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NF-κB-Dependent Induction of Cathelicidin-Related Antimicrobial Peptide in Murine Mast Cells by Lipopolysaccharide

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Key Words
Cathelicidin-related antimicrobial peptide · Inflammation · Mast cells · Transcription factors

Abstract
Background: An important aspect of the innate immune response to pathogens is the production of anti-microbial peptides such as cathelicidin-related antimicrobial peptide (CRAMP), the murine homologue of human cathelicidin LL-37. In this study, mechanisms regulating LPS-induction of CRAMP gene expression in mast cells were investigated. NF-κB and MAPK pathways were the focus of investigation. ***Methods:*** Mouse bone marrow-derived mast cells were grown in culture and stimulated with LPS. MAPKs and NF-κB were monitored by immunoblot analysis. ERK, JNK and p38 MAPK were inhibited using siRNAs or a pharmacological inhibitor. Accumulation of the p65 component of NF-κB was inhibited by siRNA and NF-κB activation was inhibited by overexpression of IκBα. MEKK2 or MEKK3 were overexpressed by transfection. The effects of all of these treatments on CRAMP gene expression were monitored by RT-PCR. ***Results:*** Inhibition of ERK, JNK or p38 MAPK had little discernible effect on LPS-inducible CRAMP gene expression. Overexpression of MEKK2 or MEKK3 likewise had little impact. However, inhibition of the accumulation of p65 NF-κB prevented LPS-induced CRAMP mRNA. An important role for NF-κB in CRAMP gene expression was confirmed by overexpression of IκBα, which reduced both basal and induced levels of CRAMP mRNA. ***Conclusions:*** NF-κB, but not MAPKs, plays an important role in LPS-mediated induction of CRAMP gene in mast cells. Defects which inhibit NF-κB activity may increase susceptibility to bacterial and viral pathogens which are sensitive to cathelicidins.

Introduction

Mast cells play important roles in immunity, chronic inflammation and allergic reactions, primarily through 2 major responses: synthesis and secretion of cytokines, including tumor necrosis factor (TNF)-α, IL-4 and IL-13, and degranulation, with the release of histamine, serotonin, proteases and proteoglycans. Induction of these responses through cross-linking of the high-affinity IgE receptor (FcεRI) on mast cells with multivalent antigen is regulated through distinct and highly overlapping signal transduction networks which have been extensively characterized [1, 2]. It has emerged from these studies that MAPK cascades play crucial roles in regulating nearly all of these FcεRI-mediated functions, with, for example, an MEKK2-MEK5-ERK5 MAPK module modulating cytokine transcription [3, 4], whereas degranulation appears to be regulated by a pathway involving MEK1 and ERK2 [5]. It is clear that Akt also plays an important role in regulating effector function in mast cells, through activation of a variety of transcription factors, including NF-κB [6, 7].

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Mast cells, which are commonly located at sites exposed to the external environment, such as the skin, the airways and the intestine, are also well placed to serve as sentinel cells in host defense [8]. Upon contact with bacteria, mast cells release a variety of molecules important in host defense, including TNF-α and leukotriene B4, which can recruit and/or activate effector cell types [8]. It has been reported that murine mast cells, upon exposure to LPS, also synthesize and secrete the cathelicidin-related antimicrobial peptide (CRAMP, the murine homolog of human LL-37), thus potentially providing an immediate and potent defense against some bacterial and viral pathogens [9]. It has also been shown that mast cell-produced cathelicidin protects against invasive group A *Streptococcus* infection of the skin [10]. Recent studies further suggest that antimicrobial peptides, in addition to possessing intrinsic antimicrobial activity, may also initiate signal transduction pathways upon binding to certain cell types, including mast cells, and thereby modulate immune responses [11].

A single cathelicidin gene appears to be present in humans and mice, giving rise to LL-37 and CRAMP, respectively [12, 13]. The proteins are translated as procathelicidin molecules comprised of a conserved cathelin domain and a C-terminal domain representing the more variable antibacterial peptide itself, and proteolytic processing is required for release of the active peptide. Cathelicidins have been identified in many cell types, including neutrophils, epithelial cells, keratinocytes, corneal fibroblasts, lymphocytes, monocytes and mast cells [14, 15]. The cathelicidins are cationic, amphipathic peptides and through interaction with bacterial membranes can disrupt and kill sensitive strains [11, 12]. They can also directly bind to and inactivate certain viruses, such as vaccinia virus [16]. The cathelicidins also possess other distinct biological activities and can, for example, induce migration of mast cells, neutrophils and T cells [17, 18] and modulate cytokine and cytokine receptor expression [19, 20]. It has also been recently reported that LL-37 can modulate the proinflammatory response of monocytes to LPS by suppressing NF-κB translocation and inhibiting the release of TNF-α [21]. Interaction of cathelicidins with cells has been reported to initiate numerous biochemical events, including activation of MAPK pathways [22, 23] and Ca^{2+} mobilization [24, 25], in addition to affecting NF-κB translocation [21, 25]. There is only limited knowledge concerning identification of the range of functional cathelicidin receptors, of the molecular pathways initiated by cathelicidin binding, and of their biological outcomes. Furthermore, little is yet known concerning the signal transduction pathways regulating cathelicidin gene expression, the object of the present study.

Like aggregation of FcεRI on murine mast cells, interaction of LPS with Toll-like receptor 4 (TLR4) on mast cells can activate all 3 major types of MAPK modules, i.e. those leading to activation of JNK, ERK and p38 MAPK [26]. NF-κB is also involved in transducing the signals initiated by ligation of TLR4 [27]. Therefore, the possible roles of these components were investigated in the LPS-mediated induction of CRAMP gene transcription in murine bone marrow-derived mast cells (BMMCs). Although an absolute dependence on NF-κB induction was observed, no apparent involvement of key MAPK module components, including JNK, ERK and p38 MAPK, was demonstrated.

