Activation and Translocation of p38 Mitogen–Activated Protein Kinase After Stimulation of Monocytes With Contact Sensitizers

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Recently we described the induction of tyrosine phosphorylation by contact sensitizers as an early molecular event during the activation of antigen-presenting cells. In this study, the role of the p38 mitogen-activated protein kinase for the activation of human monocytes after exposure to four structurally unrelated contact sensitizers was analyzed in comparison with the irritant benzalkonium chloride and an inductor of oxidative stress (H_2O_2) using immunofluorescence, Western blotting, and enzyme-linked immunosorbent assay techniques. Biochemical analysis revealed a translocation of p38 from the cytoplasm to the detergent-resistant cell fraction only upon stimulation with contact sensitizers. The activity of p38 was studied by quantification of its phosphorylated active form with a specific antibody and by kinase assay. Although all stimulants used in this study led to the activation of p38, a translocation to the detergent-resistant fraction as well phosphorylation of the mitogen-activated protein kinase dependent transcription factor Elk-1 was induced only by contact sensitizers. Evidence for a functional relevance of mitogen-activated protein kinase activation was provided by measurement of the hapten-induced production of the proinflammatory cytokine interleukin-1β. Its release was inhibited by blocking p38-mediated signaling using the imidazole compounds SB203580 and SB202190. These data show that contact sensitizers are strong activators of the p38 mitogen-activated protein kinase. Although activation of this stress-associated pathway has been reported for many other stimuli, a unique translocation of p38 from the cytoplasm to the detergent-resistant fraction seems to be a specific event during hapten-induced activation of antigen-presenting cells. *Key words: hapten/IL-1β/irritant/phosphorylation/signal transduction. J Invest Dermatol 119:99–106, 2002*

Contact hypersensitivity is defined as a delayed type hypersensitivity reaction induced by small reactive chemicals (haptens). For sensitization haptens have to bind to carrier proteins and require processing and presentation by antigen-presenting dendritic cells. As a prerequisite for antigen presentation to T cells in regional lymph nodes, epidermal dendritic cells (Langerhans cells) or dermal dendritic cells have to be activated to induce their migration to these organs (Weinlich *et al*, 1998). Although some cellular events like production of interleukin-1β (IL-1β) (Enk and Katz, 1992), upregulation of major histocompatibility complex (MHC) class II molecules (Aiba and Katz, 1990) and costimulatory molecules (Weinlich *et al*, 1998), as well as downregulation of cadherin molecules by haptens (Schwarzenberger and Udey, 1996) have been described, only little is known about the intracellular mechanisms underlying these events. In previous studies we demonstrated an induction of tyrosine phosphorylation in human blood derived dendritic cells, cultured dendritic cells, Langerhans cells, and other MHC class II positive cell populations after their stimulation with haptens (Kühn *et al*, 1998; Neisius *et al*, 1999).

These experiments were continued using enriched human monocytes as a model for cutaneous antigen-presenting cells. To investigate whether the coupling of contact sensitizers to antigen-presenting cells would induce stress response mechanisms, we analyzed the involvement of a stress-mediated signal transduction pathway and focused on the activation of the p38 mitogen-activated protein kinase (MAPK). This protein is the most recently identified member of the MAPK family of serine/threonine kinases (Han *et al*, 1994; Rouse *et al*, 1994) responsible for the transduction of signals induced by several extracellular stimuli. Activation of p38 is achieved by dual phosphorylation of threonine 180 and tyrosine 182 (within a TGY motif) (Han *et al*, 1994; Raingeaud *et al*, 1995), a common feature of the members of the MAPK family. This dual phosphorylation is known to be induced by different kinds of environmental stress such as osmotic shock (Han *et al*, 1994) and heat shock (Ito *et al*, 1997; Zu *et al*, 1997), or as a result of stimulation with proinflammatory cytokines (Ridley *et al*, 1997), ligation of CD40 (Aicher *et al*, 1999), translational inhibitors like anisomycin (Nahas *et al*, 1996), and endotoxin exposure (Han *et al*, 1994).

Recently, Arrighi *et al* (2001) showed that p38 MAPK plays an important role in the maturation of human blood derived dendritic cells. The authors demonstrated that lipopolysaccharide and tumor necrosis factor α as well as the contact sensitizers

dinitrofluorobenzene and $NiSO_4$ but not the irritants sodium dodecyl sulfate (SDS) and benzalkonium chloride (BAC) were able to induce the maturation of immature dendritic cells and that this process involved critically the activation of p38.

We are able to confirm these findings and show that a panel of chemically nonrelated contact sensitizers induce the phosphorylated, active form of p38 (p-p38) in monocytes. Furthermore, evidence for a unique hapten-specific translocation of p-p38 from the cytoplasm to the saponine-resistant cell fraction under these conditions is presented.

MATERIALS AND METHODS

Isolation of human monocytes Monocytes were enriched from peripheral blood mononuclear cells by short-term adherence to cell culture dishes. Briefly, peripheral blood mononuclear cells were isolated from leukocyte enriched buffy coats (Transfusion Center, Mainz, Germany) by Ficoll-Paque 1.077 (Biochrom, Berlin, Germany) density gradient centrifugation. The isolated cells were resuspended in culture medium [Iscove's modified Dulbecco's medium, 3% fetal bovine serum (FBS)] and seeded onto culture dishes (1×10^6 cells per cm^2) for 45 min at 37°C. Nonadherent cells were removed by washing gently three times with warm phosphate-buffered saline (PBS). The adherent cells were cultured overnight in autologous serum before their detachment by vigorous rinsing. Contaminating B lymphocytes were depleted using CD19-coupled immunomagnetic beads (Dynal, Oslo, Norway) following a standard protocol. The purity of the enriched CD14+ monocytes was 80%–90%, less than 3% of the cells expressed the CD19 antigen, and the viability routinely exceeded 95% as determined by flow cytometry.

