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ORIGINAL ARTICLE

The Role of Mitogen-Activated Protein Kinase-Activated Protein Kinase 2 in the p38/TNF-α Pathway of Systemic and Cutaneous Inflammation

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Mitogen-activated protein kinase-activated protein kinase 2 (MK2) is a downstream molecule of p38, involved in the production of TNF-α, a key cytokine, and an established drug target for many inflammatory diseases. We investigated the role of MK2 in skin inflammation to determine its drug target potential. MK2 deficiency significantly decreased plasma TNF-α levels after systemic endotoxin application. Deficient mice showed decreased skin edema formation in chronic 2-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced irritative dermatitis and in subacute 2,4-dinitrofluorobenzene (DNFB)-induced contact hypersensitivity. Surprisingly, MK2 deficiency did not inhibit edema formation in subacute 2,4-dinitrochlorobenzene (DNCB)-induced contact allergy and even increased TNF-α and IL-1β levels as well as granulocyte infiltration in diseased ears. Ear inflammation in this model, however, was inhibited by TNF-α neutralization as it was in the subacute DNFB model. MK2 deficiency also did not show anti-inflammatory effects in acute DNFB-induced contact hypersensitivity, whereas the p38 inhibitor, SB203580, ameliorated skin inflammation supporting a pathophysiological role of p38. When evaluating possible mechanisms, we found that TNF-α production in MK2-deficient spleen cells was strongly diminished after TLR stimulation but less affected after T-cell receptor stimulation. Our data suggest that MK2, in contrast to its downstream effector molecule, TNF-α, has a rather elusive role in T-cell-dependent cutaneous inflammation.

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INTRODUCTION

Mitogen-activated protein kinase (MAPK)-activated protein kinase 2 (MK2) is one of several kinases that are regulated exclusively through direct phosphorylation by p38 MAP kinase in response to stress stimuli (Gaestel, 2006). The role of the stress-activated p38 MAPK protein kinase cascade in inflammation was defined several years ago by the anti-inflammatory effect of the p38 inhibitor, SB203580, and related compounds (Lee *et al.*, 1994; Jackson *et al.*, 1998). Accordingly, it was expected that several components of this kinase cascade may have been essential for early signaling in the inflammatory response and therefore yielded targets for anti-inflammatory therapy. Targeted disruption of p38 in mice results in embryonic lethality and impaired interleukin (IL)-1 signaling (Allen *et al.*, 2000). Deletion of one of the two known specific upstream activators of p38, the dual-specific MAPK kinase 3 (MKK3), leads to a reduction in IL-12 production (Lu *et al.*, 1999) and impaired tumor necrosis factor (TNF)-alpha-induced cytokine expression (Wysk *et al.*, 1999), and heterozygosity for p38α reduces ear swelling and cell infiltration in acute DNFB-induced contact allergy (Takanami-Ohnishi *et al.*, 2002).

Mice deficient in MK2 showed a reduction in bacterial lipopolysaccharide (LPS)-induced biosynthesis of TNF-α, interferon (IFN)-γ, IL-1β, IL-6, and nitric oxide, suggesting a critical role of MK2 in inflammatory cytokine production and inflammation (Kotlyarov *et al.*, 1999). It was later shown that MK2 regulates biosynthesis of IL-6 at the levels of mRNA stability and of TNF-α mainly through an AU-rich element-dependent translational control (Neininger *et al.*, 2002). The

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Abbreviations: DNCB, 2,4-dinitrochlorobenzene; DNFB, 2,4-dinitrofluorobenzene; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MAPKAP, mitogen-activated protein kinase-activated protein; MK2, mitogen-activated protein kinase-activated protein kinase 2; TCR, T-cell receptor; TLR, toll-like receptor; TNF, tumor necrosis factor; TPA, 12-O-tetradecanoylphorbol-13-acetate

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crucial role for MK2 in regulating TNF-α and IL-6 production was also shown in a mouse arthritis model. MK2-deficient mice had significantly lower LPS-induced TNF-α and IL-6 serum levels when compared with wild-type controls and were resistant to induction of collagen-induced arthritis (Hegen *et al.*, 2006). Moreover, deficiency of MK2 has been shown to markedly reduce infarct size following cerebral ischemic injury in mice. This protective effect was attributed to significantly lower expression of IL-1β, but not TNF-α mRNA (Wang *et al.*, 2002).

A series of pyridinyl imidazole compounds, exemplified by SB203580, have been developed as specific p38 inhibitors (Badger *et al.*, 1996). *In vivo* studies using some of these compounds have suggested that they might be useful in the treatment of several inflammatory conditions including immunologically driven and irritant-associated airway inflammation, rheumatoid arthritis, and psoriasis (Badger *et al.*, 1996; Jackson *et al.*, 1998). However, the preclinical efficacy of SB203580 has been associated with potential adverse events (Rogers and Giembycz, 1998; Dambach, 2005), and targeting p38 directly might thus have liabilities for drug development. Although p38-deficient mice are lethal, MK2 knockout mice are viable and show a normal phenotype (Allen *et al.*, 2000).

Taken together, targeting MK2 as a downstream kinase in the p38 pathway might have advantages over targeting p38 directly. Moreover, in contrast for example to TNF-α, MK2 as a kinase is considered as a molecular target druggable by small molecules (Gaestel *et al.*, 2007), an attractive characteristic that is also reflected by the fact that a number of pharmaceutical companies are pursuing drug discovery and development programs with MK2 (Anderson *et al.*, 2007; Gaestel *et al.*, 2007).

The aim of this study was (i) to determine the biological role of MK2 in skin inflammation models mainly by comprehensively comparing wild-type *versus* knockout mice responses in several models of cutaneous and systemic inflammation and (ii) to determine whether MK2 represents a promising drug target for the treatment of inflammatory skin diseases.

RESULTS

MK2 is involved in acute, systemic inflammation after endotoxin challenge in mice

As TNF-α is a key mediator of inflammation and a well-established drug target in skin inflammation (Schottelius *et al.*, 2004), we wanted to test the effect of MK2 deficiency on plasma levels of TNF-α in the model of LPS-induced systemic inflammation. As expected, homozygous MK2 deficiency resulted in significantly reduced TNF-α serum levels following a systemic LPS challenge in comparison with wild-type mice ($P<0.05$) (Figure 1). Partial deficiency of MK2 (MK2 heterozygous mice) also strongly reduced TNF-α serum levels, but the reduction did not reach statistical significance. These data confirm a report published earlier (Kotlyarov *et al.*, 1999) and show that the MK2-deficient mice we used for our studies displayed a similar phenotype as described.

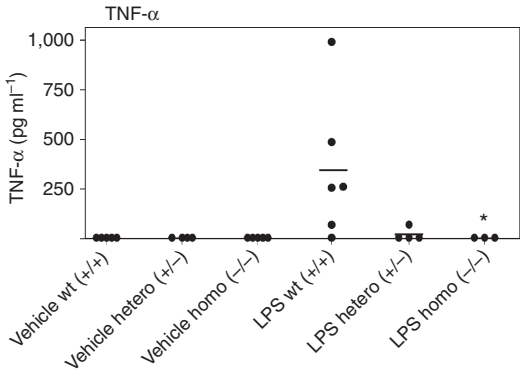

Figure 1. MK2 deficiency results in suppression of TNF-α production after systemic LPS exposure. Wild-type, MK2-heterozygous, and MK2-deficient mice were left untreated (NaCl control) or intraperitoneally challenged with LPS from *E. coli* (5 mg kg^{-1}). TNF-α serum levels as determined by Luminex measurement were strongly and significantly elevated at 1.5 hours after LPS challenge in wild-type mice ($n=6$), but were completely suppressed in mice heterozygous for MK2 ($n=4$) and in MK2-deficient mice ($n=3$; *$P<0.05$, Mann–Whitney *U*-test).

To investigate the pathophysiological role of MK2 in skin disease, we tested MK2-deficient and wild-type mice in models of skin inflammation, which display characteristics of chronic, subacute, and acute skin inflammation.

