

# IgE-dependent sensitization increases responsiveness to LPS but does not modify development of endotoxin tolerance in mast cells

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## Abstract

**Objective** Effects of immunoglobulin E (IgE)-dependent sensitization on the response to bacterial lipopolysaccharide (LPS) were analyzed in mast cells.

**Methods** Murine bone marrow-derived mast cells (BMMCs) were sensitized or not with IgE before stimulation with LPS. TLR4 and co-receptors expression was analyzed by flow cytometry and RT-PCR, TNF- $\alpha$  production by ELISA, IKK and I $\kappa$ B activation by western blot or immunoprecipitation. NF $\kappa$ B nuclear translocation was determined by EMSA.

**Results** IgE-sensitized BMMCs secreted larger amounts of TNF- $\alpha$  than non-sensitized cells shortly after LPS challenge. No change in TLR4, CD14 or MD-2 expression was detected after the IgE-dependent sensitization process, whereas TLR4-dependent phosphorylation of IKK and I $\kappa$ B was augmented. IgE-dependent sensitization increased basal NF $\kappa$ B activity. Endotoxin tolerance was not affected by the IgE-dependent sensitization process.

**Conclusions** IgE-induced sensitization primes mast cells for higher response to LPS through pre-activation of NF $\kappa$ B transcription factor. IgE-dependent sensitization does not modify events leading to endotoxin tolerance.

**Keywords** Mast cell · IgE · Fc $\epsilon$ RI · TLR4 · Endotoxin tolerance

## Introduction

Mast cells have been identified as central players in the initiation and maintenance of allergic and other inflammatory reactions [1]. Located in all the vascularized tissues in mammals, they migrate from the bone marrow as immature precursors and finish their differentiation under the influence of locally produced mediators [1, 2]. Long-term exposure of mast cells to monomeric immunoglobulin E (IgE) induces a sensitization process, consisting of a series of changes that include an increase in the high affinity IgE receptor (Fc $\epsilon$ RI) expression in plasma membrane [3, 4], augmented cell survival [5] and histamine content. Sensitization improves a subsequent response to Fc $\epsilon$ RI crosslinking, i.e., cells are able to secrete larger quantities of interleukin (IL)-4, IL-3,  $\beta$ -hexosaminidase, serotonin, tumour necrosis factor (TNF)- $\alpha$ , CCL3 and other mediators after IgE/antigen stimulation than non-sensitized cells [6, 7]. Changes attributable to mast cell sensitization augment the intensity of allergic reactions in atopic individuals [8].

Besides their role in allergic reactions, mast cells are important for innate immune responses [9], since they produce a number of pro-inflammatory cytokines after the activation of Toll-like receptors (TLRs) [10]. Lipopolysaccharide (LPS) from *Escherichia coli* is able to activate TLR4 receptor on mast cells, leading to the activation of the IKK-I $\kappa$ B-NF $\kappa$ B pathway and the secretion of pro-inflammatory mediators [11]. A number of studies utilizing diverse models of endotoxemia have shown that mast cells are the main source of TNF- $\alpha$  in the peritoneal cavity of mice and that TLR4-mediated secretion of that cytokine is

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an essential modulator of immune responses against bacterial infections [12, 13].

Prolonged exposure to LPS causes a hypo-responsive state characterized by the absence of response to a second LPS challenge (endotoxin tolerance) [14]. This condition is originated and sustained by the expression of negative regulators of the TLR4 signal transduction system and the synthesis of anti-inflammatory mediators, such as transforming growth factor (TGF)- $\beta$  [15].

Despite the number of studies that have characterized the effects of IgE-dependent sensitization on a later stimulation with IgE/antigen complexes, information on the consequences of such sensitization for the responses to other ligands, such as LPS, is scarce. We decided to explore the effects of IgE-dependent sensitization on TNF- $\alpha$  production after LPS challenge and also on the establishment of endotoxin tolerance in murine bone marrow-derived mast cells (BMMCs).

## Materials and methods

### Mice

Four- to eight-weeks-old 129S1/SvImJ mice (Jackson Laboratories Stock # 002448) were euthanized by CO<sub>2</sub> inhalation. Bone marrow was extracted from both tibias and fibulas by injection of 3 ml of complete cell culture media (see following sections) directly into the hollow of the bones. Procedures utilizing animals were approved by our Institute's Committee for Experimental Use and Animal Care (CICUAL, Protocol # 383-07).