Stimulation of monocytes with chemicals Monocytes (10^6 per ml) were treated with the strong haptens 5-chloro-2-methylisothiazolinone plus 2-methylisothiazolinone (MCI/MI, 1 μg per ml; Hermal, Reinbeck, Germany), thimerosal (mercury-o-carboxyphenyl-thioethyl sodium salt, 2 μg per ml; Sigma, St. Louis, MO), formaldehyde (200 μg per ml; Sigma), 2,4-dinitrofluorobenzene (DNFB, 4 μg per ml; Sigma), the irritant BAC (4 μg per ml; Sigma), and hydrogen peroxide (H_2O_2, 600 μM; Sigma) for 15 min at 37°C in Iscove's modified Dulbecco's medium. For inhibition of p38 MAPK, monocytes were preincubated with the imidazole compounds SB203580 (10 μM, Calbiochem-Novabiochem, Germany) or SB202190 (10 μM, Calbiochem) for 45 min.

Stock solutions of SB203580, SB202190, and DNFB were prepared in dimethylsulfoxide; the other chemicals were dissolved in culture medium immediately before use.

Quantification of signaling components by flow cytometry To perform intracellular fluorescence-activated cell sorter (FACS) analysis, monocytes were permeabilized immediately after stimulation with PBS/ 2% FBS/0.25% saponine (Roth, Karlsruhe, Germany). The cells were then stained with primary antibodies (antiphosphotyrosine PY20-FITC, Leinco Technologies, St. Louis, MO; p38, polyclonal rabbit antiserum, Santa Cruz Biotechnology, Santa Cruz, CA; Lyn, polyclonal rabbit antiserum, Santa Cruz; antiphospho-p38, New England Biolabs, Beverly, MA) or rabbit IgG control (Dianova, Hamburg, Germany) dissolved in PBS/1% FBS/0.25% saponine for 20 min at 4°C, followed in the case of rabbit primary antibodies by incubation for 15 min with a DTAF-conjugated antirabbit secondary antibody (Dianova). Between each step, samples were washed in cold washing buffer (PBS/2% FBS/0.25% saponine).

Fluorescence intensities were assessed using a FACScalibur flow cytometer (Becton Dickinson, Heidelberg, Germany) equipped with the CellQuest software without hard or soft gates. Standard calibration procedures were used following the recommendation of Becton Dickinson. The level of significance for a difference between medium treated and stimulated cells was calculated using a paired Student *t* test.

Cytospin preparation and fluorescence microscopy Stimulated monocytes were permeabilized using 0.25% saponine in PBS/2% FBS and incubated with primary antibodies directed against p38, Lyn, or rabbit IgG control for 20 min on ice. Cells were washed and labeled with antirabbit Rhodamine X (Dianova) secondary antibody for 15 min. After removing unbound antibodies by washing, the monocytes were treated with the fixative paraformaldehyde (0.7% in PBS) for 10 min at room temperature. For analysis of the cellular localization of transcription factor Elk-1 human monocytes were stained for CD14 surface expression

(clone 3C10) and for phospho-Elk-1 (New England Biolabs) simultaneously. As secondary reagents, antimouse DTAF (Dianova) and antirabbit Rhodamine X (Dianova) antibodies were used. Cytospins were performed (5 min, 600 rpm) employing a Cytospin centrifuge (Shannon) and evaluated under a fluorescence microscope.

Preparation of cell extracts Each 5×10^6 cells were stimulated as indicated in an appropriate volume of culture medium or mock stimulated with PBS. Monocytes were lyzed in 50 μl ice-cold lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM ethylenediamine tetraacetic acid, 10% glycerol, 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 10 μg per ml aprotinin, and 1 mM sodium orthovanadate) by vigorous resuspension and incubation on ice for 30 min. Insoluble material was spun down (20 min, 14,000 rpm, 4°C) and the clear supernatants were stored at −70°C.

For some experiments, 15×10^6 monocytes were permeabilized after stimulation by incubation on ice for 15 min in buffer containing saponine. Soluble proteins were removed by washing twice with saponine buffer and the remaining membrane/cytoskeleton fraction was lyzed as described above.

Protein content of the lysates was determined using the BCA protein assay (Pierce, Rockford, IL).

SDS polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot Cell lysates (150 μg per lane) were mixed with loading buffer (Roti Load, 4× concentrated; Roth, Karlsruhe, Germany), heated for 5 min at 96°C, and subjected to SDS-PAGE on a 10% polyacrylamide gel with 0.5% SDS using standard procedures (45 mA, maximum 500 V). A biotinylated M_r-marker (Boehringer Mannheim, Mannheim, Germany) and a prestained M_r-marker (Boehringer Mannheim) were run in parallel for quantification of the M_r after Western transfer and for assessment of transfer efficiency, respectively. Proteins were blotted onto PVDF membrane (Millipore, Bedford, MA) using a semidry blotting unit (Trans-Blot SD, Bio-Rad, Hercules, CA) in a Tris/glycin buffer for 50 min at 250 mA and a maximum of 25 V. After transfer, the membranes were blocked in blocking buffer [1 × TBS (137 mM NaCl, 20 mM Tris, pH 7.6), 0.1% Tween-20, 5% nonfat dry milk powder] for a minimum of 1 h.

To detect p38 or Lyn, the membranes were incubated with 2 μg of primary antibodies (anti-p38 and anti-Lyn: polyclonal rabbit antisera, Santa Cruz) for 2 h and with horseradish peroxidase conjugated secondary antibodies (Dianova) at a dilution of 1:1000 in blocking buffer for 1 h. Phosphorylated signaling components (phospho-p38; New England Biolabs, Beverly, MA) were detected using the manufacturer's instructions. Blots were developed using chemoluminescence (ECL system, Amersham, Buckinghamshire, U.K.). Sequential detection of different antigens was achieved by incubating the membranes in stripping buffer (62.5 mM Tris–HCl, 2% SDS, and 100 mM 2 mercaptoethanol, pH 6.7) for 45 min at 50°C and washing in TBS + Tween-20 to remove antibodies before further analysis.

