng and Flavell, 1997). c-Maf appears to be induced late in the differentiation of Th2 cells (Ho *et al.*, 1996). Although NFAT and AP-1 complexes are detected in both Th1 and Th2 cells, these transcription factors mediate stimulus-dependent transcription only in Th2 cells (Rincón and Flavell, 1997b; Rincón *et al.*, 1997b). An accumulation of JunB in the AP-1 complexes has been found in Th2 cells (Rincón *et al.*, 1997b). Defects in NFATp cause decreased expression of Th2-related cytokines (Schuh *et al.*, 1997). No intracellular signaling pathways triggered in Th2 cells have yet been identified.

In contrast to the progress that has been made towards understanding Th2-specific responses, little is known about the transcription factors and intracellular signals that control Th1 responses. STAT-4 is required for the initiation of Th1 responses (Kaplan *et al.*, 1996; Thierfelder *et al.*, 1996); however, it has not yet been established whether the *IFN*γ gene is regulated by STAT-4. More recent studies have demonstrated an important role of IRF-1 in Th1 responses through induction of IL-12 expression in macrophages (Lohoff *et al.*, 1997; Taki *et al.*, 1997). Here, we have shown that p38 MAP kinase is selectively activated in Th1 cells in response to both TcR-dependent and -independent stimuli, while significant induction was not observed in Th2 cells. These results indicate that not only transcription factors, but also upstream intracellular signaling pathways, are differentially regulated in Th1 and Th2 subsets. It is not clear at present whether p38 MAP kinase is selectively activated in Th1 cells or selectively repressed in Th2 cells. Th1-specific expression of specific p38 MAP kinase family members or upstream

activators represents one potential mechanism. Although the mechanism of differential regulation in Th1 and Th2 cells is unclear, our analysis strongly implicates p38 MAP kinase signaling as an important check-point in the control of Th1 responses.

Our study shows, for the first time, that the p38 MAP kinase pathway in $CD4^+$ T cells is not required for Th2 responses; however, p38 MAP kinase plays an important role in the regulation of Th1 responses. Since inhibition of p38 MAP kinase can diminish the progression of several models of arthritis, our results provide an additional mechanism that may contribute to the inflammatory effects of p38 MAP kinase signaling. Furthermore, our observations indicate a role for the p38 MAP kinase pathway in the maintenance of the balance between Th1 and Th2 responses in physiological and pathophysiological immune responses.

Materials and methods

Generation of the dn p38 and MKK6(Glu) transgenic mice

The dn p38 mutant was generated by replacing Thr^{180} and Tyr^{182} by Ala and Phe, respectively (Raingeaud *et al.*, 1995). The constitutively-activated MKK6 cDNA was generated by replacing Ser^{207} and Thr^{211} by Glu [MKK6(Glu); Raingeaud *et al.*, 1996]. These cDNA were subcloned downstream of the distal *lck* promoter (Wildin *et al.*, 1991). The DNA fragments containing the distal *lck* promoter, the dn p38 or MKK6(Glu) genes and the human growth hormone (hGH) intron and polyadenylation signals was injected into fertilized (C57BL6/×C3H) F2 eggs and transgenic mice were generated as previously described (Hogan *et al.*, 1986). Expression of the transgene was analyzed by slot blot using a *Bam*HI–*Sac*I 0.5 kb fragment from the *hGH* gene. Three founders were back-crossed into B10.BR (Jackson Laboratory, Bar Harbor, Maine) background to establish stable transgenic mouse lines.

Cell preparation and surface staining

Total $CD4^+$ T cells were isolated from spleen and lymph nodes from wild-type or transgenic littermates by negative selection as previously described (Kamogawa *et al.*, 1993; Rincón and Flavell, 1997b; Rincón *et al*., 1997a,b). Mitomycin C-treated (50 μg/ml) syngeneic splenocytes were used as the source of APC.