Impact of MK2 deficiency on the chronic TPA-induced skin inflammation model in mice

MK2 knockout mice and wild-type controls were subjected to chronic 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced irritative skin inflammation. Inflammatory edema formation after repeated TPA exposure was significantly decreased in MK2-deficient mice on days 5 ($P<0.001$) and 10 ($P<0.05$) (Figure 2a). Moreover, a significant reduction of neutrophil infiltration, as assessed by neutrophil elastase activity in skin homogenates, could be observed in MK2 knockout *versus* wild-type mice ($P<0.05$) (Figure 2c). Overall granulocyte infiltration, as assessed by peroxidase activity, however, was not different in MK2 knockout mice *versus* wild-type controls (Figure 2b).

Impact of MK2 deficiency on subacute DNFB- and DNCB-induced contact allergy models in mice

On repeated challenges with contact allergy-inducing haptens, DNFB (dinitrofluorobenzene)- or DNCB (dinitrochlorobenzene)-sensitized mice develop a T-cell-dependent skin inflammation of a pronounced Th1 phenotype. Owing to the subacute regimen with repeated challenges, these models may be particularly relevant for inflammatory skin diseases, such as psoriasis (Zollner *et al.*, 2004) in which TNF-α protein expression in lesional skin is suggested to be post-transcriptionally regulated by activated MK2 (Johansen *et al.*, 2006).

First, we tested MK2 knockout mice in a subacute model of DNFB-induced contact allergy. A significant reduction of ear edema formation was observed for MK2-deficient mice compared with the respective wild-type controls with the strongest reduction seen on day 8 (day 7 $P<0.05$; day 8

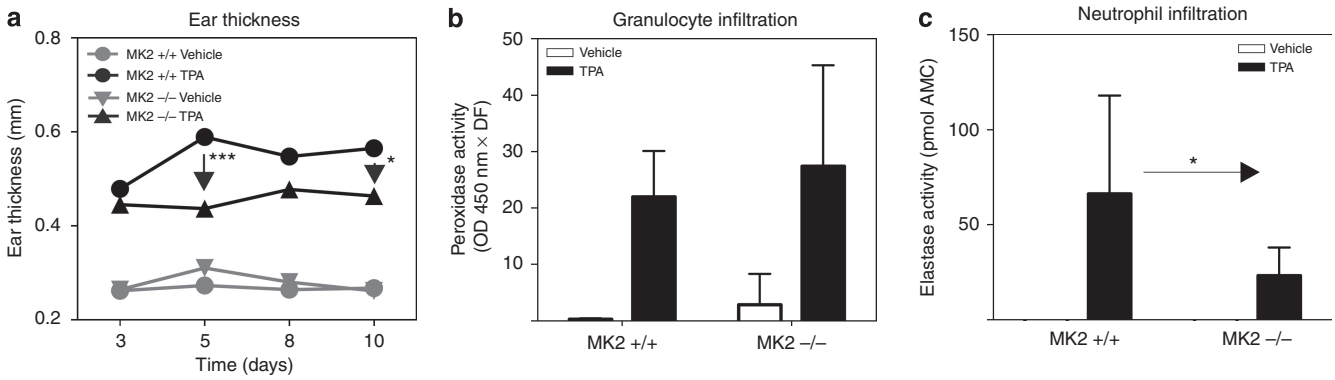

Figure 2. Skin inflammation is reduced in MK2-deficient mice in the chronic TPA-induced irritative dermatitis model. MK2-deficient and wild-type mice ($n = 10$ per group) were subjected to TPA-induced skin inflammation. Mice ears were treated with TPA on days 1, 3, 5, 8, and 10 and killed on day 10 at 6 hours after the last TPA application. (**a**) The kinetics of ear thickness was assessed with a caliper. Skin inflammation was significantly decreased on days 5 and 10 in MK2-deficient mice compared with wild-type mice (***$P<0.001$ day 5, *$P<0.05$ day 10, Mann–Whitney *U*-test) after sensitization with TPA, whereas skin inflammation was not significantly decreased on days 3 and 8 in MK2-deficient mice. Moreover, although there was a significant reduction of neutrophil infiltration as assessed by elastase activity in ear homogenates on day 10 (*$P<0.05$) (**c**), peroxidase activity as a parameter for overall granulocyte infiltration was not significantly different in MK2 knockout mice compared with wild-type controls. (**b**) DF, dilution factor.

$P<0.001$) (Figure 3a). Interestingly, as in TPA-induced chronic skin inflammation, the reduction of edema formation was not accompanied by a consistent reduction in cutaneous granulocyte infiltration. Peroxidase activity in skin homogenates, as a parameter for overall granulocyte infiltration, was even significantly increased ($P<0.05$, Figure 3b), whereas neutrophil elastase activity, as a parameter for neutrophil infiltration, was significantly decreased ($P<0.05$, Figure 3c) in MK2-deficient animals compared with wild-type controls.

To elucidate whether inflammation in the subacute DNFB model is driven by TNF-α, we tested TNF-α expression at the mRNA and protein levels and the effect of neutralization with an anti-TNF-α antibody. The TNF-α mRNA levels were significantly upregulated following repeated challenges with DNFB in this model peaking at day 8 ($P<0.05$ day 8 *vs* day 5) (Figure 3d, left panel). IL-1β mRNA levels also increased steadily up to day 8, but the increase was not statistically significant (Figure 3d, right panel). Neutralization of TNF-α with two different anti-TNF-α antibodies significantly reduced ear edema in this model ($P<0.05$ for hamster anti-mouse TNF-α *versus* hamster control on day 8; $P<0.05$ for rabbit anti-mouse TNF-α *versus* rabbit control on days 7 and 8) (Figure 3e) and also strongly and significantly suppressed protein levels of TNF-α and IL-1β ($P<0.01$ *versus* control for both) (Figure 3f). These data suggest that the pathophysiology of the subacute DNFB contact allergy model is mainly driven by the inflammatory effects of TNF-α and that MK2-deficiency leads to a significant reduction in ear inflammation, potentially through TNF-α suppression in this model.

In contrast, the formation of ear edema 8 or 9 days post challenge was not reduced when MK2 knockout mice were tested in the subacute DNCB-induced contact allergy model (Figure 4a). Surprisingly, in this model, the infiltration of inflammatory cells was even strongly and significantly increased in MK2-deficient mice compared with wild-type controls ($P<0.001$ for cutaneous peroxidase and elastase activity) (Figure 4b and c). To further investigate whether the increase of cellular infiltration in MK2-deficient mice in DNCB-induced contact allergy was accompanied by changes in cutaneous cytokine production, we analyzed ear homogenates of DNCB-challenged mice for the presence of inflammatory cytokines. TNF-α and IL-1β levels were increased at 24 hours after the last DNCB challenge in both MK2 wild-type and deficient mice compared with unchallenged mice (Figure 4d). However, MK2 knockout mice produced significantly higher levels of TNF-α and IL-1β after repeated challenges with DNCB when compared with wild-type controls ($P<0.05$ for TNF-α, $P<0.001$ for IL1β). Increased infiltration of inflammatory cells in MK2 knockout mice in DNCB-induced contact allergy was thus accompanied by a higher production of TNF-α and IL-1β.

As MK2 deficiency caused different effects on skin inflammation in DNFB- *versus* DNCB-induced contact allergy, we further wanted to elucidate whether TNF-α also has a pathophysiological role in the subacute DNCB-induced contact allergy model, that is, whether TNF-α levels are incrementally increased and whether neutralization of TNF-α would reduce inflammation equally in this model. In line with the results from the subacute DNFB model, we observed increasing TNF-α and IL-1β mRNA expression in the subacute DNCB model ($P<0.05$ for TNF-α for days 7 and 9 *vs* day 6 and for IL-1β for day 9 *vs* day 6) (Figure 4e). Most importantly, neutralization of TNF-α with an anti-TNF-α antibody reduced ear thickness significantly and with comparable efficacy as in subacute DNFB-induced contact allergy (Figure 3e) in the subacute DNCB-induced contact allergy ($P<0.05$ for days 7, 8, and 9) (Figure 4f). Also, in the DNCB-induced contact allergy model, neutralization of TNF-α strongly and significantly suppressed protein levels of TNF-α and IL-1β ($P<0.01$ for TNF-α and $P<0.05$ for IL-1β) (Figure 4g).