P38 kinase assay For p38 MAPK activity, 20×10^6 monocytes were stimulated for 15 min as described, permeabilized in 0.25% saponine for 15 min on ice, and washed twice with PBS. Lysates of the resulting detergent-resistant cell fractions were prepared as described. The kinase assay was performed using a commercial kit (New England Biolabs). In short, phosphorylated p38 (p-p38) was precipitated from 150 μg total lysate protein with anti-p-p38 antibody coupled to protein A sepharose overnight at 4°C with head over end rotation. Sepharose/p-p38 complexes were washed twice with lysis buffer and twice with kinase buffer (20 mM HEPES, 2 mM dithiothreitol, 20 mM p-nitrophenylphosphate, 20 mM β-glycerolphosphate, 20 mM $MgCl_2$, 0,1 mM sodium vanadate). Sepharose beads were resuspended in 50 μl kinase buffer supplemented with 200 μM ATP and 1.5 μg ATF-2 fusion protein and incubated for 30 min at 30°C. Kinase reaction was stopped by addition of loading buffer (Roti Load, 4× concentrated) and boiling for 5 min. The kinase reactions were then subjected to SDS-PAGE on a 10% SDS-polyacrylamide gel and phosphorylated p38 substrate (phospho-ATF-2) was detected by Western blotting with a phospho-specific anti-ATF-2 antibody.

Measurement of IL-1β production by enzyme-linked immunosorbent assay (ELISA) Peripheral blood mononuclear cells were resuspended in Iscove's medium/1% autologous plasma and seeded onto a 12-well plate (8×10^6 cells per well). After 45 min the nonadherent cells were removed by washing with warm PBS. Adherent cells were pretreated with p38 inhibitors (SB203580, SB202190) for 45 min. After stimulation with MCI/MI (1 μg per ml) or

lipopolysaccharide (100 ng) for 15 min, the medium was aspirated and replaced by 1.5 ml fresh medium containing 1% autologous plasma.

Supernatants were collected after 24 h and assessed for IL-1β production by ELISA according to the recommendations of the distributor of the pair of antibodies employed (R&D Systems, Wiesbaden, Germany). Briefly, Maxisorp Nunc Immunoplates (Nunc, Wiesbaden, Germany) were coated overnight at 4°C with 2 μg per ml capture antibody diluted in 0.1 M NaHCO$_3$, pH 8.2. After washing with PBS/0.05% Tween-20, the plates were blocked for 2 h at 37°C with PBS/1% bovine serum albumin (BSA). Serial dilutions of the provided standards (R&D Systems) and collected supernatants were incubated overnight at 4°C. The next day the plates were washed and incubated at 37°C for 1 h with 10 ng per ml biotinylated detection antibody, diluted in PBS/0.1% BSA. After washing, 2.5 μg per ml peroxidase-conjugated avidin (Sigma) was added for 30 min. The plates were then developed with ABTS and read in a microplate reader Model 450 (Bio-Rad) at 405 nm. IL-1β concentration was calculated according to the standard curves with a detection limit of 16 pg per ml. Statistical analysis was performed using a paired Student t test.

RESULTS

Potent contact sensitizers induce significant tyrosine phosphorylation in enriched monocytes Incubation of human monocytes with the contact sensitizers MCI/MI, thimerosal, formaldehyde, and DNFB resulted in a similar strong increase in tyrosine phosphorylation as seen for human dendritic cells and murine Langerhans cells before (**Fig 1**). An irritant like BAC or the use of H$_2$O$_2$ as inductor of oxidative stress failed to increase the level of phosphotyrosine. In pilot experiments different concentrations of chemicals were tested and for haptens optimal activating but strictly subtoxic concentrations were defined. BAC and H$_2$O$_2$ were tested at maximal, still subtoxic, concentrations, as analyzed by staining with propidium iodide and FACS analysis. From these findings we conclude that monocytes can be employed as a model for the reactivity of cutaneous antigen–presenting cells to stimulation with contact sensitizers.

Stimulation of monocytes with a contact sensitizer results in a translocation of p38 MAPK To analyze the expression of p38 MAPK flow cytometric studies on monocytes permeabilized with saponine were performed and compared with the expression of the src kinase Lyn. This kinase is strongly expressed in human monocytes in a membrane-associated fashion. Medium treated cells revealed only a weak expression of p38. Surprisingly, stimulation with the strong contact sensitizer MCI/MI raised the detectable amount of p38 (**Fig 2a**). In contrast, almost constant levels were shown for the src kinase Lyn. This augmented detection of p38 was prominent after stimulation with haptens, whereas an irritant or oxidative stress (H$_2$O$_2$) failed to induce a convincing increase (**Fig 2b**).

In order to confirm our flow cytometric data, the expression of p38 and Lyn was analyzed in permeabilized cells by Western blotting (**Fig 2c**). No serious differences were seen for the expression of Lyn whereas p38 was significantly more associated with the saponine-resistant cell fraction as a result of treatment with contact sensitizers but not BAC or H$_2$O$_2$. This suggests a translocation of p38 from the cytoplasm to the detergent-resistant fraction during stimulation with contact sensitizers, thereby preventing the loss of this protein by permeabilization as seen in the unstimulated control (**Fig 2c**).