Total or naïve $CD4^+$ T cells were cultured at 10^6 cells/ml in the presence of syngeneic APC (5×10^5 cells/ml) with ConA (Boehringer Mannheim, Germany) or synthetic moth Cyt *c* peptide (Yale University) plus IL-12 (kindly provided by Genetics Institute) or IL-4 (kindly provided by DNAX). After 4 days, effector Th cells were exhaustively washed, counted and restimulated at 10^6 cells/ml with ConA (in the absence of APC) or Cyt *c* peptide plus APC (5×10^5 cells/ml).

The distribution of populations in the thymus and in lymph nodes was examined by cell surface staining and flow-cytometry (EPICS, Coulter). A red^{613}-conjugated anti-CD4 mAb and a fluorescein isothiocyanate (FITC)-conjugated anti-CD8 mAb (Pharmingen, San Diego, CA) were used.

The proliferative response was examined after 2 days by measurement of [^{3}H]thymidine (Amersham Corp.) incorporation for 18 h.

RT–PCR

To determine the expression of transgenes, isolated cytoplasmic RNA (Tugores *et al.*, 1992) was treated with RNase-free DNase I (Promega, Madison, WI) and used (5 μg) to generate single strand cDNA by using Superscript RT (Gibco-BRL, Gaithersburg, MD). One-fifth of the reaction was used for PCR amplification of the dn p38 transgene cDNA and 1/25 was used to amplify γ-actin as a control in a total volume of 50 μl. A primer located in dn p38 MAP kinase (5′ primer, 5′-GCCCTTGCACATGCCTACTTTGC-3′) and a second primer corresponding to the human growth hormone polyadenylation sequences (Brinster *et al.*, 1988) of the transgene (3′ primer, 5′-CAGAACCCCCA-GACCTCCCTC-3′) were used to analyze the expression of the dn p38 MAP kinase transgene. A γ-actin 5′ primer (5′-CACCCTGTGCT-CACCCGAGGCC-3′) and 3′ primer (5′-CCACACAGATGACTTGCG-CTCAGG-3′) were used to examine γ-actin expression by RT–PCR. Fifteen μl/sample were examined by gel electrophoresis and detected by ethidium-bromide staining.

To examine cytokine gene expression, total RNA was extracted as described (Chomczynski and Sacchi, 1987) from 2×10^6 cells. Briefly, after dilution (1:5) of reverse transcription reaction mixture, 1 μl was assayed for levels of IFNγ or HPRT transcripts using the competitor (5 pg) in the presence of specific primers by competitive RT–PCR as described (Reiner *et al.*, 1993). The percentage of inhibition by SB203580 was stimulated by the ratio of IFNγ:competitor transcript levels, as determined by densitometry.

Cytokine production

ELISAs were performed using purified anti-IL-4, anti-IL-5 and anti-IFNγ mAbs (3 μg/ml) as primary antibodies and the corresponding biotinylated anti-IL-4, anti-IL-5 and anti-IFNγ mAbs (1 μg/ml) (Pharmingen), horseradish peroxidase-conjugated avidin D (2.5 μg/ml; Vector Laboratories, Burlingame, CA), the TMB microwell peroxidase substrate and stop solution (Kirkegaard and Perry Laboratories, Gaithersburg, MD), following the recommended protocol (Pharmingen). Recombinant IL-4 (DNAX) and IL-5 and IFNγ (Gibco-BRL) were used as standards.

p38 and JNK kinase assays

T cells were lysed with buffer A (20 mM Tris pH 7.5, 10% glycerol, 1% Triton X-100, 0.137 M NaCl, 25 mM β-glycerophosphate, 2 mM EDTA, 0.5 mM dithiothreitol, 1 mM sodium orthovanadate, 2 mM Na pyrophosphate, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride), as described (Dérijard *et al.*, 1994; Rincón *et al*., 1997b). Endogenous p38 and JNK were immunoprecipitated using anti-p38 polyclonal antibody (Raingeaud *et al.*, 1995) or anti-JNK mAb (Santa Cruz Biotechnology, Santa Cruz, CA). The immunoprecipitates were washed twice with buffer A and twice with kinase buffer (25 mM HEPES pH 7.4, 25 mM β-glycerophosphate, 25 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1 mM sodium orthovanadate). The protein kinase reactions were initiated by addition of 1 μg of recombinant substrate proteins (GST–ATF2 for p38 and GST–Jun for JNK) and 50 μM [γ-^{32}P]ATP (10 Ci/mmol). The reactions were terminated after 30 min at 30°C by addition of Laemmli sample buffer. Phosphorylation of the substrate proteins was examined after SDS–PAGE by autoradiography and PhosphorImager analysis (Molecular Dynamics Inc.).