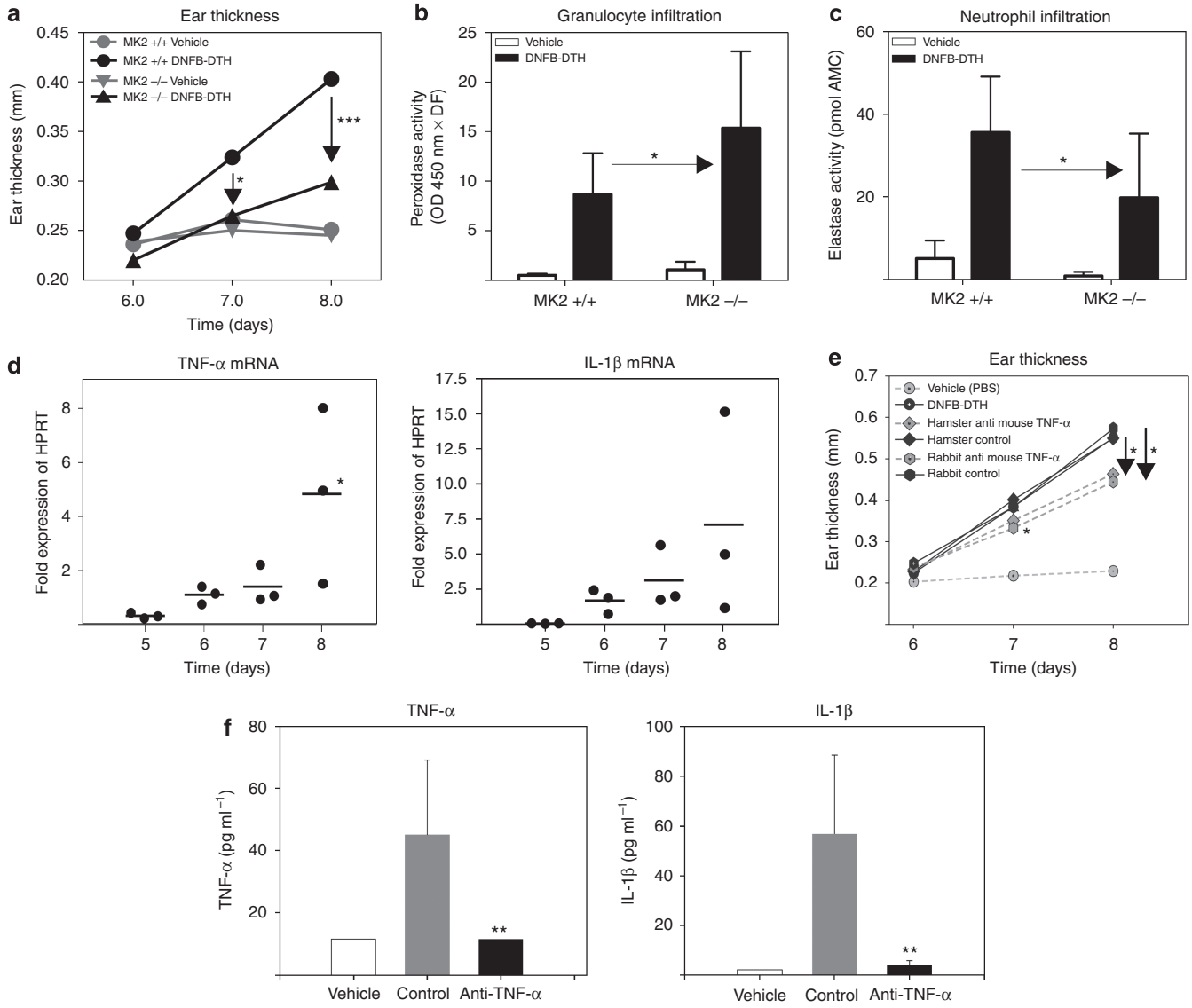

Figure 3. MK2-deficient mice show decreased skin inflammation in the subacute model of DNFB-induced contact allergy. MK2-deficient and wild-type mice ($n=10$ per group) were challenged on the ears with DNFB on days 5, 6, and 7 after sensitization. (**a**) Ear thickness was measured with a caliper on days 6, 7, and 8 after DNFB sensitization. MK2-deficient mice developed significantly less ear edema when compared with wild-type controls (*$P<0.05$ day 7; ***$P<0.001$ day 8, Mann–Whitney *U*-test). (**b**, **c**) Ears were homogenized on day 8 after sensitization and assayed for peroxidase activity as a parameter for granulocyte infiltration (**b**) and elastase activity for neutrophil infiltration (**c**). Both parameters were significantly increased after challenge with DNFB. Although granulocyte infiltration was significantly increased (*$P<0.05$), neutrophil infiltration was significantly decreased in MK2-deficient *versus* wild-type mice (*$P<0.05$, Mann–Whitney *U*-test). The subacute model of DNFB-induced contact allergy is mainly driven by TNF-α. (**d**) Wild-type mice were challenged with DNFB on days 5, 6, and 7 after DNFB sensitization. mRNA was extracted from ear homogenates of mice killed on days 5, 6, 7, and 8 after sensitization ($n=3$ for each time point). TNF-α and IL-1β mRNA levels steadily increased up to day 8 (*$P<0.05$ day 8 *vs* day 5 for TNF-α, Mann–Whitney *U*-test). (**e**, **f**) Treatment with either hamster or rabbit anti-mouse TNF-α antibodies before challenges (day 5) significantly reduced ear inflammation in subacute DNFB-induced contact allergy in C57Bl/6 wild-type mice ($n=7$ for anti-TNF-α antibodies; $n=10$ for control IgGs). (**e**) TNF neutralization significantly diminished ear thickness on day 7 (*$P<0.05$ for rabbit anti-mouse TNF-α *versus* rabbit control, Mann–Whitney *U*-test) and on day 8 after sensitization (*$P<0.05$ for hamster anti-mouse TNF-α *versus* hamster control and for rabbit anti-mouse TNF-α *versus* rabbit control, Mann–Whitney *U*-test). (**f**) Neutralization of TNF-α by hamster anti-mouse TNF-α significantly reduced protein levels of TNF-α and IL-1β (**$P<0.01$ *versus* hamster control IgG, Mann–Whitney *U*-test) in ear homogenates on day 8 after DNFB sensitization. DF, dilution factor.

Taken together, our data establish the central role of TNF-α in the pathogenesis of both the subacute DNFB- and DNCB-induced contact allergy models and further show that the differential functional effects of MK2-deficiency in these models cannot be explained by the lack of a role for TNF-α in inducing inflammation in one model *versus* the other.

Differing effect of MK2 deficiency and p38 inhibition on the acute DNFB-induced contact allergy model in mice

As MK2 knockout mice showed the most prominent and significantly reduced ear edema formation in the subacute DNFB contact allergy model, we wanted to investigate whether this anti-inflammatory effect of MK2-deficiency