Haptens induce an intracellular reorganization of signaling components For many cell types a rapid translocation of p38 MAPK from the cytosol to the nucleus upon stimulation has been described (Treisman, 1996). As the nucleus is a part of the saponin-resistant cell fraction, we investigated whether haptens induce the accumulation of p38 within the nucleus. Therefore cytospins of permeabilized monocytes were stained with antibodies specific for p38 and analyzed using a fluorescence microscope. In accordance with the flow cytometric and biochemical data presented, the detectable amount of p38 was markedly increased only in hapten-treated cells as a result of the translocation of this MAPK, whereas it
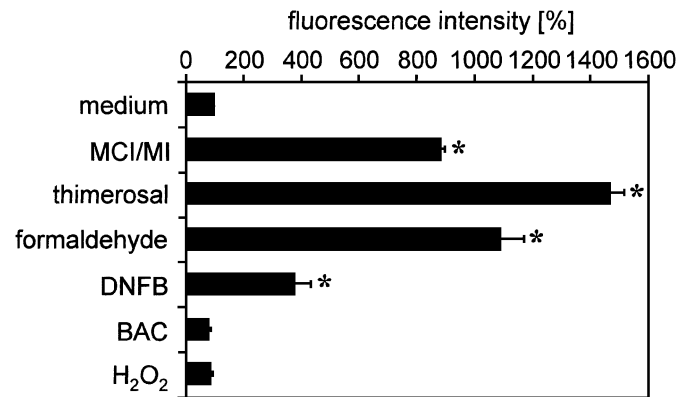

Figure 1. FACS analysis of tyrosine phosphorylation in monocytes induced by contact sensitizers but not irritants. Human monocytes were incubated for 15 min with MCI/MI (1 μg per ml), thimerosal (2 μg per ml), formaldehyde (200 μg per ml), DNFB (4 μg per ml), BAC (4 μg per ml), and H$_2$O$_2$ (600 μM). Subsequently, cells were permeabilized, stained with the phosphotyrosine-specific antibody PY20 and analyzed by FACS. Mean ± SEM of four independent experiments are shown as a percentage of medium treated cells (*p < 0.01).

was almost completely lost during the process of permeabilization in the medium control as well as in cells treated with BAC and H$_2$O$_2$ (**Fig 3**). No pronounced accumulation of p38 was visible within the nuclear region; instead aggregates clearly distinct from the nucleus became apparent (**Fig 3**). When the expression of src kinase Lyn was investigated with the same technique, the total amount of Lyn was unaffected by the stimulation with contact sensitizers but this kinase was translocated into distinct aggregates, too (**Fig 3**). Controls performed with rabbit IgG were completely negative (data not shown).

Translocation of p38 correlates with its activity As the translocation of p38 to the detergent-resistant fraction upon stimulation does not necessarily imply an increased activity of this kinase, the amount of phosphorylated active p38 was analyzed in permeabilized monocytes by flow cytometry and was found to be significantly increased after stimulation with contact sensitizers, but not following treatment with BAC or H$_2$O$_2$ (**Fig 4a, b**).

As a control for the validity of these flow cytometric data, the effect of contact sensitizers on the translocation of phospho-p38 in permeabilized cells was determined by Western blot analysis. Again the amount of the active form in the detergent insoluble cell fraction increased only after stimulation with contact sensitizers (**Fig 4c**).

In order to measure the activity of p38 MAPK directly within the detergent-resistant cell fraction, kinase assays were performed. In contrast to MCI/MI used as the model hapten in this experiment, neither the irritant BAC nor H$_2$O$_2$ were able to induce the phosphorylation of the p38 kinase substrate ATF-2 when permeabilized monocytes were investigated (**Fig 4d**).

For analysis of the total amount of p-p38 without any influence of permeabilization, whole cell lysates of monocytes treated with our panel of stimulants were analyzed by Western blotting. Whereas the total expression of p38 was found to be constant with or without stimulation, p-p38 was hardly detectable in medium treated monocytes (**Fig 4e**). Treatment with different haptens but also BAC and H$_2$O$_2$ induced a distinct phosphorylation of this kinase (**Fig 4e**).

Taken together, these results demonstrate that stimulation of monocytes with haptens, an irritant, or oxidative stress was sufficient to activate p38, but its translocation to the detergent-resistant fraction was induced only by contact sensitizers.
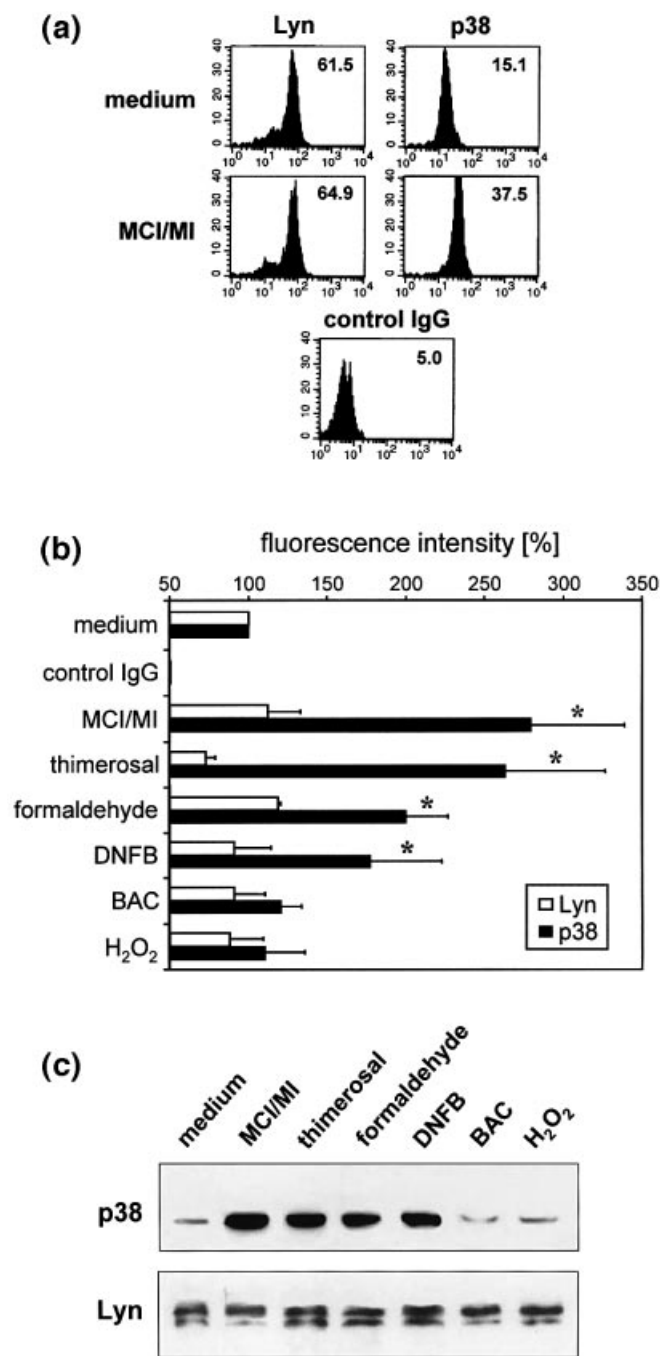
Figure 3. Accumulation of p38 and Lyn in aggregates after stimulation with contact sensitizers but not BAC or H_2O_2. Monocytes were treated as outlined in **Fig 2** and stained with antibodies directed against p38 and Lyn as described for FACS analysis. Cytospins were performed and evaluated using a fluorescence microscope.