IFNγ promoter activity

Jurkat T cells were transiently transfected with *IFN*γ (–538 bp) promoter luciferase reporter plasmid by electroporation as described (Penix *et al.*, 1993, 1996). After transfection, the cells were stimulated with PMA and ionomycin for 18 h, harvested and lysed to examine luciferase activity as recommended by the manufacturer (Promega).

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References

Alberola-Ila,J., Forbush,K.A., Seger,R., Krebs,E.G. and Perlmutter,R.M. (1995) Selective requirement for MAP kinase activation in thymocyte differentiation. *Nature*, **373**, 620–623.

Aune,T.L., Penix,L., Rincón,M. and Flavell,R.A. (1996) Differential expression of transcription directed by discrete IFNγ promoter elements in naïve and memory (effector) CD4 T cells, and CD8 T cells. *Mol. Cell. Biol.*, **17**, 199–208.

Badger,A.M., Bradbeer,J.N., Votta,B., Lee,J.C., Adams,J.L. and Griswold,D.E. (1996) Pharmacological profile of SB 203580, a selective inhibitor of cytokine suppressive binding protein/p38 kinase, in animal models of arthritis, bone resorption, endotoxin shock and immune function. *J. Pharmacol. Exp. Ther.*, **279**, 1453–1461.

Beyaert,R., Cuenda,A., Vanden Berghe,W., Plaisance,S., Lee,J.C., Haegeman,G., Cohen,P. and Fiers,W. (1996) The p38/RK mitogen-activated protein kinase pathway regulates interleukin-6 synthesis response to tumor necrosis factor. *EMBO J.*, **15**, 1914–1923.

Boulton,T.G., Yancopoulos,G.D., Gregory,J.S., Slaughter,C., Moomaw,C., Hsu,J. and Cobb,M.H. (1990) An insulin-stimulated protein kinase similar to yeast kinase involved in cell cycle control. *Science*, **249**, 64–67.

Boulton,T.G. *et al.* (1991) ERKs: family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell*, **65**, 663–675.

Brinster,R.L., Allen,J.M., Behringer,R.R., Gelinas,R.E. and Palmiter,R.D. (1988) Introns increase transcriptional efficiency in transgenic mice. *Proc. Natl Acad. Sci. USA*, **85**, 836–840.

Chomczynski,P. and Sacchi,N. (1987) Single-step method of RNA extraction by acid guanidinium thiocyanate-phenol-chloroform. *Anal. Biochem.*, **162**, 156–159.

Cippitelli,M., Sica,A., Viggiano,V., Ye,J., Ghosh,P., Birreer,M.J. and Young,H.A. (1995) Negative transcriptional regulation of the interferon-γ promoter by glucocorticoids and dominant negative mutants of c-Jun. *J. Biol. Chem.*, **270**, 12548–12556.

Crawley,J.B., Rawlinson,L., Lali,F.V., Page,T.H., Saklatvala,J. and Foxwell,B.M. (1997) T cell proliferation in response to interleukins 2 and 7 requires p38MAP kinase activation. *J. Biol. Chem.*, **272**, 15023–15027.

Croft,M., Bradley,L.M. and Swain,S.L. (1994) Naive versus memory CD4 T cell response to antigen. *J. Immunol.*, **152**, 2675–2685.

Cuenda,A., Rouse,J., Doza,Y.N., Meier,R., Cohen,P., Gallagher,T.F., Young,P.R. and Lee,J.C. (1995) SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett.*, **364**, 229–233.

Dérijard,B., Hibi,M., Wu,I.-H., Barret,T., Su,B., Deng,T., Karin,M. and Davis,R.J. (1994) JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell*, **76**, 1025–1037.