### Cell culture

BMMCs were generated by culturing bone marrow in RPMI 1640 culture media with L-glutamine and 25 mM HEPES buffer (Sigma, San Louis, MO, USA). Media was also supplemented with 10% Fetal Calf Serum (FCS), 100 U/ml penicillin–100 g/ml streptomycin (GIBCO, Foster City, CA, USA), nonessential amino acids (Biofluids, Camarillo, CA, USA), 100 mM sodium pyruvate (Biofluids), 5  $\mu$ M  $\beta$ -mercaptoethanol (Sigma) and 20 ng/ml murine IL-3 (Peprotech, Rocky Hill, NJ, USA). Cell cultures were grown at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. Four weeks later, expression of the Fc $\epsilon$ RI receptor was assayed by flow cytometry, and only the cultures showing more than 95% positive cells were considered mature and used for the assays.

### Flow cytometry

Routine flow cytometry for detection of Fc $\epsilon$ RI receptor in mature cultures was performed by incubating  $2 \times 10^6$  mast

cells for 1 h with 300 ng/ml of a monoclonal anti-dinitrophenol (DNP)-IgE (Sigma, clone SPE-7) in staining buffer (PBS pH 7.4, 5 g BSA and 0.5 g of sodium azide, Sigma). After this incubation, cells were washed once and incubated for 30 min in the presence of a FITC-labeled anti-IgE antibody (BD Biosciences, San Jose, CA, USA). Cells were washed again and resuspended in staining buffer until FACS analysis. For TLR4 receptor detection in sensitized and non-sensitized mast cells,  $2 \times 10^6$  BMMCs were incubated with an anti-TLR4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in staining buffer for 30 min and, after washing the cells, a secondary PE-labelled anti-IgG2A (Santa Cruz Biotechnology) was added for 30 min. After a second wash, stained cells were analyzed in a FACScan apparatus (BD Biosciences).

### Cell sensitization and stimulation

Mature BMMCs were diluted in complete BMMC media to  $10^6$  cells/ml. In the case of sensitized mast cells, 300 ng/ml of a monoclonal anti-DNP-IgE (Sigma) was added for 18 h. After the sensitization step, cells were washed once and resuspended in fresh cell culture media in the presence of 500 ng/ml LPS. Cells were kept at 37°C for different times as needed. After stimulation, cell supernatant was collected and TNF- $\alpha$  secretion was determined by a specific ELISA kit (see following sections).

### RNA isolation and RT-PCR

After cell stimulation, cells were collected and used for total RNA extraction with TRI-Reagent (Sigma). RNA pellets were resuspended in 50  $\mu$ l of RNA secure solution (Ambion, Foster City, CA, USA) and quantified by light absorption at 260 nm.

First strand cDNA synthesis was performed using the First Strand Synthesis Kit from Invitrogen (Carlsbad, CA, USA). Two microliters from each final reaction were used to amplify murine TLR-4, MD-2, CD14 and GAPDH mRNAs. Primers used for RT-PCR were as follows: TLR-4 sense 5' GCA-ATG-TCT-GGC-AGG-TGT-A 3' and antisense 5' CAA-GGG-ATA-AGA-ACG-CTG-AGA 3' [16]; MD-2 sense 5' ATG-TTG-CCA-TTT-ATT-CTC-TTT-TCG-ACG 3' and antisense 5' ATT-GAC-ATC-ACG-GCG-GTG-AAT-GAT-G 3' [17]; CD-14 sense 5' CGT-CTA-GAA-GAA-CAC-CAT-CGC-TGT-AAA-G 3' and antisense 5' GCT-CTA-GAA-TTC-GTT-GAC-GAG-GAC-CCG-C 3' [18]; GAPDH sense 5' TGA-AGG-TCG-GTG-TGA-ACG-GAT-TTG-GC 3' and antisense 5' CAT-GTA-GGC-CAT-GAG-GTC-CAC-CAC 3' [19].

Amplification conditions were as follows: hot start at 94°C (5 min), denaturation at 94°C (1 min), annealing at 60°C (2 min 30 s), and extension at 72°C (4 min). The

optimal number of cycles was experimentally determined for each of the genes amplified and it was found that at 28 cycles all of them were on the linear range of the amplification curve. PCR products were separated by 2% agarose gel electrophoresis and were visualized by ethidium bromide staining. A 100-bp DNA ladder was included every time as a molecular weight marker.

## ELISA

Secreted TNF- $\alpha$  was analyzed with a commercial mouse TNF- $\alpha$  ELISA kit (Invitrogen) according to the manufacturer's instructions. The limit of detection of the assay was 3 pg/ml. All measurements were performed in triplicate. This assay is reported to show no detectable cross-reactivity with a range of other growth factors and cytokines.