Figure 2. Increased detection of p38 but not Lyn following stimulation with contact sensitizers. Monocytes were treated as indicated (MCI/MI 1 μg per ml, thimerosal 2 μg per ml, formaldehyde 200 μg per ml, DNFB 4 μg per ml, BAC 4 μg per ml, and H_2O_2 600 μM). The cells were then permeabilized and analyzed for p38 and Lyn expression by FACS and Western blot analysis. (*a*) Representative histograms for the expression of p38 and Lyn in unstimulated and MCI/MI-stimulated monocytes in comparison to the rabbit IgG control. The median for the absolute fluorescence intensity is indicated. (*b*) Relative fluorescence intensities calculated as a percentage of untreated monocytes. Mean values ± SEM of 12 independent experiments are shown (*p < 0.01). The relative fluorescence intensities of the rabbit IgG control were 31.2% ± 17.1% of p38 expression of untreated cells and 5.7% ± 2.4% of Lyn expression, respectively. (*c*) Western blot analysis of p38 and Lyn expression in monocytes permeabilized prior to lysis after stimulation as outlined above. The blot is representative for three independent experiments.
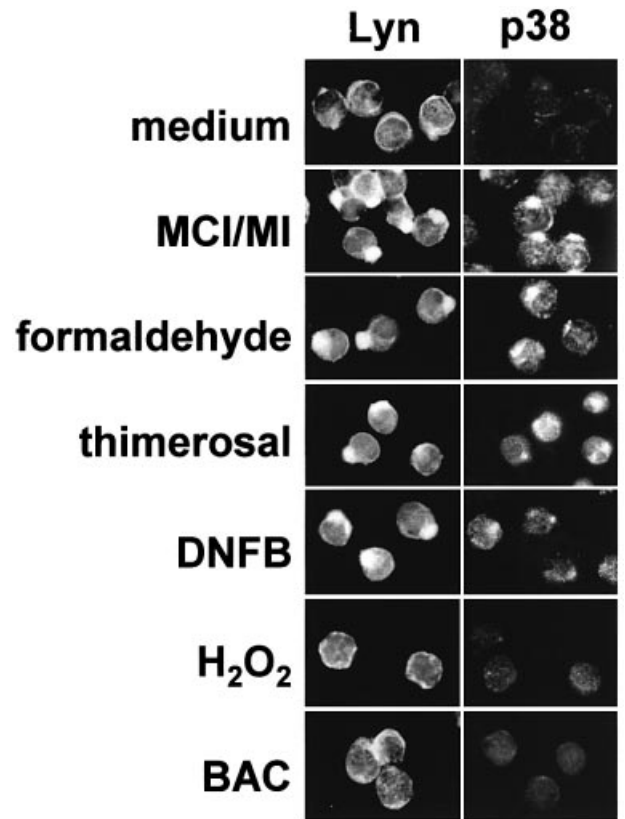
Contact sensitizers induce rapid phoshorylation of transcription factor Elk-1   To analyze the relevance of p38 translocation after stimulation with contact sensitizers for downstream signaling events, we investigated the activation of the MAPK-dependent transcription factor Elk-1 (Raingeaud *et al*, 1996; Whitmarsh *et al*, 1997; Ogura and Kitamura, 1998). Within 15 min after haptenization with different haptens, a significant amount of phosphorylated Elk-1 was detectable in monocytes as shown by cytologic analysis (**Fig 5**). In contrast BAC and H_2O_2 stimulated monocytes revealed no evidence for a significant phoshorylation of Elk-1.