Dérijard,B., Raingeaud,J., Barret,T., Wu,I.-H., Han,J., Ulevitch,R.J. and Davis,R.J. (1995) Independent human MAP kinase signal transduction pathways defined by MEK and MKK isoforms. *Science*, **267**, 683–685.

Enslen,H., Raingeaud,J. and Davis,R.J. (1998) Selective activation of p38 MAP kinase isoforms by the MAP kinase kinases MKK3 and MKK6. *J. Biol. Chem.*, **273**, 1741–1748.

Foltz,I.N., Lee,J.C., Young,P.R. and Schrader,J.W. (1997) Hemopoitic growth factors with the exception of interleukin-4 activate the p38 mitogen-activated protein kinase pathway. *J. Biol. Chem.*, **272**, 3296–3301.

Freshney,N.W., Rawlinson,L., Guesdon,F., Jones,E., Cowley,S., Hsuan,J. and Saklatvala,J. (1994) Interleukin-1 activates a novel protein kinase cascade that results in the phosphorylation of the Hsp27. *Cell*, **78**, 1039–1049.

Fukunaga,R. and Hunter,T. (1997) MNK1, a new MAP kinase-activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates. *EMBO J.*, **16**, 1921–1933.

Griswold,D.E., Hillegass,L.M., Neunier,P.C., DiMartino,M.J. and Hanna,N. (1988) Effect of inhibitors of eicosanoid metabolism in murine collagen-induced arthritis. *Arthritis Rheum.*, **31**, 1406–1412.

Han,J., Lee,J.-D., Bibbs,L. and Ulevitch,R.J. (1994) A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science*, **265**, 808–811.

Han,J., Lee,J.-D., Jiang,Y., Li,Z., Feng,L. and Ulevitch,R.J. (1996) Characterization of the structure and function of a novel MAP kinase kinase (MKK6). *J. Biol. Chem.*, **271**, 2886–2891.

Han,J., Jian,Y., Li,Z., Kravchenko,V.V. and Ulevitch,R.J. (1997) Activation of the transcription factor MEF2C by the MAP kinase p38 in inflammation. *Nature*, **386**, 296–299.

Ho,I.-C., Hodge,M.R., Rooney,J.W. and Glimcher,L.H. (1996) The proto-oncogene c-*maf* is responsible for tissue-specific expression of interleukin-4. *Cell*, **85**, 973–983.

Hogan,B., Costantini,F. and Lacy,E. (1986) *Manipulating the Mouse Embryo. A Laboratory Manual*. Cold Spring Harbor Press, Cold Spring Harbor, NY.

Hsieh,C.S., Macatonia,S.E., Tripp,C.S., Wolf,S.F., O'Garra,A. and Murphy,K.M. (1993) Development of Th1 $CD4^+$ T cells through IL-12 produced by *Listeria*-induced macrophages. *Science*, **260**, 547–549.

Huang,S., Jiang,Y., Li,Z., Nishida,E., Mathias,P., Lin,S., Ulevitch,R.J., Nemerow,G.R. and Han,J. (1997) Apoptosis signaling pathway in T cells is composed of ICE/Ced-3 family proteases and MAP kinase kinase 6b. *Immunity*, **6**, 739–749.

Ip,Y.T. and Davis,R.J. (1998) Signal transduction by the c-Jun N-terminal kinase (JNK)—from inflammation to development. *Curr. Opin. Cell. Biol.*, **10**, 205–219.

Jiang,Y., Chen,C., Lie,Z., Guo,W., Gegner,J.A., Lin,S. and Han,J. (1996) Characterization of the structure and function of a new mitogen-activated protein kinase (p38β). *J. Biol. Chem.*, **271**, 17920–17926.

Juo,P., Kuo,C.J., Reynolds,S.E., Konz,R.F., Raingeaud,J., Davis,R.J., Biemann,H.-P. and Blenis,J. (1997) Fas activation of the p38 mitogen-activated protein kinase signaling pathway required ICE/CED-3 family proteases. *Mol. Cell. Biol.*, **17**, 24–35.