Materials and Methods

Cell Culture

Bone marrow cells were prepared from mouse femurs (C57BL/6 and BALB/c) and grown in IMDM supplemented with 10% FCS, 50 μM β-mercaptoethanol, 2 mM glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin. The medium also contained 1% (v/v) of conditioned medium prepared from cultures of IL-3-producing cells and 1% (v/v) of conditioned medium prepared from cultures of stem-cell-factor-producing cells, as described previously [4, 5, 28]. For some mast cell preparations, the medium contained purified, recombinant IL-3 (15 ng/ml; Biosource, Camarillo, Calif., USA) and stem cell factor (25 ng/ml; Biosource) instead of the conditioned mediums. Growth medium was changed after each 3 days in culture. After 4 weeks in culture, homogeneous cultures of BMMCs were confirmed as described previously [5] and were used for experiments at 4–6 weeks after initiation of culture. For stimulation with LPS (Sigma Inc., St. Louis, Mo., USA), mast cells at a density of 2×10^6/ml were incubated with 200 ng/ml of LPS (or indicated doses) for 2 h. For passive sensitization and stimulation [28], mast cells at a density of 3×10^6/ml were incubated overnight with 500 ng/ml of anti-ovalbumin (OVA)-specific IgE. After washing with warm complete growth medium 2 times, they were incubated at 37°C for 2 h. The cells were then challenged with OVA (10 μg/ml) or vehicle (PBS) for 15 min and harvested for assays. In experiments where cells were pre-treated with PD98059, the inhibitor was added 30 min before the addition of OVA or LPS.

Flow Cytometry

BMMCs were processed and stained for flow cytometric analysis as described previously [29], using a phycoerythrin-labeled monoclonal anti-TLR4 (Toll-like receptor 4) antibody (Santa Cruz Biotechnology Inc., Santa Cruz, Calif., USA). Cells were also analyzed using the appropriate isotype control (normal rat IgG2a antibody) obtained from Santa Cruz Biotechnology. Flow cytometry was performed using a FACScan (BD Biosciences, Rockville, Md., USA) and data were analyzed using FlowJo software (Tree Star, Ashland, Oreg., USA).

Plasmid and siRNA Transfection

For plasmid transfections, cells (1.2×10^7 BMMCs) were washed twice with pre-warmed IMDM medium (without antibiotics) and then suspended in 600 μl of IMDM with 10 μg of plasmid (as indicated in the text). The mixture was incubated at room temperature for 15 min and electroporation was performed as described previously [28]. In brief, the conditions for electroporation were 350 V, 1,180 μF, high Ω and fast charge. After electroporation, the cells were permitted to recover at room temperature for 30 min and were then transferred into normal growth medium at a density of 1×10^6 cells/ml and incubated for 48 h at 37°C. The MEKK2 and MEKK3 plasmids were described previously [3, 4]. The plasmid encoding IκBα was a kind gift from Dr. Hong-Bing Shu (National Jewish Health, Denver, Colo., USA) and encodes a mutant form of IκBα which has an extended half-life due to phosphorylation-site mutations (S32A/S36A) which protect it from ubiquitin-dependent degradation [30].

For siRNA transfection, cells (1.2×10^7 BMMCs) were washed 2 times with pre-warmed PBS and then suspended in 600 μl of siPORT™ siRNA electroporation buffer (Ambion Inc., Austin, Tex., USA) with 100 nM of specific siRNA. The mixture was incubated at room temperature for 15 min and then electroporation was performed at 350 V, 1,180 μF, high Ω and fast charge. After electroporation, the cells were incubated at 37°C for 10 min and then were transferred into growth medium at a density of 1×10^6 cells/ml and incubated for 72 h at 37°C. Specific siRNAs targeting JNK, p38 MAPK and the p65 component of NF-κB were purchased from Santa Cruz Biotechnology; control siRNAs were obtained from SiRNA Inc. (Boulder, Colo., USA).

Immunoblot Analysis

Immunoblot analysis was performed as described previously [31]. Cells (5 to 10×10^6) were pelleted by centrifugation, washed with PBS and re-suspended in 100 μl of lysis buffer. The samples were kept on ice for 30 min and then the extracts were clarified by centrifugation, mixed with gel electrophoresis loading buffer and placed in a boiling water bath for 5 min. Samples were resolved by 10% polyacrylamide gel electrophoresis and proteins were transferred to nitrocellulose membranes. Proteins were detected using protein-specific antibodies by a chemilumenescent detection method, as described previously. Specific monoclonal antibodies (mAbs) to IκBα, IκBβ and p65 were purchased from Santa Cruz Biotechnology, mAbs to phospho-ERK1/2, p-p38 and p-ERK5 were purchased from Cell Signaling Technology Inc. (Danvers, Mass., USA), the mAb to p-JNK was purchased from Upstate Inc. (Lake Placid, N.Y., USA) and the mAb to β-actin was purchased from Sigma-Aldrich Corp. (St. Louis, Mo., USA).