Hapten-induced IL-1β release of monocytes is inhibited by SB203580 and SB202190   Rapid induction of IL-1β mRNA is a well-known reaction of cutaneous antigen-presenting cells upon stimulation with contact sensitizers. To obtain evidence for the functional relevance of p38 activation by a hapten for this response, we analyzed the effect of the specific p38 inhibitors SB203580 and SB202190 on MCI/MI-induced IL-1β release by monocytes using a specific ELISA. Lipopolysaccharide-treated cells served as positive control for optimal IL-1β production. Preincubation with subtoxic concentrations of SB203580 and SB202190 resulted in a nearly complete inhibition of MCI/MI- as well as lipopolysaccharide-induced IL-1β release (**Fig 6**). MCI/MI was chosen for these experiments because of its reliable capacity to induce IL-1β release by monocytes. The other contact sensitizers used in this paper were reactive only in some but not all experiments, whereas BAC and H_2O_2 completely failed to provoke the release of this cytokine (data not shown).
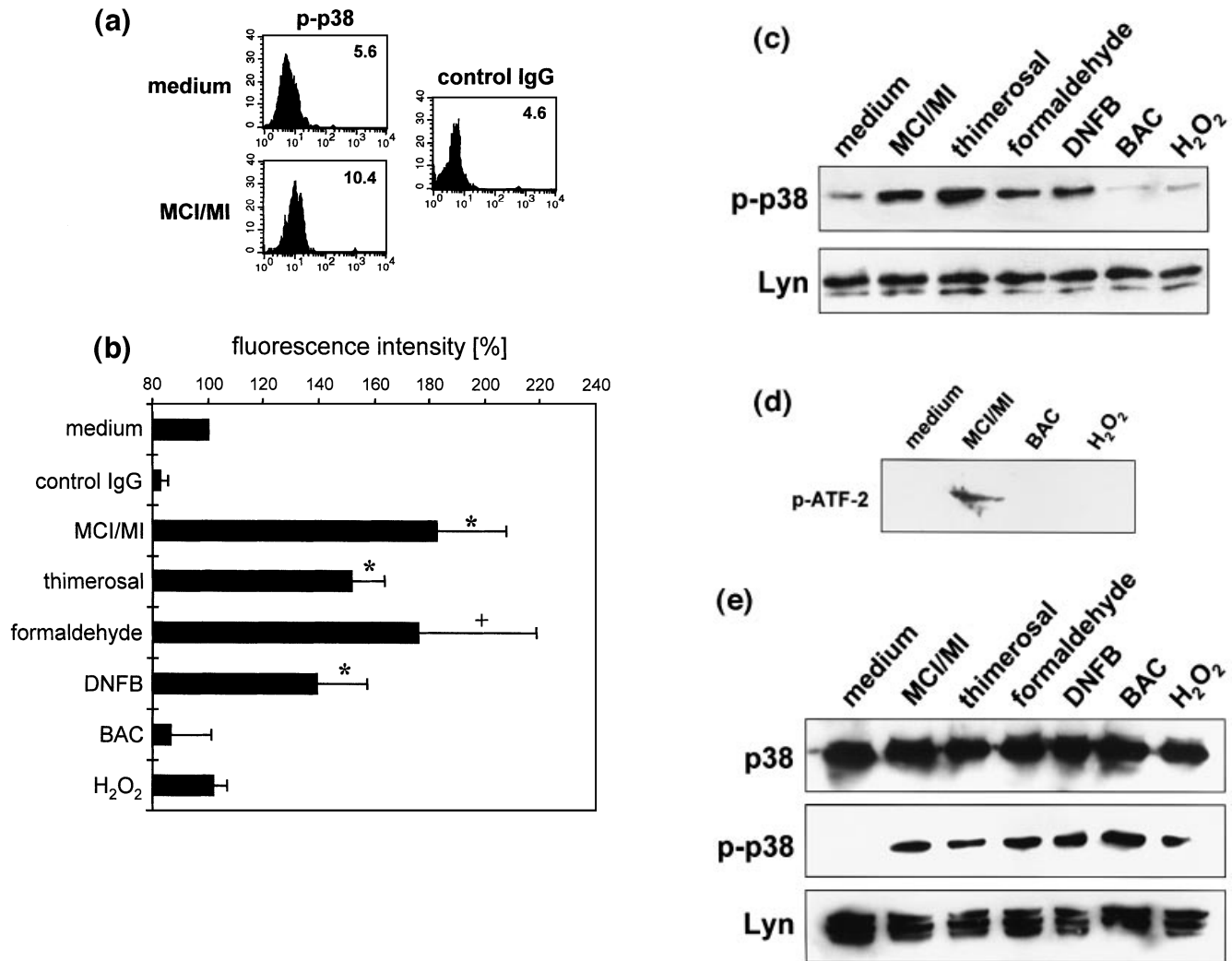
Figure 4. Contact sensitizers induce the phosphorylation of p38 MAPK and translocation of active p38 to membrane-associated compartments. (*a*) Representative histograms for the expression of p-p38 in unstimulated and MCI/MI-stimulated monocytes in comparison to the rabbit IgG control. The median for the absolute fluorescence intensity is indicated. (*b*) Flow cytometric quantification of active phospho-p38 in permeabilized monocytes stimulated for 15 min as indicated in **Fig 2**. Data are calculated as a percentage of the medium control; mean ± SEM of six independent experiments are shown (*, $p < 0.01$; +, $p < 0.05$). (*c*) Western blot analysis of phospho-p38 in permeabilized monocytes after stimulation with contact sensitizers, BAC and H_2O_2. The expression of Lyn was analyzed in sequence as a control for comparable protein loading. The blot is representative for three experiments with similar results. (*d*) Assay for p38 kinase activity in permeabilized monocytes. Cells were treated with medium, MCI/MI (1 µg per ml), BAC (4 µg per ml), or H_2O_2 (600 µM) for 15 min and lyzed. p38 MAPK activity was measured as described in *Materials and Methods*. A Western blot of p38-mediated phosphorylation of transcription factor ATF-2 is shown and representative for two independent experiments. (*e*) Western blot analysis of p38 and phospho-p38 expression in whole monocytes. Cells were treated with four contact sensitizers (MCI/MI 1 µg per ml, thimerosal 2 µg per ml, formaldehyde 200 µg per ml, DNFB 4 µg per ml) as well as BAC (4 µg per ml) and H_2O_2 (600 µM) for 15 min and lyzed without permeabilization. Phospho-p38 as well as total p38 is shown in comparison to the expression of Lyn. The blot is representative for four independent experiments.