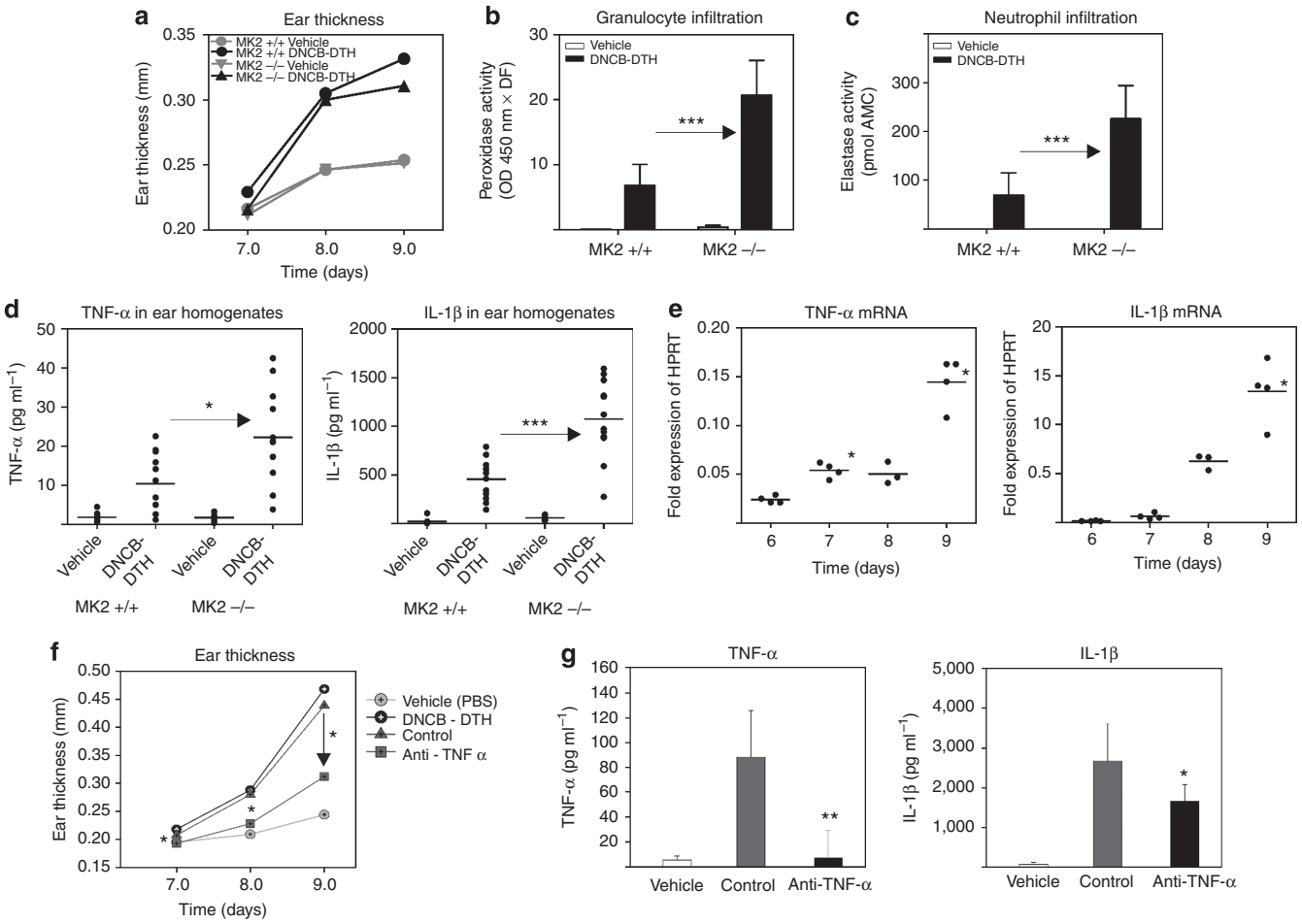

Figure 4. Skin inflammation is not reduced in MK2-deficient mice in the subacute DNCB-induced contact allergy model. MK2-deficient and wild-type mice ($n=10$ per group) were challenged with DNCB on days 6, 7, and 8 after sensitization. (**a**) Ear thickness was measured with a caliper on days 7, 8, and 9 after DNCB sensitization. No significant differences in ear thickness were detected between MK2-deficient and wild-type mice. (**b**, **c**) Ears were homogenized on day 9 after sensitization and assayed for peroxidase activity as a parameter for granulocyte infiltration (**b**) and elastase activity for neutrophil infiltration (**c**). Both parameters were significantly strongly increased in MK2-deficient compared with wild-type mice (***$P<0.001$ for granulocyte and neutrophil infiltration, Mann–Whitney *U*-test). (**d**) TNF-α and IL-1β protein levels in ear homogenates were determined on day 9 after sensitization ($n=6$–12 for each group). Both TNF-α and IL-1β were significantly increased in MK2-deficient compared with wild-type mice (*$P<0.05$ for TNF-α, ***$P<0.001$ for IL1β). The subacute model of DNCB-induced contact allergy is driven by TNF-α. (**e**) Wild-type mice were challenged with DNCB on days 6, 7, and 8 after DNCB sensitization. mRNA was extracted from ear homogenates of mice killed on days 6, 7, 8, and 9 after sensitization ($n=4$ for each time point). TNF-α and IL-1β mRNA levels steadily increased up to day 9 (*$P<0.05$ days 7 and 9 *vs* day 6 for TNF-α and day 9 *vs* day 6 for IL-1β, Mann–Whitney *U*-test). (**f**, **g**) C57Bl/6 wild-type mice ($n=10$ for rabbit anti-TNF-α and $n=8$ for rabbit IgG controls) were challenged with DNCB on days 6, 7, and 8 after sensitization. Treatment with rabbit anti-mouse TNF-α antibodies was performed before the challenges (day 6). (**f**) TNF-α neutralization significantly reduced ear inflammation as assessed by ear thickness (*$P<0.05$ *versus* rabbit IgG control on days 7, 8, and 9, Mann–Whitney *U*-test). (**g**) TNF-α neutralization significantly reduced protein levels of TNF-α and IL-1β (**$P<0.01$ and *$P<0.05$ *versus* rabbit IgG control, respectively, Mann–Whitney *U*-test) in ear homogenates on day 9 after sensitization. DF, dilution factor.

could also be observed in the acute model of DNFB-induced contact allergy using a single challenge of the same hapten in sensitized mice. In contrast to the effects seen in the subacute DNFB-induced contact allergy model after 3 DNFB challenges, no significant anti-inflammatory effect was seen in MK2 knockout mice in the acute DNFB contact allergy model compared with controls (Figure 5a–c). Comparable induction of ear edema formation (Figure 5a) and granulocyte (Figure 5b) as well as neutrophil infiltration (Figure 5c) was seen in MK2 knockout and wild-type mice in this model.

To further explore whether the p38 axis has any role in the pathophysiology of this acute contact allergy model, we tested the effects of the potent p38 inhibitor, SB203580, in MK2 wild-type mice compared with the potent glucocorticoid, dexamethasone, as a positive control. The pharmacological inhibition of p38 strongly and dose-dependently decreased skin inflammation and blocked ear edema formation and cell infiltration as potently as dexamethasone ($P<0.01$ for 0.3 and 1 mg kg^{-1} dexamethasone and for 3 and 10 mg kg SB203580; inhibition of granulocyte infiltration: $P<0.01$ for 1 mg kg^{-1} dexamethasone and for 3 and 10 mg kg^{-1} SB203580; inhibition of neutrophil infiltration: $P<0.05$ for 1 mg kg^{-1} dexamethasone and $P<0.01$ for 3 and 10 mg kg^{-1} SB 203580) (Figure 6a–c), showing that the p38

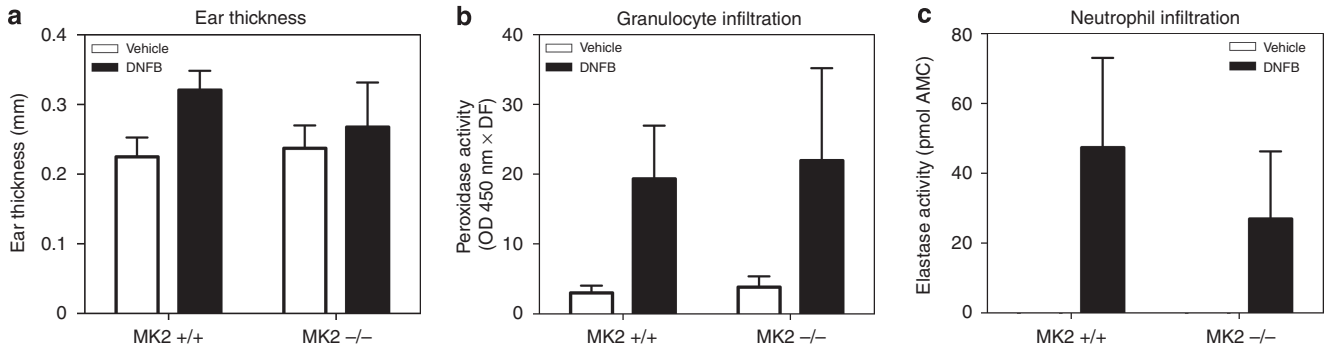

Figure 5. Skin inflammation is not reduced in MK2-deficient mice in the acute model of DNFB-induced contact allergy. MK2-deficient mice (vehicle $n=6$, DNFB $n=6$) and wild-type mice (vehicle $n=10$, DNFB $n=11$) were challenged on the ears with DNFB on day 5 after sensitization and killed on day 6. (**a**) As assessed by the ear thickness measurements with a caliper, MK2-deficient and wild-type mice developed similar degrees of ear edema. Moreover, cell infiltration was not different between MK2-deficient and wild-type mice as determined by peroxidase (**b**) and elastase (**c**) activities in ear homogenates as parameters for granulocyte and neutrophil infiltration, respectively. DF, dilution factor.