Determination of mRNA Levels

Total RNA was extracted from BMMCs using an RNeasy mini kit (Qiagen Inc., Valencia, Calif., USA). Two micrograms of total RNA were used in each reaction primed with oligo-dT to obtain c-DNA. Then, 3 μl of the synthesized c-DNA was used as the template for CRAMP, MEKK2, MEKK3 and β-actin mRNA amplification reactions. Primer sequences were designed for amplification of CRAMP, MEKK2, MEKK3 and β-actin and were synthesized by Biosource. They were as follows: CRAMP forward CGA GCT GTG GAT GAC TTC AA, reverse ACC AAT CTT CTC CCC ACC TT (product size 340 bp). MEKK2 forward ATG CCT AGG GCA CAG AGC TA, reverse TCT CTG GGC TCT CAG GGT TA (product size 487 bp). MEKK3 forward AGT ATA TGC CAG GGG GCT (product size 381 bp). β-actin forward GTG GGC CGC TCT AGG CAC CAA, reverse CTC TTT GAT GTC ACG CAC GAT TTC (product size 500 bp).

CRAMP gene expression was also measured by quantitative real-time RT-PCR. RNA was isolated as above and 1 μg was converted into cDNA using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, Calif., USA). The primer sequences and TaqMan TAMRA probe (Applied Biosystems, Foster City, Calif., USA) that were used to determine CRAMP expression were as follows: CRAMP forward TCTCCCAGACCCCCAGCTA, reverse AGGGACTGCTGGTTGAAGTCA; CRAMP probe 6FAMAGGGATGCTGTGCCCGAGCTGTGTAMRA.

Quantitative real-time PCR was performed on an ABI 7700 Sequence Detection System (Applied Biosystems). The PCR reaction mixture contained 2× TaqMan Master Mix, 900 nM forward primer, 900 nM reverse primer, 200 nM probe and 1 μg cDNA in a final volume of 25 μl, and was subjected to the following thermal cycle conditions: 50°C for 2 min and 95°C for 10 min for 1 cycle, followed by 40 cycles of amplification at 94°C for 15 s and 60°C for 1 min.

Results

CRAMP Expression Is Inducible by LPS in BMMCs Derived from BALB/c Mice

BMMCs were isolated from BALB/c and C57BL/6 mice. As shown in figure 1a, low basal levels of CRAMP mRNA were seen in BMMCs from both strains of mice. However, only BMMCs from BALB/c mice showed increased levels of CRAMP after treatment with LPS. Since LPS acts through TLR4 [32, 33], TLR4 levels on the cell surface of both kinds of BMMC were examined by flow cytometric analysis. As shown in figure 1b, there was no significant difference in TLR4 levels on the cell surface of the 2 kinds of BMMCs. BMMCs derived from BALB/c were used in all of the following experiments. As shown in figure 1c, stimulation by LPS occurred in a dose-dependent manner with maximal induction occurring between 100 and 200 ng/ml. The time course of CRAMP mRNA induction was also examined by quantitative real-time PCR. As shown in figure 1d, increased CRAMP mRNA levels were seen at 90 min after LPS treatment, with maximal amounts present at 2 h, followed by a decrease in amount.

LPS Treatment Induces MAPK and NF-κB Activities

As described above, pathways involving MAPKs and NF-κB are known to play important roles in the expression of many functions by activated mast cells, and LPS treatment of mast cells will activate JNK, ERK and p38