## Western blot

Protein expression and phosphorylation were detected using  $2 \times 10^6$  sensitized and non-sensitized BMMCs resuspended in 100  $\mu$ l of Tyrode's BSA buffer (130 mM NaCl, 5 mM KCl, 1 mM MgCl<sub>2</sub>, 1.4 mM CaCl<sub>2</sub>, 5.6 mM glucose, HEPES 1 M, and 0.1% BSA). Cells were challenged with LPS (500 ng/ml) at 37°C for the times indicated in the Figures. After this, cell lysates were obtained by adding 100  $\mu$ l of 2 $\times$  Laemmli solution (Sigma), supplemented with 5 mM *O*-vanadate (Sigma) and 1 mM  $\beta$ -mercaptoethanol (Sigma). Proteins were separated by SDS-PAGE electrophoresis following the instructions of the XCell SureLock™ mini-cell from Invitrogen, including sucrose (8% final concentration) in the final gel mix. Fifty microliters of protein extracts were separated in a 10% bis-acrylamide-acrylamide gel, running for 1.5 h at 25 V. Proteins were transferred to a PVDF (Perkin Elmer, Boston, MA, USA) membrane using the XCell II Blot Module (Invitrogen) according to the manufacturer's instructions. After blocking of the membrane (non-fat milk, 4% in TBS-T/1 h), incubation with specific antibodies was carried out for 18 h at 4°C. The primary antibodies used were the goat anti-phospho IKK  $\alpha/\beta$ , Ser 181, sc 23470 from Santa Cruz Biotechnology and the mouse anti-actin, MAB1501R from Millipore. After this time, blots were washed three times during 20 min in TBS-T buffer (20 mM Tris HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20) at room temperature and the appropriate secondary antibodies (HRP-rabbit anti-mouse IgG or HRP-rabbit anti-goat IgG, from Zymed, Carlsbad, CA, USA) were added. Bands were detected using the Chemiluminescence Western Blotting Kit (Roche, Mannheim, Germany).

## Immunoprecipitation

For p-I $\kappa$ B immunoprecipitation, 30 million BMMCs were resuspended in a lysis buffer containing 1% NP-40

(Igepal), 20  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml pepstatin, 10  $\mu$ g/ml aprotinin, 1 mM PMSF, 5 mM sodium pyrophosphate, 1 mM orthovanadate and 60 mM  $\beta$ -octylglucoside dissolved in PBS pH 7.4. All detergents, inhibitors and salts were from Sigma. Lysates were placed on ice for 1 h and spun down at 20,000g for 15 min in a refrigerated centrifuge. Supernatants were taken and incubated for 3 h with 50  $\mu$ l of protein A-Sepharose beads loaded with a mouse monoclonal anti-Ser 32 phospho-I $\kappa$ B $\alpha$  antibody from Santa Cruz (sc-8404, B-9 clone) in a cold room. Sepharose beads were collected by gentle centrifugation and washed twice with 500  $\mu$ l of 0.1% NP-40 lysis buffer. A final wash was performed with 500  $\mu$ l of 0.01% NP-40 lysis buffer and, finally, beads were incubated with 50  $\mu$ l Laemmli buffer and boiled for 5 min. After centrifugation to remove the beads, 40  $\mu$ l of each sample were separated in a 10% acrylamide gel and transferred to a PVDF membrane, in order to detect immunoprecipitated phospho-I $\kappa$ B utilizing a different antibody (rabbit polyclonal anti-Ser 32/36, sc-101713) from Santa Cruz by western blot, as described in previous sections but using as a secondary antibody the HRP-anti-rabbit/mouse IgG included in the Chemiluminescence Western Blotting Kit (Roche).

## Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were obtained from  $10 \times 10^6$  BMMCs. Non-sensitized and sensitized BMMCs were stimulated with LPS as described in the previous section for 20 min. After this time, nuclear extracts were obtained following the instructions provided in the transcription factor activity (Trans-Am) analysis kit (Active Motif, Carlsbad, CA, USA). EMSA assays were performed using the LightShift Kit (Pierce Biotechnology, Rockford, IL, USA), following the manufacturer's instructions. The biotin-labelled oligonucleotide sequences for NF $\kappa$ B were: sense 5' Biotin-CAA-ATG-TGG-GAT-TTT-CCC-ATG-AGT-CTC-AAA-ATT-AGA-GAG 3' and antisense 5' Biotin-CTC-TCT-AAT-TTT-GAG-ACT-CAT-GGG-AAA-ATC-CCA-CAT-TTG 3' [20]. Protein-DNA complexes were resolved in an acrylamide-bisacrylamide (29/1%) gel in 0.5 $\times$  TBE for 1.5 h at 25 V. After this time, samples in the gel were transferred to a nylon membrane (Amersham, Pittsburgh, PA, USA) accordingly to the LightShift Kit instructions. Bands were developed using the Chemiluminescent Nucleic Acid Detection Module Kit (Pierce).