DISCUSSION

Strong contact sensitizers have the unique capacity to induce an early and vigorous tyrosine phosphorylation in human dendritic cells and other MHC class II positive cell populations (Kühn *et al*, 1998; Neisius *et al*, 1999). In all experiments performed so far, blood-derived dendritic cells, monocyte-derived cultured dendritic cells, and monocytes showed a similar reaction pattern upon stimulation with contact sensitizers. Because monocytes reacted most vigorously to stimulation with contact sensitizers and allowed combined biochemical and flow cytometric analysis without subculture in the presence of cytokines or stimulating agents,

short-term-cultured enriched monocytes were employed as a model for cutaneous antigen-presenting cells in this study. The strong tyrosine phosphorylation following stimulation with haptens might involve a large number of different kinases and their substrates and stimulates further studies on the role of defined signal transduction elements. The interaction of contact sensitizers with antigen-presenting cells is clearly distinct from protein allergens in that it involves a chemical coupling of the hapten to cellular proteins. This process might induce cellular stress; thus we investigated the role of stress response signaling mechanisms and focused on the p38 MAPK.

Flow cytometric studies of permeabilized monocytes revealed a weak expression of p38 and high amounts of the protein tyrosine
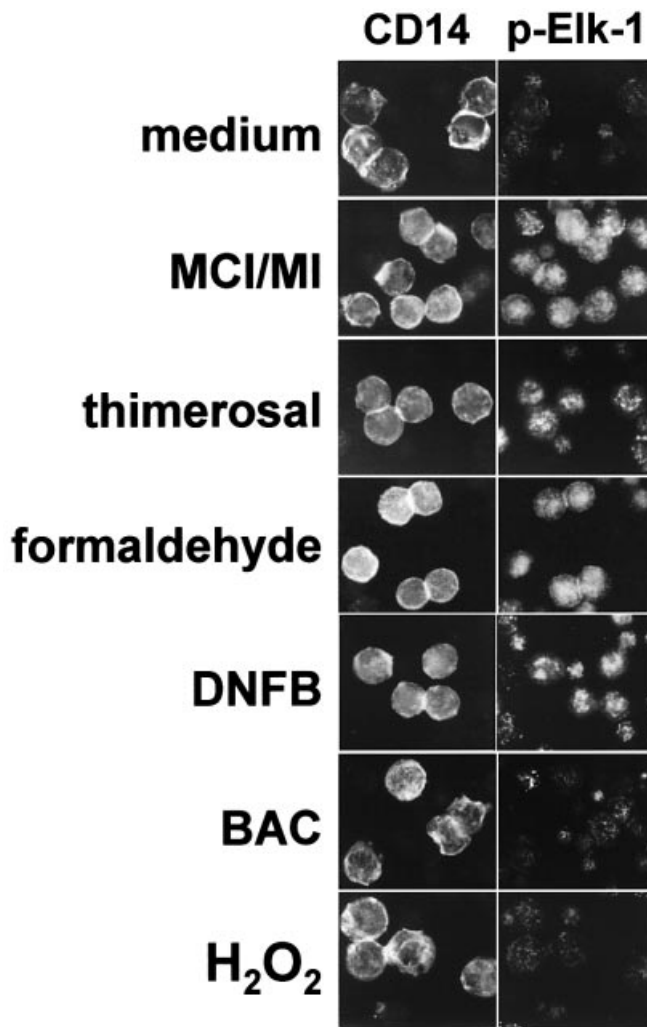
Figure 5. Phosphorylation of transcription factor Elk-1 after stimulation with contact sensitizers but not BAC and H$_2$O$_2$. Monocytes were either medium treated or stimulated as indicated for 15 min. Following permeabilization, cells were stained with antibodies specific for CD14 and p-Elk-1. After staining with the appropriate secondary reagents, cytospin preparations were evaluated using a fluorescence microscope. A representative result of four experiments is shown.

kinase Lyn in unstimulated cells. Treatment with contact sensitizers resulted in an obvious increase in detectable p38 compared to almost constant levels of Lyn, reflecting the different intracellular distribution of these kinases. In unstimulated monocytes, most Lyn is membrane associated (Stanley *et al*, 1991), making it resistant to detergent treatment. In contrast, p38 is distributed throughout the cytoplasm (Raingeaud *et al*, 1995) and therefore lost upon permeabilization. As a direct result of haptenization, p38 seems to be translocated from the cytoplasm to the detergent-resistant fraction, consisting of the plasma membrane, the cytoskeleton, and the nucleus (Chen *et al*, 1987; Mochly Rosen *et al*, 1990; Gregorio *et al*, 1992). Comparison of lysates from whole and permeabilized monocytes by Western blot analysis confirmed that increased detection of p38 relies on this translocation rather than upregulation by *de novo* synthesis.

Translocation of MAP kinases from the cytoplasm to the cell nucleus has been described in the literature (Treisman, 1996; Blanco-Aparicio *et al*, 1999). Although our cytologic analysis cannot exclude the presence of p38 in the nucleus, the accumulation of p38 and Lyn in aggregates located in the cell periphery is a unique pattern induced only by haptens but not by irritants. Several other signaling elements were found to be present in these aggregates, including tyrosine phosphorylated proteins, protein kinase C (PKC) β-isoforms and transcription factor NF-κB (data not shown). A similar aggregation has been reported for PKC βII in murine T lymphocytes activated by phorbol myristate acetate or T cell receptor stimulation (Gregorio *et al*, 1992). Although the mechanism and functional role of this translocation and aggregation is unknown, it is likely that accumulation of different signaling structures into aggregates should support cascades of subsequent phosphorylation and dephosphorylation.