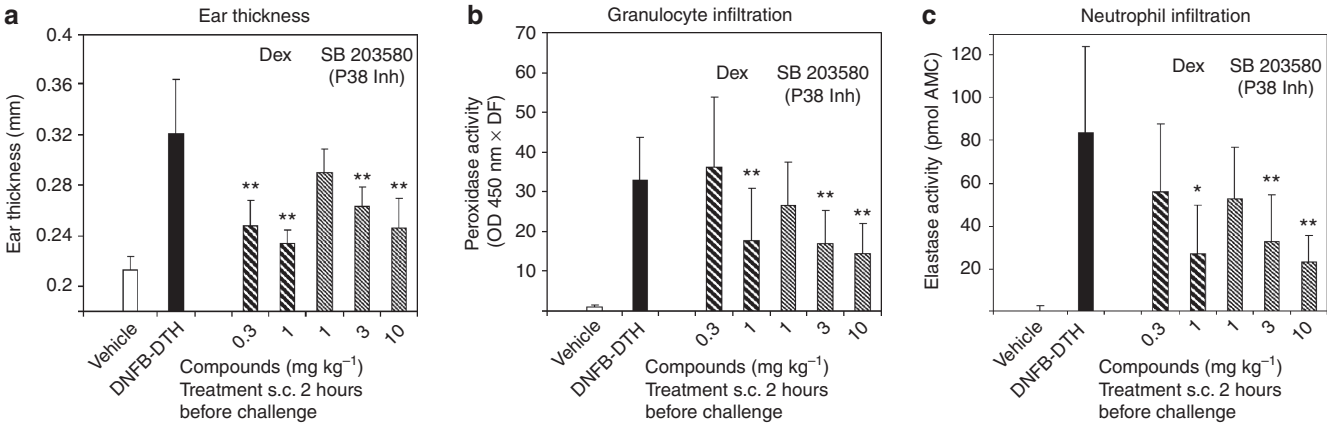

Figure 6. Pharmacological inhibition of p38 strongly and dose-dependently inhibits skin inflammation in the acute DNFB-induced contact allergy model in wild-type mice. DNFB-sensitized female NMRI mice ($n=10$ per group) were s.c. treated with different dosages of the p38 inhibitor, SB 203580, or vehicle at 2 hours before DNFB challenge on both ears at day 5 after sensitization and killed on day 6. Treatment with the glucocorticoid dexamethasone (Dex) served as positive control. Both compounds, SB 203580 and dexamethasone, significantly and dose-dependently inhibited (**a**) ear edema formation as determined by caliper measurement on day 6 after DNFB sensitization (**$P<0.01$ for 0.3 & 1 mg kg^{-1} dexamethasone and for 3 and 10 mg kg^{-1} SB203580, Mann–Whitney *U*-test) as well as (**b**) granulocyte infiltration (peroxidase activity) (**$P<0.01$ for 1 mg kg^{-1} dexamethasone and for 3 and 10 mg kg^{-1} SB203580, Mann–Whitney *U*-test) and (**c**) neutrophil infiltration (elastase activity in ear homogenates) (*$P<0.05$ for 1 mg kg^{-1} dexamethasone and **$P<0.01$ for 3 and 10 mg kg^{-1} SB 203580, Mann–Whitney *U*-test). DF, dilution factor.

axis needs to be intact for inflammation to develop in acute DNFB-induced contact allergy.

Collectively, these data show that specific MK2 deficiency does not exert a significant anti-inflammatory effect in this model of DNFB-induced acute skin inflammation, whereas the upstream regulatory molecule p38 is clearly involved, showing the relevance of the p38 pathway in this acute contact allergy model.

The TNF-α regulatory role of MK2 is consistent for TLR stimulation but may be restricted in T-cell stimulation

For a better mechanistic understanding of the observed differential *in vivo* effects, we further wanted to elucidate the role of MK2 signaling for the production of TNF-α in the most prominent pathways in relevant immune cells *ex vivo*/*in vitro*. Stimulation of different toll-like receptors (TLRs) in splenocytes with their respective ligands led to a strong induction of TNF-α, which was consistently and significantly reduced in the absence of MK2 ($P<0.01$ for LPS, $P<0.05$ for zymosan and imiquimod) (Figure 7a). The LPS *in vitro* data are consistent with our results observed in the model of LPS-induced systemic inflammation (Figure 1). Moreover, *in vitro* TNF-α production was comparably inhibited after the pharmacological blockade of p38 in wild-type splenocytes as in MK2-deficient splenocytes.

Remarkably, a quite similar inhibition of TNF-α production was seen in splenocytes stimulated with LPS alone or in combination with IFN-γ or anti-CD40 co-stimulation ($P<0.05$ for all stimuli) (Figure 7b, left panel) showing the robustness of the effects. However, in isolated splenic CD4$^+$

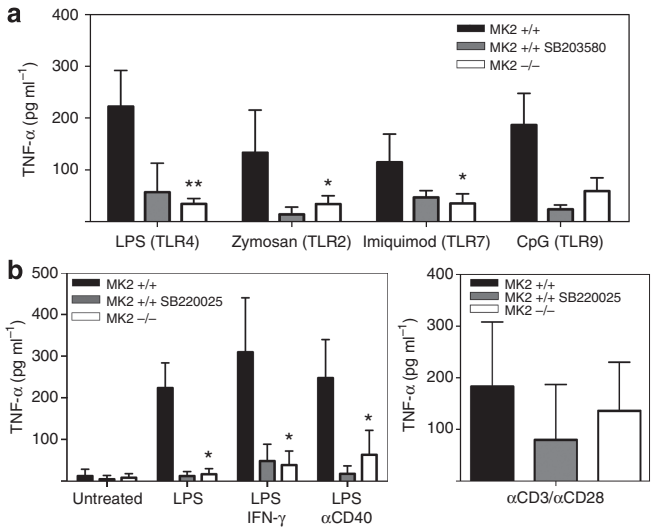

Figure 7. (a) TNF-α secretion after stimulation with different TLR stimuli is MK2-dependent. Spleen cells (5×10^6 ml^{-1}) from C57Bl/6 mice (wt; dark gray bars) or MK2-deficient mice (white bars) were stimulated for 4 hours with several TLR stimuli (LPS at 5 μg ml^{-1}, zymosan at 10 μg ml^{-1}, imiquimod at 1 μg ml^{-1}, or CpG ODNs at 10 μg ml^{-1}) as indicated. As a control, wild-type spleen cells were also stimulated in the presence of the p38 MAP kinase inhibitor, SB203580, at 10 μM (light gray bars). TNF-α content in supernatants was determined by ELISA. Absolute values are depicted from two (CpG) or 3–6 (all others) mice as means with error bars indicating SD. Significant differences between cell cultures from wild-type and MK2-deficient mice were determined by the Mann–Whitney *U*-test (*$P<0.05$, **$P<0.01$). (**b**) MK2 deficiency significantly diminishes TNF-α secretion after LPS stimulation with and without co-stimuli, but not after TCR stimulation. Splenocytes from C57Bl/6 (dark gray bars) and MK2-deficient mice (white bars) were left untreated or activated with LPS alone or in combination with co-stimulation (IFN-γ or anti-CD40) for 4 hours (left panel). As a control, wild-type spleen cells were also stimulated in the presence of the p38 MAP kinase inhibitor, SB220025, at 10 μM (light gray bars). TNF-α levels in supernatants were analyzed by ELISA, and the results are shown as the means from four mice (± SD). CD4$^+$ T cells separated from the spleens of either C57Bl/6- (dark gray bars) or MK2-deficient mice (white bars) were activated with plate-bound anti-CD3 and anti-CD28 antibodies for 72 hours (right panel). As a control, wild-type CD4 + T cells were also stimulated in the presence of the p38 MAP kinase inhibitor, SB220025, at 10 μM (light gray bars). TNF-α levels in supernatants were analyzed by ELISA. Results are shown as the means from four mice (± SD). Significant differences between cell cultures from wild-type and MK2-deficient mice were determined by Mann–Whitney *U*-test (*$P<0.05$).