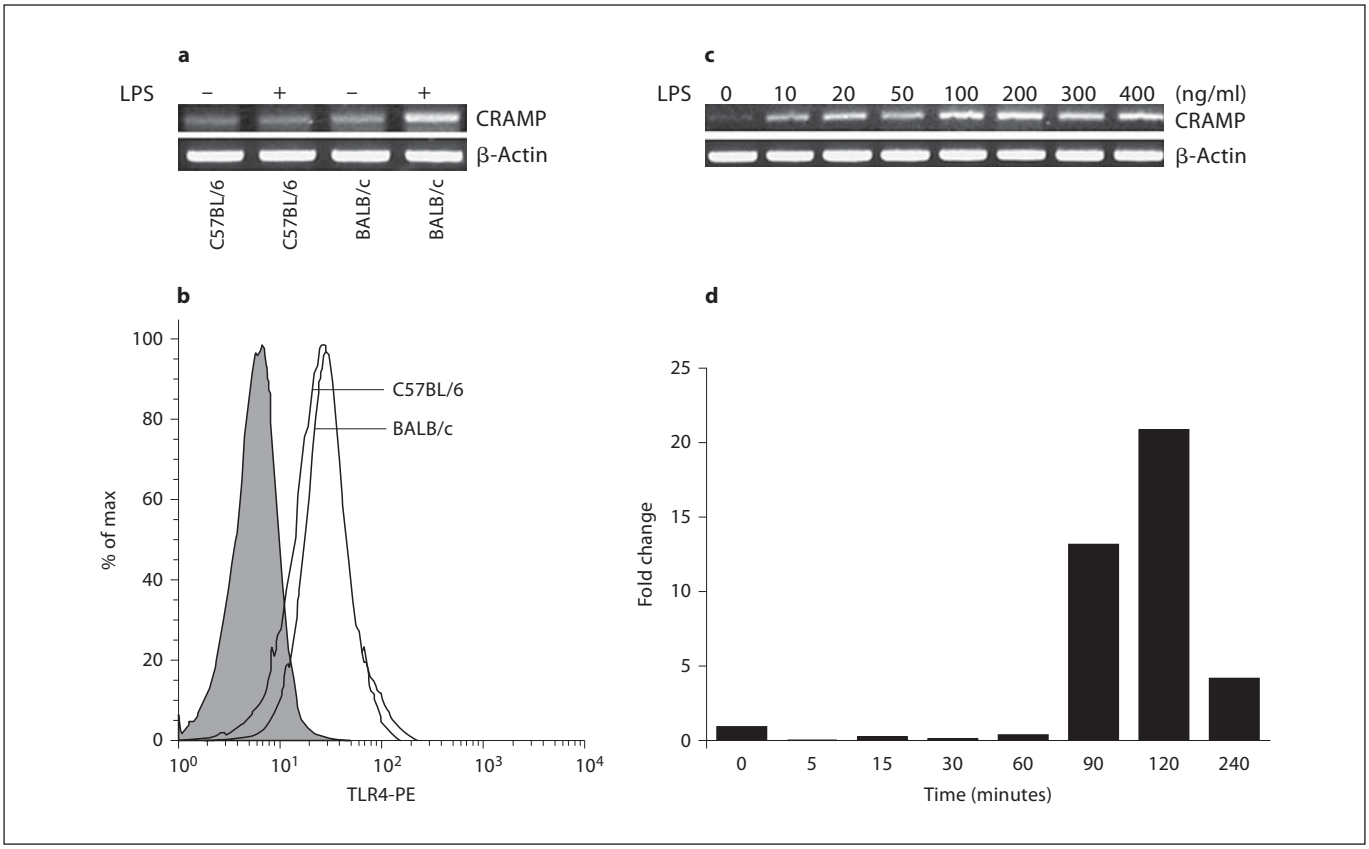

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Fig. 1. Induction of CRAMP expression by LPS. **a** BMMCs derived from C57BL/6 or BALB/c mice were stimulated with LPS (200 ng/ml) for 2 h. CRAMP levels were determined by RT-PCR. **b** TLR4 levels on the surfaces of BMMC derived from C57BL/6 and BALB/c mice were compared by flow cytometry using a PE-conjugated monoclonal anti-TLR4 antibody. Staining using the isotype control antibody is also shown, in grey. **c** BMMCs were stimulated with the indicated doses of LPS. Expression of CRAMP was detected by RT-PCR. The induction of CRAMP mRNA expression shows a dose-dependent response to LPS. Data are representative of 3 independent experiments. **d** Levels of CRAMP mRNA at various times after LPS addition were determined by quantitative real-time PCR, as described in 'Materials and Methods'. Data are representative of 2 experiments.

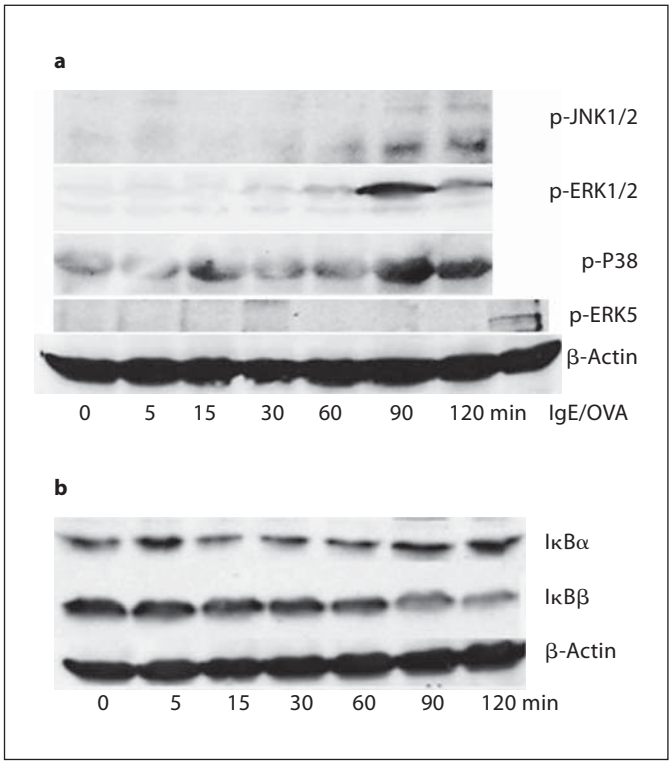

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Fig. 2. LPS stimulation induces MAPK phosphorylation and IκBα and IκBβ degradation. **a** Time courses of LPS-induced MAPK phosphorylations were determined using phospho-specific antibodies for the indicated kinases. The last lane in the p-ERK5 analysis shows phospho-ERK5 levels after stimulation with IgE/OVA, as a positive control. Data are representative of 2 independent experiments. **b** Time course of IκBα and IκBβ degradation as indicated by immunoblot analysis using antibodies specific for the proteins. There is a decrease in IκBα levels at 15–60 min after LPS addition and a subsequent decrease in IκBβ levels beginning at 90 min. Data are representative of 3 independent experiments.

MAPKs and also NF-κB. Under the conditions used here, LPS stimulation (with 200 ng/ml) clearly induced activation of JNK, p38 and ERK kinases, as indicated by immunoblot analysis using phospho-specific antibodies to these kinases (fig. 2a). MAPK (JNK, ERK and p38) activation was apparent at about 60–90 min after addition of LPS, which was much later and less robust than that normally seen with IgE/OVA stimulation of mast cells, which occurs by 15 min after addition of antigen [5, 28]. Furthermore, LPS did not appear to activate ERK5, as normally occurs after IgE/OVA stimulation (last lane of ERK5 immunoblot in fig. 2a) [3, 4, 28].