We found evidence for *de novo* phosphorylation of p38 and functional activity of this kinase in membrane-associated compartments after stimulation with contact sensitizers. On the other hand, biochemical analysis of whole cell lysates also demonstrated phosphorylation of p38 after stimulation with an irritant as well as H$_2$O$_2$, a mechanism already described for the latter stimulus (Huot *et al*, 1997; Clerk *et al*, 1998; Ogura and Kitamura, 1998). In monocyte-derived dendritic cells stimulation with SDS and BAC did not induce phosphorylation of p38 (Arrighi *et al*, 2001). This difference might be explained by a higher sensitivity of monocytes to chemical irritation. Nevertheless, it is not the capacity of a compound to mediate increased phosphorylation of p38 as part of a stress response mechanism (Ogura and Kitamura, 1998) but its capacity to induce the translocation of p38 in its active and inactive form
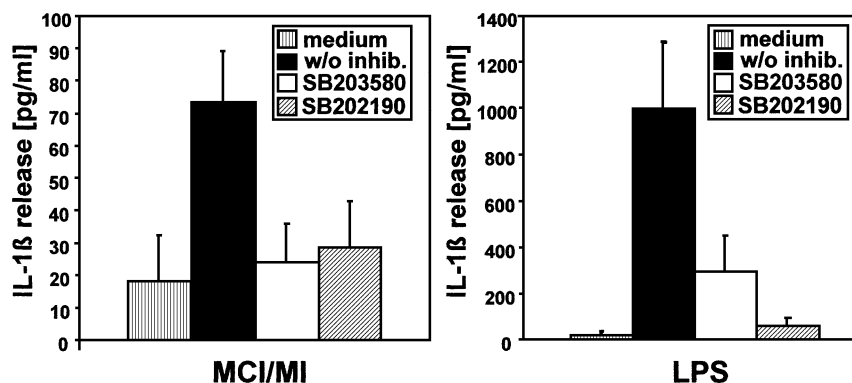

Figure 6. Hapten-induced IL-1β production depends on p38 activity. Adherent monocytes were pretreated for 45 min with SB203580 (10 μM) or SB202190 (10 μM) or mock treated (without inhibitor) prior to stimulation for 15 min with MCI/MI (1 μg per ml) or lipopolysaccharide (100 ng per ml). Cells were then incubated with fresh medium for 24 h. Supernatants were collected and IL-1β release was measured using a specific ELISA. Mean ± SEM of eight independent experiments is shown. The difference between medium control and MCI/MI or lipopolysaccharide stimulated cells as well as the difference between stimulated cells in the presence or absence of inhibitor was highly significant (p < 0.001).

from the cytoplasm to the detergent-resistant cell fraction that seems to be the main difference between haptens and nonsensitizing compounds. This conclusion is supported by the fact that although H_2O_2 and BAC induce detectable phosphorylation of p38 in monocytes, this does not result in phosphorylation of the MAPK-dependent transcription factor Elk-1 as noticed for hapten-stimulated cells. Any further conclusions from our cytologic analysis regarding the exact role of p-p38 for this activation of Elk-1 are highly speculative and not addressed by our study.

The rapid induction of IL-1β mRNA following stimulation with strong contact sensitizers has been described in Langerhans cells in vivo (Enk and Katz, 1992) as well as in monocyte-derived human dendritic cells (Kühn et al, 1998). Release of IL-1β by monocyte-derived dendritic cells stimulated with different contact sensitizers was demonstrated by Aiba et al (1997). For some murine monocytic cell lines the lipopolysaccharide-induced release of IL-1β has been shown to be dependent on p38-activated transcription factors C/EBPβ and C/EBPδ (Baldassare et al, 1999). To address the functional relevance of MAPK activation by contact sensitizers, the capacity of p38 specific inhibitors (Cuenda et al, 1995) to block the production of IL-1β was investigated. We demonstrate a significant increase in IL-1β secretion of human monocytes upon stimulation with our model hapten MCI/MI. As subtoxic concentrations of the p38 specific inhibitors SB203580 and SB202190 almost completely blocked this increase, p38 seems to be of central importance for this response of monocytes to stimulation with a strong hapten. This is in good accord with the data of Arrighi et al who demonstrated a decisive role of p38 for the maturation of monocyte-derived dendritic cells after stimulation with DNFB and $NiSO_4$ (Arrighi et al, 2001). On using SB203580 during stimulation with contact sensitizers we found no evidence for an influence of this inhibitor on the translocation or phosphorylation of p38 in the flow cytometric analysis (data not shown).

In aggregate, our data show that hapten treatment activates the p38 MAPK as reported before for stress-related stimuli. On the other hand, exposure to H_2O_2 at a concentration known to induce oxidative stress (Joseph et al, 1997; Clerk et al, 1998) or to an irritant did not resemble the effect of haptens in several aspects. The most striking difference between contact sensitizers and the nonsensitizing compounds tested was the translocation of p38 from the cytoplasm to the detergent-resistant cell fraction. Further results from our laboratory indicate that this process of a rapid translocation to the membrane fraction was not restricted to p38 MAPK but was also observed for extracellular-regulated kinase 1/2 (ERK) and c-Jun N-terminal kinase 1/2 (JNK) after treatment with a contact sensitizer (data not shown). This translocation may be an important process for the transduction of signals induced primarily by binding of contact sensitizers to as yet unidentified cellular structures.

To elucidate the upstream events involved in the process of translocation future studies have to focus on two MAPK kinases that specifically phosphorylate p38 (MKK3, MKK6) (Raingeaud et al, 1996) as well as a further MAPKK, termed MKK4 or JNKK, with a dual specificity for p38 and c-Jun N-terminal kinase (Derijard et al, 1995; Lin et al, 1995). MKK4 provides a possible link between the two stress-associated signal transduction pathways. Actually, further data from our group (not shown) suggest a complex activation of all three MAPK families, but here we demonstrate that at least the augmented release of IL-1β by MCI/MI-stimulated monocytes is under the control of p38. Given the important functional role of this cytokine during the sensitization phase of allergic contact dermatitis, it will be very interesting to study the capacity of p38-specific inhibitors to block IL-1β production in this situation and subsequently the process of sensitization. Therefore, uncovering the molecular details of the activation of antigen presenting cells by contact sensitizers might help to develop new strategies for prevention of contact sensitization.

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