Kamogawa,Y., Minasi,L.-A.E., Carding,S., Bottomly,K. and Flavell,R.A. (1993) The relationship of IL-4 and IFNγ-producing T cells studied by lineage ablation of IL-4-producing cells. *Cell*, **75**, 985–995.

Kaplan,M.H., Schindler,U., Smiley,S.T. and Grusby,M.J. (1996) Stat6 is required for mediating responses to IL-4 and for the development of Th2 cells. *Immunity*, **4**, 313–319.

Kawasaki,H., Morooka,T., Shimohama,S., Kimura,J., Hirano,T., Gotoh,Y. and Nishida,E. (1997) Activation and involvement of p38 mitogen-activated protein kinase in glutamate-induced apoptosis in rat cerebellar granule cells. *J. Biol. Chem.*, **272**, 18518–18521.

Kaye,J., Hsu,M.-L., Sauron,M.-E., Jameson,S.C., Gascoigne,N.R.J. and Hedrick,S.M. (1989) Selective development of CD4+ T cells in transgenic mice expressing a class II MHC-restricted antigen receptor. *Nature*, **341**, 746–749.

Kummer,J.L., Rao,P.K. and Heidenreich,K.A. (1997) Apoptosis induced by withdrawal of trophic factors is mediated by p38 mitogen-activated protein kinase. *J. Biol. Chem.*, **272**, 20490–20494.

Kyriakis,J.M. (1994) The stress-activated protein kinase subfamily of c-Jun kinases. *Nature*, **369**, 156–160.

Le Gros,G., Ben-Sasson,S.Z., Seder,R., Finkelman,F.D. and Paul,W.E. (1990) Generation of interleukin 4 (IL-4)-producing cells *in vivo* and *in vitro*: IL-2 and IL-4 are required for *in vitro* generation of IL-4 producing cells. *J. Exp. Med.*, **172**, 921–929.

Lee,J.C. and Young,J.L. (1996) Role of CSBP/p38/RK stress response kinase in LPS and cytokine signaling mechanisms. *J. Leukoc. Biol.*, **59**, 152–157.

Lee,J.C., Badger,A.M., Griswold,D.E., Dunnington,D., Trunech,A., Votta,B., White,J.R., Young,P.R. and Bender,P.E. (1993) Bicyclic imidazoles as a novel class of cytokine biosynthesis inhibitors. *Ann. N. Y. Acad. Sci.*, **696**, 149–170.

Lee,J.C. *et al.* (1994) A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature*, **372**, 739–746.

Lohoff,M., Ferrick,D., Mittrücker,H.-W., Duncan,G.S., Bischof,S., Röllinghoff,M. and Mak,T.W. (1997) Interferon regulatory factor-1 is required for T helper 1 immune response *in vivo*. *Immunity*, **6**, 681–689.

Mclaughlin,M.M., Kumar,S., McDonnell,P.C., Van Horn,S., Lee,J.C., Livi,G.P. and Young,P.R. (1996) Identification of mitogen-activated protein (MAP) kinase-activated protein kinase-3, a novel substrate of CSBP p38 MAP kinase. *J. Biol. Chem.*, **271**, 8488–8492.

Moriguchi, T. *et al.* (1996) A novel kinase cascade mediated by mitogen-activated protein kinase kinase 6 and MKK3. *J. Biol. Chem.*, **271**, 13675–13679.

Paul,W.E. and Seder,R.A. (1994) Lymphocyte response and cytokines. *Cell*, **76**, 241–251.

Penix,L., Weaver,W.M., Pang,Y., Young,H.A. and Wilson,C.B. (1993) Two essential regulatory elements in the human interferon γ promoter confer activation specific expression in T cells. *J. Exp. Med.*, **178**, 1483–1496.

Penix,L.A., Sweetser,M.T., Weaver,W.M., Hoey,T., Hoeffler,J.P., Kerpolla,T.K. and Wilson,C.B. (1996) The proximal regulatory element of the interferon-γ promoter mediates selective expression in T cells. *J. Biol. Chem.*, **271**, 31964–31972.