T cells stimulated with anti-CD3/anti-CD28 antibodies, MK2 deficiency did not significantly alter the production of TNF-α (Figure 7b, right panel).

In summary, these *in vitro* data show that the p38/MK2 axis has a pivotal role in TLR-stimulated production of TNF-α in splenocytes, whereas anti-CD3/anti-CD28-driven TNF-α production in splenic T cells appears to be less dependent on MK2 signaling.

DISCUSSION

The p38 MAP kinase pathway has been shown to be a central regulator of inflammation (Lee *et al.*, 1994). MK2 is exclusively regulated by p38 and controls key inflammatory cytokines such as TNF-α and IL-6 (Kotlyarov *et al.*, 1999; Neininger *et al.*, 2002) as well as chemokines and adhesion molecules (Gorska *et al.*, 2007). Both kinases have thus been identified as potentially promising drug targets and have become the focus of several drug discovery programs (Gaestel, 2006; Duraisamy *et al.*, 2008). Targeting MK2 as a downstream kinase in the p38 pathway might have advantages over targeting p38 directly, because pharmacological inhibition of p38 was implicated with potential adverse events (Dambach, 2005). The relevance of MK2 as a drug target was elucidated for certain inflammatory diseases such as rheumatoid arthritis in relevant animal models (Hegen *et al.*, 2006); however, its target relevance in inflammatory skin disease has remained elusive. Although a recent report shows a role of MK2 in the oxazolone-induced contact allergy model (Funding *et al.* 2009), here, we provide a comprehensive characterization of the pathophysiological role of MK2 in a broad panel of murine models of skin inflammation, contributing to its evaluation as a drug target in inflammatory skin disease.

Here, we show that MK2 deficiency exerts anti-inflammatory effects in chronic TPA-induced irritative skin inflammation and in the subacute model of DNFB-induced contact allergy leading to a significant reduction in the development of ear edema (Figures 2a and 3a). However, the reduction in edema formation was accompanied by an only partial reduction of inflammatory cell infiltration parameters in these models. Although neutrophil infiltration, assessed by elastase activity, was significantly reduced in MK2-deficient mice (Figures 2c and 3c), general granulocyte infiltration, as assessed by cutaneous peroxidase activity, was in tendency or significantly enhanced (Figures 2b and 3b, respectively). Moreover, we did not observe any inhibitory effect of MK2 deficiency on inflammation in the subacute DNCB contact allergy model. Here, a lack of significant inhibition of ear edema formation was accompanied by even significantly increased cell infiltration parameters (Figure 4a–c). It is noted that this increase in cutaneous cell infiltration in MK2 knockout mice was also accompanied by a significant increase in TNF-α and IL-1β production in the inflammatory skin lesions of MK2-deficient mice compared with wild-type controls (Figure 4d). This result was surprising, because experiments with MK2 knockout mice used in our studies were able to confirm that LPS-induced plasma levels of TNF-α were drastically reduced as reported earlier (Figure 1) (Kotlyarov *et al.*, 1999).

Remarkably, the differential effects of MK2 deficiency in the subacute DNFB- *versus* the subacute DNCB-induced contact allergy model cannot be explained by a different role for TNF-α in the pathogenesis of these models, because in both models cutaneous TNF-α expression significantly increased with inflammation (Figures 3d and 4e) and neutralization of TNF-α exerted a potent and comparable anti-inflammatory effect (Figures 3e and 4f). Although both DNFB and DNCB are considered Th1-inducing haptens, a variety of studies have shown a number of differences in the cellular and molecular mechanisms elicited by these compounds, all of which may have contributed to the

differential effects observed in contact allergy in mice in the absence of MK2, namely the different effects on CD86 and HLA-DR expression in dendritic cells (Manome *et al.*, 1999) indicating a more potent role of DNCB over DNFB in dendritic cells and the distinct ability of DNFB to induce IL-10-producing mast cells and NK cell-mediated memory responses (O'Leary *et al.*, 2006; Grimbaldeston *et al.*, 2007) where the p38/MK2 pathway may have a differential role *versus* contact allergy elicited by DNCB.

When tested in the acute model of DNFB-induced contact allergy, MK2 knockout mice did not develop significantly less skin inflammation than their wild-type controls (Figure 5a–c). In contrast, the pharmacological inhibition of p38 potently and dose-dependently abrogated both edema formation and infiltration of inflammatory cells in acute DNFB-induced contact allergy (Figure 6a–c). These results confirm the reported role of p38 MAP kinase in the pathophysiology of acute DNFB-induced contact hypersensitivity (Takanami-Ohnishi *et al.*, 2002). The lack of a significant anti-inflammatory effect by MK2 deficiency in acute DNFB-induced contact allergy thus cannot be attributed to a lesser pathophysiological role of the p38 pathway in this skin inflammation model. The p38 kinase has also been shown to be activated in lesional psoriatic skin (Johansen *et al.*, 2005), supporting its relevance in human inflammatory skin disease.

One possible explanation for the inconsistent impact of MK2 deficiency in T-cell-dependent contact allergy models might be provided by the results of our mechanistic studies in mouse splenocytes. We show that, as *in vivo*, MK2 has a pivotal role in TLR-triggered TNF-α production in splenocytes. In contrast, however, the CD3/CD28-mediated TNF-α production in splenic T cells appears to be less dependent on MK2 signaling.

The fact that TLR-dependent, but not TCR-dependent, TNF production is significantly decreased in MK2-deficient and SB22025-treated splenocytes is in agreement with the established role of the p38/MK2 module in signal transduction located directly downstream to the canonical TLR4/IRAK4/TAK1/MKK3/6 pathway (summarized in Gaestel, Kotlyarov, and Kracht, 2009). In contrast, stimulation of TNF-α production in response to TCR stimulation could proceed in a non-canonical manner through non-receptor protein tyrosine kinases, such as Lck (Salvador *et al.*, 2005), Itk, or Syk, in a more p38/MK2-independent manner. This non-canonical stimulation of TNF-α generation downstream of TCR stimulation could in turn explain that the pharmacological blockade of p38 by the compound SB220025 and MK2 deficiency shows much lesser effects on TNF production in splenocytes on anti-CD3/anti-CD28 stimulation.

One could further hypothesize that the autoimmune suppressor Gadd45a may have some function in the differentiated role of TCR-mediated TNF production in splenocytes. Gadd45a can inhibit p38 Tyr323 phosphorylation, which is specific for the non-canonical or alternative pathway of p38 activation in T cells (Salvador *et al.*, 2005).

Taken together, our results confirm the crucial function of MK2 in LPS-induced TNF-α production in systemic inflammation and further support the observation that this axis has distinct roles for different inflammatory pathways in different cell types and target organs.

Here, we show that MK2 deficiency exerts limited anti-inflammatory effects or can even enhance inflammation in specific, more chronic mouse models of skin inflammation in which TNF-α has a central role in the development of disease, and, in contrast to p38 inhibition, did not exert inhibitory effects in an acute T-cell-dependent DNFB-induced contact allergy model. These results may indicate that MK2 is bypassed in skin inflammation or that MK2 function is compensated for by a different kinase. One such kinase candidate may be MK3, another MAPKAP kinase downstream of p38, which has been described to be also involved in TNF-α production and which might compensate for the function of MK2 (Hegen *et al.*, 2006; Ronkina *et al.*, 2007).