In nonstimulated cells, inhibitors of NF-κB, including IκBα and IκBβ, form complexes with NF-κB, which prevent NF-κB translocation from cytoplasm to nucleus where it functions as a transcription factor. Extracellular stimuli which activate NF-κB pathways, such as LPS, trigger the phosphorylation and subsequent proteasome-mediated degradation of IκBs, thus releasing NF-κB and permitting its translocation into nuclei [34]. Therefore, stimulation-induced IκB degradation can be taken as an indication of NF-κB activation. As shown in figure 2b, under the conditions used here IκBα levels showed a modest decrease by about 15 min after LPS stimulation and then returned to normal high levels at about 90 min. IκBβ levels showed a modest decrease beginning by 15 min after stimulation and a more dramatic decrease at about 90 min after LPS addition. Taken together, the results presented in figure 2 confirm that LPS treatment of BMMCs results in activation of MAPK and NF-κB pathways.

siRNA-Mediated Inhibition of JNK and p38 MAPK Accumulation Has No Effect on CRAMP Inducibility by LPS, but p65 Knockdown Abrogates It

Since JNK, p38 and ERK MAP kinases and NF-κB were all activated by LPS in BMMCs, their possible role in CRAMP inducibility was next investigated. BMMCs were transfected with specific siRNAs targeting JNK, p38 or the p65 component of NF-κB. After 72 h of transfection, the cells were isolated by centrifugation and each sample was divided into two parts. One part was used to examine specific siRNA-mediated protein knockdown by immunoblot analysis, and the other part was re-suspended in growth medium and stimulated by LPS or vehicle for 2 h. Figure 3a clearly shows that the 3 specific siRNAs resulted in substantial although not complete reductions in their target proteins without affecting overall β-actin levels. The JNK-specific siRNA reduced levels of both JNK1 and JNK2. Figure 3b shows that reduction of p65 NF-κB levels effectively eliminated CRAMP induction by LPS. In contrast, reduction of JNK or p38 kinase levels had little discernable effect on CRAMP induction by LPS. The role of ERK1/2 in CRAMP inducibility was not examined by the siRNA approach because a sequence which specifically and effectively knocked down ERK1/2 levels in BMMCs was not identified, despite repeated efforts. However, the effect of PD98059, an inhibitor of ERK1/2 phosphorylation [35], on the induction of CRAMP transcription in response to LPS stimulation was examined. It was first determined that ERK phosphorylation could be effectively abrogated in BMMCs using 6.0–8.0 μM PD98059, doses which did not affect activation-induced phosphorylation of JNK or p38 MAPK (data not shown). Therefore, BMMCs were pretreated with 8.0 μM PD98059 for 30 min before LPS stimulation. As shown in figure 3c, PD98059 had no effect on the induction of CRAMP transcription by LPS.

As shown above, reduction of p65 NF-κB levels effectively eliminated CRAMP induction by LPS. To examine the possibility that reduction of p65 NF-κB levels could lower TLR4 levels on the cells, thereby preventing effective LPS signaling, TLR4 levels on cells treated with either control or siRNA targeting the p65 component of NF-κB were compared by flow cytometry. As shown in figure 3d, no decrease in TLR4 levels was seen despite a substantial reduction of p65, which was demonstrated by the immunoblot analysis shown in figure 3e.

Effects of Overexpression of Mutant IκBα on CRAMP Induction by LPS Stimulation

In order to further investigate the requirement of NF-κB function in LPS-induced CRAMP expression, a phosphorylation-defective (S32A/S36A) IκBα which is resistant to activation-induced degradation was transfected into BMMC. As shown in figure 4a, transfection resulted in a substantial increase in IκBα levels. Figure 4b shows that expression of the phosphorylation-defective IκBα dramatically inhibited CRAMP inducibility by LPS in BMMCs, supporting the conclusion that activation of NF-κB is essential for LPS-stimulated induction of CRAMP gene transcription. Presence of the stable IκBα form also appeared to reduce the basal level of CRAMP mRNA in nonstimulated cells.

Effects of Overexpression of MEKK2 and MEKK3 on CRAMP Transcription by LPS Stimulation

It has been suggested that the IKK-NF-κB pathway could be an important downstream target for certain MAP3-kinases [36]. A possible role for MEKK2 or