Pietersman,A., Tilly,B.C., Gaestel,N., Dejong,N., Lee,J.C., Koster,J.F. and Sluiter,W. (1997) p38 mitogen activated protein kinase regulates endothelial VCAM-1 expression at the post-transcriptional level. *Biochem. Biophys. Res. Commun.*, **230**, 44–48.

Prichett,W., Hand,A., Sheilds,J. and Dunnington,D. (1995) Mechanism of action of bicyclic imidazoles defines a translational regulatory pathway for tumor necrosis factor α. *J. Inflamm.*, **45**, 97–105.

Raingeaud,J., Gupta,S., Roger,J., Dickens,M., Han,J., Ulevitch,R.J. and Davis,R.J. (1995) Pro-inflammatory cytokines and environmental stress cause p38 MAP kinase activation by dual phosphorylation on tyrosine and threonine. *J. Biol. Chem.*, **270**, 7420–7426.

Raingeaud,J., Whitmarsh,A.J., Barret,T., Dérijard,B. and Davis,R.J. (1996) MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. *Mol. Cell. Biol.*, **16**, 1247–1255.

Reddy,M.P., Webb,E.F., Cassat,D., Maley,D., Lee,J.C., Griswold,D.E. and Truneh,A. (1994) Pyridinyl imidazoles inhibit the inflammatory phase of delayed type hypersensitive reactions without affecting T-dependent immune responses. *Int. J. Immunopharmacol.*, **16**, 795–804.

Reiner,S.L., Zheng,S., Corry,D.B. and Locksley,R.M. (1993) Constructing polycompetitor cDNAs for quantitative PCR. *J. Immunol. Methods*, **165**, 37–46.

Rincón,M. and Flavell,R.A. (1994) AP-1 transcriptional activity requires both T-cell receptor-mediated and co-stimulatory signals in primary T lymphocytes. *EMBO J.*, **13**, 4370–4381.

Rincón,M. and Flavell,R.A. (1997a) Transcriptional control of Th1/Th2 polarization. *Curr. Biol.*, **7**, R729–R732.

Rincón,M. and Flavell,R.A. (1997b) Transcription mediated by NFAT is highly inducible in effector $CD4^+$ Th2 cells but not in Th1 cells. *Mol. Cell. Biol.*, **17**, 1522–1534.

Rincón,M., Anguita,J., Nakamura,T., Fikrig,E. and Flavell,R.A. (1997a) IL-6 directs the differentiation of IL-4-producing $CD4^+$ T cells. *J. Exp. Med.*, **185**, 461–469.

Rincón,M., Dérijard,B., Chow,C.-W., Davis,R.J. and Flavell,R.A. (1997b) Reprogramming the signaling requirement for AP-1 (activator protein-1) activation during differentiation of precursor $CD4^+$ T cells to effector Th1 and Th2 cells. *Genes Function*, **1**, 51–68.

Rouse,J., Cohen,P., Trigon,S., Morange,M., Alonso-Llamazares,A., Zamanillo,D., Hunt,T. and Nebreda,A.R. (1994) A novel kinase cascade triggered by stress and heat shock that stimulateds MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. *Cell*, **78**, 1027–1037.

Schuh,K. *et al.* (1997) NF-ATp plays a prominent role in the transcriptional induction of Th2-type lymphokines. *Immunol. Lett.*, **57**, 171–175.

Seder,R.A., Gazzinelli,R., Sher,A. and Paul,W.E. (1993) IL-12 acts directly on $CD4^+$ T cells to enhance priming for IFNγ production and diminish IL-4 inhibition of such priming. *Proc. Natl Acad. Sci. USA*, **90**, 10188–10192.

Sen,J., Kapeller,R., Fragoso,R., Sen,R., Zon,L.I. and Burakoff,S.J. (1996) Intrathymic signals in thymocytes are mediated by p38 mitogen-activated protein kinase. *J. Immunol.*, **156**, 4535–4538.

Shimoda,K. *et al.* (1996) Lack of IL4-induced Th2 response and IgE class switching in mice with disrupted *Stat6* gene. *Nature*, **380**, 630–633.