Our results are in partial contrast to other reports that concluded a general potent anti-inflammatory effect of MK2 inhibition, based on MK2 deficiency in other cytokine-driven diseases such as LPS-induced systemic inflammation or mouse arthritis models (Kotlyarov *et al.*, 1999; Hegen *et al.*, 2006) and also on a recent report showing that MK2 deficiency diminishes inflammation in an oxazolone-induced acute allergic contact dermatitis model (Funding *et al.* 2009). Interestingly, MK2 deficiency has been shown to exacerbate TNF-dependent inflammatory bowel disease in the mouse (Kontoyiannis *et al.*, 2002). The observed differences may be based on the distinct role for the p38/MK2/TNF-α axis in the pathogenesis of these different inflammation models in different target organs.

From a methodological point of view, our data show that drawing general conclusions from a single skin inflammation model alone may not be reliable. The data we obtained in the subacute DNFB-induced contact allergy (and also the chronic TPA-induced irritative skin inflammation model), suggesting an impact of MK2 in skin inflammation, were neither confirmed in a different subacute contact allergy model nor in the acute DNFB model, when the hapten was applied in a more acute setting. Therefore, both the kinetics of the model (acute *versus* subacute) and the resulting dominating stimulatory pathways (for example, TCR stimulation *versus* cytokine stimulation) as well as the choice of hapten (DNFB *versus* DCNB) appear to have an impact on the biological role of MK2 in skin inflammation and its downstream effects on TNF-α. Investigating the effects of compounds or genetic alterations in several different contact allergy models comprehensively may thus be required before general conclusions can be drawn from a data set.

In conclusion, our studies confirm the role of MK2 in an acute TLR-driven systemic inflammation, whereas MK2 seems to have at least a less prominent and more complex role in skin inflammation. However, the entire role of MK2 in chronic relapsing T-cell-dependent skin diseases in man is still not fully understood and awaits further analysis. As expression analysis of MK2 in human lesional skin from patients with chronic plaque-type psoriasis revealed an activation of MK2 protein in inflamed psoriatic skin (Johansen

et al., 2006), the role of MK2, for example, in psoriasis, is still subject to further investigations.

As neutralization or inhibition of TNF-α has been validated as a potent therapy for rheumatoid arthritis, inflammatory bowel disease, and psoriasis in the clinic (Schottelius *et al.*, 2004), disruption or dampening of TNF-α production would be expected to yield good therapeutic effects. It would thus seem conceivable that MK2 is an attractive target for a pharmacological inhibition in treating a variety of inflammatory diseases. With the data available to date, including our findings reported here, this approach might yield more therapeutic promise in rheumatoid arthritis than in inflammatory skin diseases such as psoriasis or atopic dermatitis. Future studies with MK2-deficient mice in other animal models of disease may be able to further elucidate the role of MK2 for other inflammatory conditions such as Crohn's disease or ulcerative colitis.

MATERIALS AND METHODS

Mice

C57Bl/6 mice (purchased from Charles River, Sulzfeld, Germany), NMRI female mice (from ''Schoenwalde NMRI colonies'' exclusively bred for Bayer Schering Pharma AG at Charles River, Germany), and MK2 knockout mice (provided from Hannover Medical School, Hannover, Germany) in the age group of 8–12 weeks were housed in the animal facility at the Bayer Schering Pharma AG. All animal studies were approved by the competent authority for labor protection, occupational health, and technical safety for the state and city of Berlin, Germany, and were performed in accordance with the ethical guidelines of Bayer Schering Pharma AG.

Systemic endotoxin-induced inflammation in mice

C57Bl/6 wild-type, MK2-heterozygous, and MK2-deficient mice were treated by intraperitoneal injection with 5 mg kg^{-1} LPS (*E. coli* 0111:B4; Sigma L-4391, Steinheim, Germany) or NaCl control. At 1.5 hours after LPS challenge, the mice were killed, and the serum levels of TNF-α were determined by Luminex assay (Biorad, Munich, Germany).

Acute DNFB-induced contact allergy model

For sensitization, female WT and MK2-deficient mice were topically treated on day 0 and day 1 with 25 μl of 0.5% (w/v) 2,4-dinitrofluorobenzene (DNFB) (Sigma) in acetone/olive oil 4:1 (v/v) on the shaved abdomen. After 5 days, groups of 10 mice were challenged by the topical application of 20 μl of 0.3% (w/v) DNFB in acetone/olive oil 4:1 (v/v) on both sides of one ear. Small molecule inhibitors were applied subcutaneously (s.c.) in a volume of 0.1 ml per 20 g body weight in 0.9% NaCl solution containing 0.085% Myrj 53 (ICI, UK) and 5% ethanol 2 hours before DNFB challenge of the animals on day 5. On day 6 (24 hours after challenging animals), the thickness of the inflamed ears were determined, and myeloperoxidase (for granulocyte infiltration) and granulocyte elastase activities (a parameter for neutrophil infiltration) were measured in homogenates of inflamed ears as described earlier (Schottelius *et al.*, 2002; Zugel *et al.*, 2002).

Subacute DNFB- and DNCB-induced contact allergy models

To examine the role of MK2 in the subacute DNFB model, female and male WT and MK2 knockout mice were topically sensitized on day 0 and day 1 with 25 μl of a 0.5% (w/v) DNFB (Sigma) solution in acetone/olive oil 4:1 (w/v) on the shaved abdomen. On days 5, 6, and 7, contact allergy was induced by repeated challenge of the ears with topically applied DNFB (10 μl of a 0.15% [w/v] DNFB solution in acetone/olive oil 4:1 [w/v]). Ear thickness was monitored during the study as follows: day 0, day 5 (before challenge), day 6 (24 hours after first challenge), day 7 (24 hours after second challenge), and day 8 (24 hours after third challenge). In the subacute DNCB model, female and male WT and MK2 knockout mice were topically sensitized on day 0 with 25 μl of a 0.5% (w/v) 2,4-dinitrochlorobenzene (DNCB) (Merck, Darmstadt, Germany) in acetone/olive oil 4:1 (w/v) on the shaved abdomen. On days 6, 7, and 8, contact allergy was induced by repeated challenge of the ears with topically applied DNCB (10 μl of a 0.15% [w/v] DNCB solution in acetone/olive oil 4:1 [w/v]). Ear thickness was monitored during the study as follows: day 0, day 6 (before challenge), day 7 (24 hours after first challenge), day 8 (24 hours after second challenge), and day 9 (24 hours after third challenge). On day 8 (DNFB) or day 9 (DNCB), animals were killed, and peroxidase activity (for granulocyte infiltration) and elastase activity (a parameter for neutrophil infiltration) were measured in homogenates of inflamed ears as described earlier (Schottelius *et al.*, 2002; Zugel *et al.*, 2002). Genders have been combined in all figures as no significant gender differences have been observed for any of the skin inflammation models investigated.

TNF-α neutralization in subacute DNFB- and DNCB-induced contact allergy

Female C57Bl/6 mice were sensitized with 25 μl of 1% DNCB in acetone/olive oil (4:1) on the shaved flank skin on day 0. Challenges with 10 μl 1% DNCB in acetone/olive oil (1:9) on the dorsum of both ears were performed on days 6, 7, and 8. As treatment in both contact allergy models purified rabbit polyclonal anti-mouse-TNF-α antibodies (endogen) and in an additional treatment group in the subacute DNFB model purified hamster polyclonal anti-mouse TNF-α (Abcam Cambridge, UK) and as isotype controls purified rabbit and purified hamster IgG (AbD Serotec, Munich, Germany) was used.

The proteins were prepared for *in vivo* use with SLIDE-A-LYZER dialysis cassettes (Serotec). Treatment was applied 1 hour before the first challenge i.p. Ear thickness was determined over the time course with a custom-built automated caliper/micrometer (Bayer Schering Pharma AG, Berlin, Germany). At day 9, animals were killed and the ears were cut, weighed, and mechanically homogenized in 2 ml homogenization buffer (hexadecyltrimethylic ammonium bromide/morpholinopropan sulfonic acid (Sigma)), centrifuged at 25,000 *g* for 20 minutes at 12°C, and the supernatant was used for determination of immune mediators. Cutaneous granulocyte infiltration was assessed by myeloperoxidase activity assay in ear homogenates as described earlier (Schottelius *et al.*, 2002). Cytokine levels in ear homogenates were measured using MSD 96-Well MULTY-ARRAY and MULTI-SPOT technology (Meso Scale Discovery, Gaithersburg, MD) according to the manufacturer's instructions.