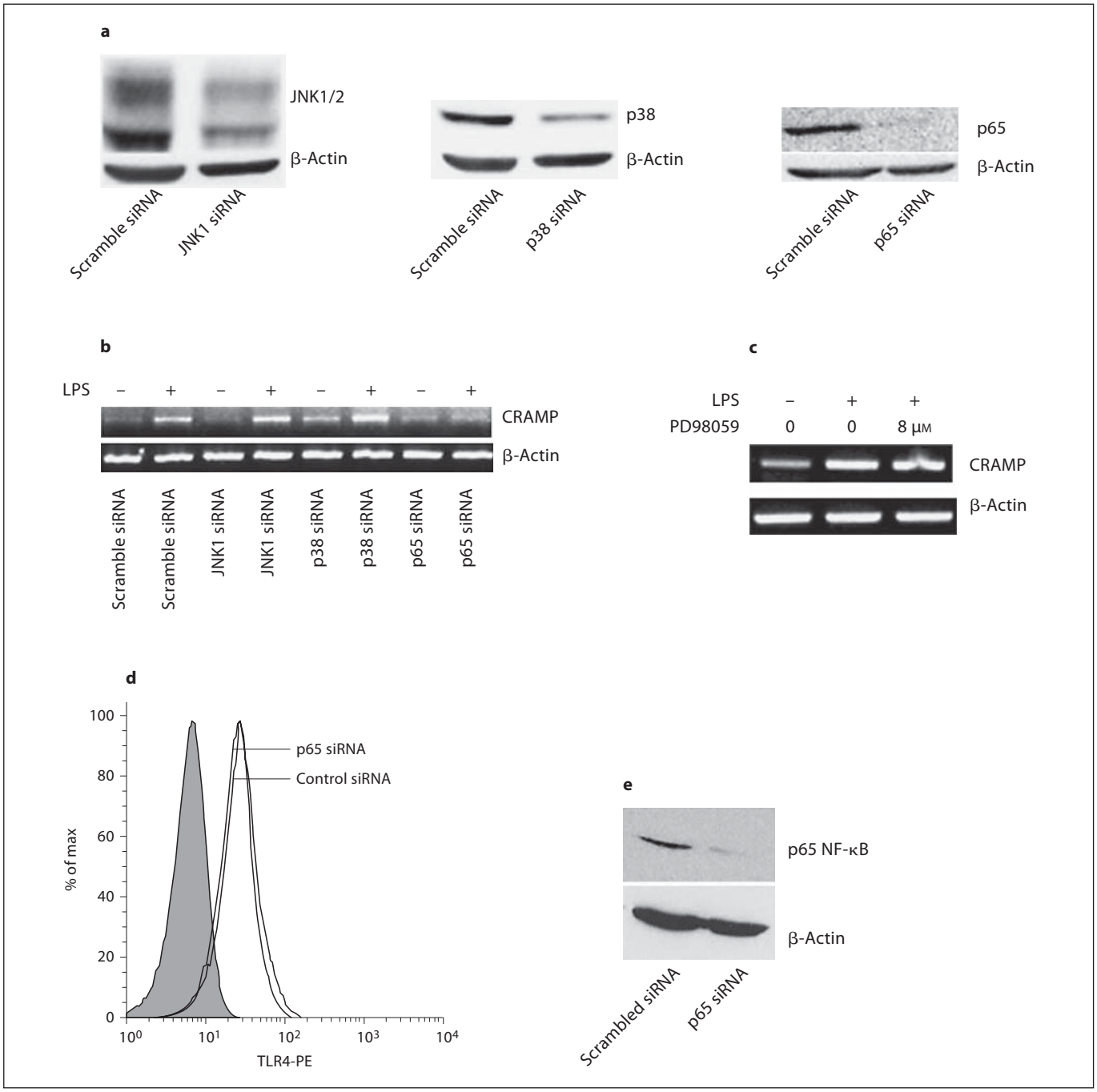

Fig. 3. Reducing levels of the p65 component of NF-κB, using siRNA, abrogates LPS-induced expression of CRAMP; reducing levels of JNK1/2 or p38, using siRNAs, or inhibiting ERK1/2 activation with PD98059, has no effect on LPS-induced expression of CRAMP. BMMCs were transfected with specific siRNAs (**a**). After 72 h of transfection, the cells were divided into 2 parts. One part was used to examine target protein levels by immunoblot analysis (**a**), the other part was divided in 2 and either stimulated with LPS or left untreated (**b**). Expression of CRAMP was detected by RT-PCR. The data are representative of 2 independent experiments. **c** BMMCs were pretreated with PD98059 for 30 min and then stimulated with LPS for 2 h and expression of CRAMP was examined by RT-PCR. The data are representative of 3 independent experiments. **d** TLR4 levels on the surface of BMMC which were transfected with the control siRNA or siRNA targeting the p65 component of NF-κB were compared by flow cytometry using a PE-conjugated monoclonal anti-TLR4 antibody at 72 h after transfection. Staining using the isotype control antibody is also shown (gray). **e** The immunoblot shows the reduction of p65 protein levels in the siRNA transfected cells, as compared to the level in cells transfected with the 'scrambled' control siRNA. Data are representative of 3 independent experiments.

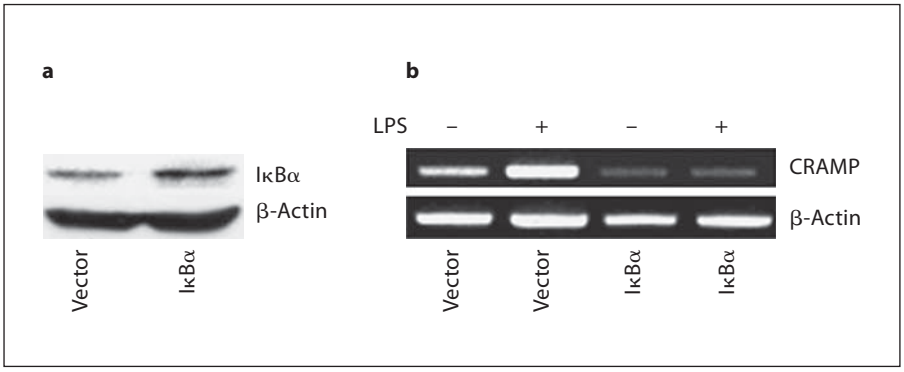

Fig. 4. Expression of phosphorylation defective (S32A/S36A) IκBα inhibits expression of CRAMP in response to LPS stimulation. BMMCs were transfected with a plasmid encoding phosphorylation-defective (S32A/S36A) IκBα or empty vector (control), respectively. After 48 h of transfection, the cells were divided into 2 parts. One part was used to assess transfection efficiency by immunoblot analysis with indicated specific antibodies (**a**). The other part was further divided into 2 parts and the cells were treated with LPS or vehicle (without LPS), as indicated. Expression of CRAMP was detected by RT-PCR (**b**). The data are representative of 2 independent experiments.