Stein,B., Brady,H., Yang,M.X., Young,D.B. and Barbosa,M.S. (1996) Cloning and characterization of MEK6, a novel member of the mitogen-activated protein kinase kinase cascade. *J. Biol. Chem.*, **271**, 11427–11433.

Su,B., Jacinto,E., Hibi,M., Kallunki,T., Karin,M. and Ben-Neriah,Y. (1994) JNK is involved in signal integration during costimulation of T lymphocytes. *Cell*, **77**, 727–736.

Swain,S.L., Weinberg,A.D., English,M. and Huston,G. (1990) IL-4 directs the development of Th2-like helper effectors. *J. Immunol.*, **145**, 3796–3806.

Swan,K.A., Alberola-Ila,J., Gross,J.A., Appleby,M.W., Forbush,K.A., Thomas,J.F. and Perlmutter,R.M. (1995) Involvement of p21ras distinguishes positive and negative selection in thymocytes. *EMBO J.*, **14**, 276–285.

Takeda,K. *et al.* (1996) Essential role of Stat6 in IL-4 signalling. *Nature*, **380**, 627–630.

Taki,S. *et al.* (1997) Multistage regulation of Th1-type immune responses by the transcription factor IRF-1. *Immunity*, **6**, 673–679.

Thierfelder,W.E. *et al.* (1996) Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. *Nature*, **382**, 171–174.

Tong,L., Pav,S., White,D.M., Rogers,S., Crane,K.M., Cywin,C.L., Brown,M.L. and Pargellis,C.A. (1997) A highly specific inhibitor of human p38 MAP kinase binds in the ATP pocket. *Nature Struct. Biol.*, **4**, 311–306.

Tugores,A., Alonso,M.A., Sánchez-Madrid,F. and de Landázuri,M.O. (1992) Human T cell activation through the activation-inducer molecule/CD69 enhances the activity of transcription factor AP-1. *J. Immunol.*, **148**, 2300–2306.

Wang,X.-Z. and Ron,D. (1996) Stress-induced phosphorylation and activation of the transcription factor CHOP (GADD153) by p38 MAP kinase. *Science*, **272**, 1347–1349.

Waskiewicz,A.J., Flynn,A., Proud,C.G. and Cooper,J.A. (1997) Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2. *EMBO J.*, **16**, 1909–1920.

Whitmarsh,A.J. and Davis,R.J. (1996) Transcription factor AP-1: regulation by mitogen activated protein kinases signal transduction pathways. *J. Mol. Med*, **17**, 2360–2371.

Whitmarsh,A.J., Yang,S.H., Su,M.S., Sharrocks,A.D. and Davis,R.J. (1997) Role of p38 and JNK mitogen-activated protein kinases in the activation of ternary complex factors. *Mol. Cell. Biol.*, **17**, 2360–2371.

Wildin,R.S., Garvin,A.M., Pawar,S., Lewis,D.B., Abraham,K.M., Forbush,K.A., Ziegler,S.F., Allen,J.M. and Perlmutter,R.M. (1991) Developmental regulation of *lck* gene expression in T lymphocytes. *J. Exp. Med.*, **173**, 383–393.

Wilson,K.P. *et al.* (1997) The structural basis for the specificity of pyridinylimidazole inhibitors of p38 MAP kinase. *Chem. Biol.*, **4**, 423–431.

Xia,Z., Dickens,M., Raingeaud,J., Davis,R.J. and Greenberg,M.E. (1995) Opposing effects of ERK and JNK-p38 MAP kinase on apoptosis. *Science*, **270**, 1326–1331.

Young,P.R. *et al.* (1997) Pyridinyl imidazole inhibitors of p38 mitogen-activated protein kinase bind in the ATP site. *J. Biol. Chem.*, **271**, 12116–12121.

Zheng,W.-P. and Flavell,R.A. (1997) The transcription factor GATA-3 is necessary and sufficient for Th2 gene expression in $CD4^+$ T cells. *Cell*, **89**, 587–596.

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