Chronic TPA-induced skin inflammation model

Ten microliters of the phorbol ester TPA (0.01 w/v) were applied to the inner and outer surfaces of each mouse ear using a micropipette. TPA was applied over a 10-day course on alternate days (days 1, 3, 5, 8, and 10). Ear thickness was assessed with a caliper starting

6 hours after TPA application (Alford *et al.*, 1992). Animals were killed on day 10 at 6 hours after last TPA application, and their ears were cut off, snap frozen, and later analyzed for peroxidase and elastase activity as described above.

Determination of elastase and peroxidase activities in ear homogenates

Peroxidase activity assay. Peroxidase activity as a measure of total granulocyte infiltration was measured as described earlier (Schottelius *et al.*, 2002). Briefly, tetramethylbenzidine (TMB) dihydrochloride was used as a sensitive chromogenic substrate for peroxidase. To convert TMB into TMB dihydrochloride, 34 µl of 3.7% hydrochloric acid (equimolar) was added to 5 mg of TMB. Then, 1 ml of DMSO was added. This stock solution was slowly added to sodium acetate–citric acid buffer (0.1 mol l^{-1}, pH 6.0) in a ratio of 1:100. Two hundred microliters of this TMB solution, 40 µl of the homogenized sample, and 25 µl of 1 mM H_2O_2 were added to a microtiter plate to start the reaction. The reaction was stopped after 30 minutes with 45 µl of 1 N H_2SO_4. Changes in OD were monitored at 450 nm at 25°C against the mixture of all solutions without the added sample homogenate. Absolute extinction numbers multiplied by respective dilution factors were used to express peroxidase activity.

Elastase activity assay. Elastase activity was measured by fluorescence of 7-amino-4-methyl-coumarin (AMC) that is released from the substrate MeO-Succ-Ala-Ala-Pro-Val-AMC (Bachem, Torrance, CA). Homogenized samples in HTAB were diluted 1/10 in cetrimide buffer (0.3% cetrimide, 0.1 M Tris, and 1 M NaCl, pH 8.5). The substrate MeO-Succ-Ala-Ala-Pro-Val-AMC (300 mM in DMSO) was diluted 1/100 in cetrimide buffer to a working concentration of 3 mM. In cetrimide buffer, diluted samples were pipetted in multiwell plates, and the reaction was started by addition of the AMC substrate at 37 °C. The reaction was stopped after 1 hour with ice-cold 100 mM Na_2CO_3, and samples were measured in a Spectra Max Gemine (Molecular Devices, Menlo Park, CA) at 380 nm and compared against a standard curve with the AMC standard 7-amino-4-methylcoumarin (5 mM in ethanol).

Cytokine analyses in serum from LPS-triggered systemic inflammation model and in ear homogenates from skin inflammation models

Inflamed ears from mouse skin inflammation models were mechanically homogenized in 2 ml homogenization buffer (hexadecyltrimethylic ammonium bromide/morpholinopropan sulfonic acid, Sigma, Deisenhofen, Germany), centrifuged at 25,000 *g* for 20 minutes at 12 °C, and supernatants were used for determination of immune mediators. Cytokine levels in ear homogenates were determined using a multiplex chemiluminescence method and MesoScale SI 6000 equipment (MesoScale Discovery, Gaithersburg, MD).

Gene expression analyses in mouse skin

For purification of RNA from mouse skin, ears were homogenized in 800 µl lysis buffer and digested with 2 mg ml^{-1} Proteinase K (BD Biosciences, Erembodegem, Belgium) for 1 hour. Purified RNA from mouse skin was qualified with the Agilent Bioanalyzer 2100 system with RNA 6000 Nano Assay Kit (Agilent Technologies, Santa Clara, CA). cDNA was synthesized using Reverse Transcription Reagents and GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). RT-PCR was performed in 12.5 µl using a universal PCR Master Mix without UNG (Eurogentec, Cologne, Germany) on a 7900 HT Sequence Detection System (Applied Biosystems) under thermal conditions: 40 cycles at 95°C for 10 minutes, 95°C for 15 seconds, and 60°C for 1 minute. Expression of target genes was quantified as the fold expression of the housekeeping gene, hypoxanthine phosphoribosyltransferase (HPRT). The expression of the following mouse genes was determined in triplicated analyses using Assays on demand from Applied Biosystems: IL-1β (Mm 00434228_m1) and TNF-α (Mm 00443258_m1).

Cell culture

Spleen cells from C57Bl/6 wild-type or $Mapkapk2^{tm1Mgl}$ (C57Bl/6) mice were brought into suspension after squeezing through a cell strainer in RPMI-1640 and supplemented with 10% FCS, 1 mM L-glutamine, 100 U ml^{-1} penicillin, and 100 µg ml^{-1} streptomycin at a density of 5×10^6 cells per ml. For TLR stimulation, cells were then directly stimulated for 4 hours with either 5 µg ml^{-1} LPS (TLR4 grade, Alexis, Lörrach, Germany) alone or in combination with co-stimulation: either 10 ng ml^{-1} of IFN-γ (R&D Systems, Wiesbaden, Germany) or activating anti-CD40 antibody (clone 3/23, 1 µg ml^{-1}; BD Bioscience, Heidelberg, Germany). Furthermore, splenocytes were stimulated for 4 hours with other TLR stimuli such as 10 µg ml^{-1} zymosan (Sigma, Steinheim, Germany), 1 µg ml^{-1} imiquimod (Sequoia Research Product, Reading, UK), or 10 µg ml^{-1} CpG ODN (R&D Systems, Germany). As a control, wild-type spleen cells were treated additionally with p38 MAP kinase inhibitors SB203580 (10 µM; Merck Biosciences, Darmstadt, Germany) or SB220025 (10 µM; Axxora, Lörrach, Germany), respectively.

For TCR stimulation, CD4+ T cells were positively separated from splenocytes by using anti-CD4 (L3T4)-coated magnetic MACS beads (Miltenyi Biotech, Bergisch Gladbach, Germany) following the manufacturer's protocol. T cells were cultured at a density of 2×10^6 cells per ml in RPMI-1640 medium supplemented with FCS (10% v/v), L-glutamine (2 mM), and penicillin/streptomycin (each at 10,000 U ml^{-1}) (all Biochrom KG, Berlin, Germany) and stimulated with plate-bound anti-CD3 (clone 17A2, 10 µg ml^{-1}; BD Bioscience) and anti-CD28 (clone 37.51, 10 µg ml^{-1}; BD Bioscience). As a control, wild-type spleen cells were treated additionally with SB203580 (Merck Biosciences) at a concentration of 10 µM.

Supernatants were collected after stimulation of the respective cells and cytokine concentrations were determined with TNF-α ELISA (OptEIA set; BD Biosciences) according to the manufacturer's description.

Statistical methods

Statistical methods used for murine skin inflammation models (comparison of groups in kinetic measurements of skin thickness) were the Kruskal–Wallis and Mann–Whitney *U*-tests by SPSS software (SPSS). Statistics for end point parameters were collected by Fieller's test using a program from Bayer Schering Pharma AG based on the SAS System for Windows 6.12 (SAS Institute) (Schottelius *et al.*, 2002). Statistical methods used for *ex vivo* and *in vitro* investigations were the Mann–Whitney *U*-test and Wilcoxon's matched-pairs signed-ranks test, respectively, using SPSS software. If not otherwise indicated, mean values ± SD are shown. Statistical significances at $P<0.05$, <0.01, and <0.001 are indicated by one, two, and three asterisks, respectively.

CONFLICT OF INTEREST

The authors UZ, WDD, TMZ, LR, AM, BB, AB, and KA are employees and shareholders of Bayer Schering AG. Bayer Schering Pharma AG is not actively pursuing MK2.

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