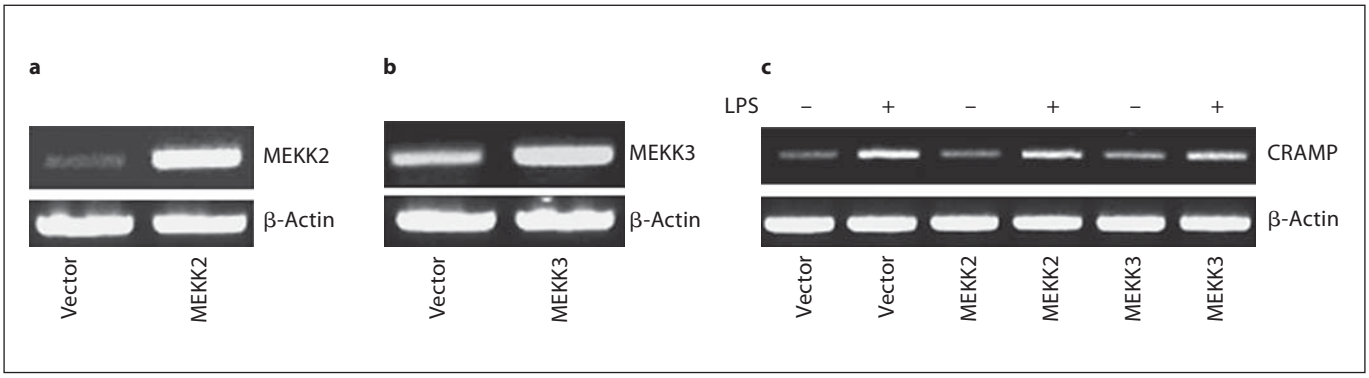

Fig. 5. Effects of overexpression of MEKK2 and MEKK3 on expression of CRAMP in response to LPS stimulation. BMMCs were transfected with plasmids encoding wild-type MEKK2, MEKK3 or empty vector, respectively. After 48 h of transfection, the cells were divided into 2 parts. One part was used to examine transfection efficiency as indicated by RT-PCR with indicated specific probes (**a**, **b**). The other part was further divided into 2 parts and the cells were treated with LPS or vehicle (without LPS), as indicated. Expression of CRAMP was detected by RT-PCR (**c**). Data are representative of 2 independent experiments.

MEKK3 function in LPS-mediated CRAMP induction in BMMCs was therefore investigated. BMMCs were transfected with wild-type MEKK2 and MEKK3, and after 48 h of transfection the cells were stimulated with LPS for 2 h. Figures 5a and b show dramatic increases of MEKK2 and MEKK3 mRNA content in the transfected cells. However, no clear change in LPS-induced CRAMP expression was seen in these cells (fig. 5c); that is, both basal and LPS-inducible levels of CRAMP mRNA in MEKK2- or MEKK3-transfected cells were similar to those seen in cells transfected with the empty vector.

Discussion

Mast cells are resident in tissues throughout the body, but are most common at sites that are exposed to the external environment, such as the skin, the airways, and the intestine. As such, mast cells are positioned to play a direct role in host defense against invading pathogens, in addition to their roles as modulators of other effector cell types [8]. Recent studies have indicated that mast cells can function to kill bacteria or inhibit their growth by expression and secretion of the cathelicidin-related anti-

microbial peptide CRAMP [9, 10]. As confirmed here, mast cell production of CRAMP is inducible by LPS [9]. The aim of the present study was to define the signal transduction pathways regulating LPS-mediated induction in BMMCs. Since LPS-mediated induction of key mast-cell effector molecules is known to involve both MAPK and NF-κB signal transduction pathways [26, 27], components of these pathways were investigated.

It was found that BMMCs prepared from 2 strains of mice, C57BL/6 and BALB/c, contained easily detectable levels of CRAMP gene transcripts. Surprisingly, CRAMP expression was only inducible by LPS in the latter strain. It appeared that this lack of inducibility in C57BL/6-derived BMMCs was not due to a deficit of TLR4, the receptor through which LPS acts [32, 33]. The molecular basis for this differential inducibility in the 2 strains is currently under further investigation. In particular, it will be determined if the signal transduction pathways initiated by LPS binding differ in the 2 mouse strains. It will also be of interest to determine basal levels of CRAMP peptide in the medium of the 2 types of BMMCs, since some cathelicidins have been reported to bind to and hence neutralize LPS [13, 37]. It has also been proposed that LL-37 modulates the LPS/TLR4-mediated inflammatory response by inhibiting nuclear translocation of NF-κB, thus decreasing production of TNF-α and other cytokines [21].

It was determined that CRAMP expression in BALB/c-derived mast cells was inducible by LPS, which also induces production of certain cytokines, including IL-13 [38, 39]. This is of interest since IL-13 (and IL-4) can reportedly *suppress* induction of cathelicidin production by some cell types, such as antigen-exposed keratinocytes [40]. In contrast, activation of mast cells with IL-4 appears to increase accumulation of cathelicidin protein [10]. It was also reported that skin obtained from patients with atopic dermatitis have decreased cathelicidin LL-37 levels compared to normal skin and thus supports high levels of vaccinia virus replication, as is characteristic of eczema vaccinatum [40]. Atopic dermatitis skin is characterized by overexpression of IL-4 and IL-13 [41]. Thus, although mast cells may be a source of cathelicidins, as described above, their presence and activation in skin could in fact, through production of certain cytokines, result in suppression of production of the antimicrobial peptides by other cell types.

As noted above, LPS stimulation through TLR4 binding can activate both MAPKs and NF-κB, which then mediate specific gene expression [26, 27]. It was determined that LPS treatment (at 200 ng/ml) of BALB/c-derived BMMCs activated the 3 major MAPK modules, involving JNK1/2, ERK1/2 and p38 MAPK. LPS treatment did not appear to activate ERK5, a kinase which clearly plays an important role in mast cell activation through FcεRI [4, 42]. However, treatment with pharmacological inhibitors or specific siRNAs to decrease levels of the kinases indicated that JNK, ERK and p38 MAPK do not play significant roles in the LPS-mediated inducibility of CRAMP gene expression. The MEKK inhibitor PD98059, which prevents phosphorylation and activation of ERK, was used because specific siRNA constructs which specifically reduced ERK1/2 levels in BMMCs could not be identified. Finally, it was shown that overexpression of 2 upstream components of MAPK activation, MEKK2 and MEKK3, had no significant effect on CRAMP gene induction by LPS. The present study did not rule out the possibility that MAPKs might be involved in the translation, processing, or secretion of CRAMP peptides, all aspects of active CRAMP production which require further investigation.

In contrast to these results, examination of the butyrate-induced activation of LL-37 expression in colon epithelial cells showed that the MEK inhibitor U0126 blocked LL-37 expression whereas the p38 inhibitor SB203580 did not [43], indicating a stimulatory role for ERK, but not p38 MAPK, in cathelicidin gene expression. A more recent study [44] suggests that butyrate-induced expression of CRAMP is dependent on both ERK and p38 MAPK activities. However, it has also been noted that proinflammatory mediators, including LPS, do not upregulate LL-37 production in colon epithelial cells [45], suggesting that the pathway described here in LPS-treated mast cells may differ from the butyrate-induced pathway in colonic epithelial cells. Cell type-specific differences in the pathways leading to cathelicidin gene induction have also been suggested by experiments showing that the MEK-ERK inhibitor U0126 blocked the butyrate-induced expression of LL-37 in gastric and colon carcinoma cell lines, but not in hepatocellular carcinoma cells [46]. Other studies indicate that the induction of LL-37 expression in epidermal keratinocytes by enforced expression of ASK1, a regulator of keratinocyte differentiation, is dependent on p38 activity [47] and that the induction of LL-37 expression in an epithelial cell line by exposure to *Mycobacterium bovis* BCG requires MEK1/2 and p38 activation [48].

The involvement of NF-κB activation in LPS-mediated induction of CRAMP was also investigated. The change in abundance of IκBα and IκBβ after LPS treatment of BALB/c BMMCs indicated that NF-κB activation

occurred in the system used here. Degradation of IκBs permits release and translocation of NF-κB to the nucleus, where it acts as a transcription factor [34]. The essential role of NF-κB activation in LPS-mediated CRAMP induction was then demonstrated by 2 approaches: (1) decreased abundance of the p65 component of NF-κB through the use of a specific siRNA effectively abrogated CRAMP induction, and (2) overexpression of an IκBα construct resistant to phosphorylation-dependent degradation also effectively abrogated CRAMP induction. In this regard, it is of interest that butyrate, an inhibitor of histone deacetylases which upregulates LL-37 transcription in colonic epithelial cells [43, 45, 46], reportedly prevents NF-κB activation by suppression of proteosome activity and stabilization of IκBα in these cells [49]. However, it has been noted that protein acetylation is involved in NF-κB regulation at multiple levels, including through direct acetylation of NF-κB p65 and p50 subunits, indirect modulation of IKK activity, and regulation of NF-κB-dependent gene accessibility, through histone modifications [50]. Thus, in some systems, deacetylase inhibitors, including butyrate, have been found to potentiate NF-κB activation [51]. The effects of histone deacetylase inhibitors on CRAMP gene induction in mast cells is under investigation; preliminary results indicate that the potent histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) [52] does not induce CRAMP gene expression in mast cells (data not shown).

It is also informative to compare the kinetics of LPS-mediated induction of CRAMP mRNA accumulation with the degradation of the IκBα and IκBβ inhibitors of NF-κB. As shown in figure 1d, increased CRAMP mRNA levels are first seen at about 90 min after LPS addition, which follows the transient decrease in IκBα levels seen in figure 2b and correlates with the major decrease observed in IκBβ levels beginning at about 90 min. Taken together with the observation that overexpression of the degradation-resistant form of IκBα abrogates the LPS-induced increase in CRAMP mRNA (fig. 4), the results suggest that degradation of IκBα is necessary but not sufficient for CRAMP mRNA induction and that full induction requires the subsequent degradation of IκBβ.

As noted above, studies of the involvement of MAPKs in cathelicidin gene expression have yielded conflicting results, which may reflect different modes of regulation dependent on species, cell or tissue type and/or the method of cell stimulation. Differences in the involvement of NF-κB in cathelicidin gene expression may also exist. Thus, despite the demonstration here of a clear role for NF-κB in LPS-inducible CRAMP gene expression in BMMCs, Buchau et al. [53] have recently suggested that NF-κB may play a negative regulatory role in cathelicidin gene expression. Their studies were performed with human keratinocytes treated with pimecrolimus and TLR2/6 ligand. A possible negative regulatory role for NF-κB in LL-37 expression would also be consistent with studies with corneal epithelial cells which were exposed to *Pseudomonas* after induction of tolerance by low-dose treatment with flagellin, which resulted in impaired activation of NF-κB (and also of p38 and JNK) pathways yet augmented production of LL-37 and β-defensin-2 [54]. In contrast, it has been reported that defensin expression can be induced by NF-κB in both intestinal epithelial cells and keratinocytes [55, 56]. Clearly, resolution of these different observations will require further comparative analyses.

The results presented here indicate that LPS-mediated CRAMP induction in mast cells is strongly dependent on NF-κB activity, but not on activation of MAPK modules involving JNK1/2, ERK1/2, ERK5 or p38 MAPK. This is of interest as most other major effector functions expressed by mast cells appear to be at least partially dependent on MAPK modules [1, 2, 28, 57]. The strong dependence on NF-κB activity which was observed is also noteworthy as defects in the NF-κB signal transduction pathway can lead to immunodeficiencies and increased susceptibility to infection [58], the latter of which could be due, at least in part, to decreased CRAMP production.

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