## Drugs

LPS from *E. coli* (serotype 026:B6) was obtained from Sigma. BAY 117085 was from Calbiochem (La Jolla, CA, USA).

## Statistical analysis

All data shown are expressed as mean  $\pm$  SEM of at least three independent experiments. Statistical analysis was performed using SigmaStat software (SYSTAT, 2004), ANOVA test. Differences between groups were considered significant at  $p < 0.05$ .

## Results

### IgE-dependent sensitization induces higher TNF- $\alpha$ secretion soon after LPS challenge in mast cells

In order to evaluate the effects of monomeric IgE-dependent sensitization on LPS-evoked TNF- $\alpha$  secretion in mast cells, murine BMMCs were incubated for 18 h with a monoclonal anti-DNP-IgE and TNF- $\alpha$  release after LPS challenge was evaluated at different times. As can be seen in Fig. 1, sensitization with IgE provoked an increased secretion of TNF- $\alpha$  that was mainly observed at the first hours after LPS addition. Non-sensitized cells produced  $5.1 \pm 2.0$ ,  $36.3 \pm 7.9$  and  $235 \pm 22$  pg/million cells at 1, 2 and 4 h after LPS (500 ng/ml) addition, respectively, whereas IgE-sensitized cells secreted  $263 \pm 19$ ,  $298 \pm 32$  and  $363 \pm 30$  pg/million cells in the same conditions. After 4 h of stimulation, TNF- $\alpha$  secretion in non-sensitized cells was, as reported [21], clearly detectable, but it was  $\sim 50\%$  lower than in sensitized BMMCs. At later times (8–24 h), the amount of secreted TNF- $\alpha$  was very similar between both groups of cells.

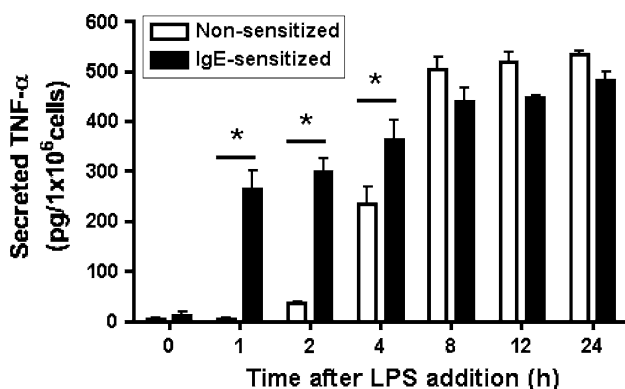


**Fig. 1** IgE-dependent sensitization increases early secretion of TNF- $\alpha$  after LPS challenge in mast cells.  $10 \times 10^6$  sensitized and non-sensitized BMMCs were plated in 6-well plates with 5 ml of fresh RPMI media and stimulated with LPS for the times indicated. At each time, supernatant was collected to evaluate TNF- $\alpha$  secretion mediated by ELISA. Values represent mean  $\pm$  SEM of at least three experiments. \* $p < 0.05$

IgE-dependent sensitization does not modify TLR4 receptor expression in plasma membrane or MD-2 and CD14 mRNA in mast cells; sensitization effects depend on Fc $\epsilon$ RI crosslinking

Since increased Fc $\epsilon$ RI receptor expression after IgE-dependent sensitization has been reported in mast cells, we decided to analyze whether pre-incubation with IgE could also cause a change in TLR4 receptor expression. As can be seen in Fig. 2a, non-sensitized and IgE-sensitized BMMCs showed similar TLR4 receptor expression in plasma membrane. Also, no detectable modification in the number of mast cells was obtained after the sensitization process (data not shown). No change was detected in the synthesis of either CD14, MD-2 or TLR4 mRNAs on IgE-sensitized mast cells (Fig. 2b).

To test the role of Fc $\epsilon$ RI crosslinking on the effects of monomeric IgE on TNF- $\alpha$  secretion, cells were sensitized with IgE in the presence of DNP-glycine, a widely utilized hapten able to separate IgE-induced Fc $\epsilon$ RI receptor aggregates, before LPS challenge. We observed that addition of monovalent hapten during the sensitization process was able to prevent the effect of monomeric IgE on TNF- $\alpha$  production after LPS challenge (Fig. 2c) and, as expected, did not modify LPS-induced TNF- $\alpha$  secretion in non-sensitized BMMCs.

Sensitization with IgE modifies the inhibitory effects of BAY 117085 on LPS-induced TNF- $\alpha$  secretion in BMMCs

TNF- $\alpha$  production in mast cells has been related to the activation of NF $\kappa$ B transcription factor [22]. In order to test whether IgE-dependent sensitization could modify the activity of the NF $\kappa$ B signaling pathway, the IKK selective inhibitor BAY 117085 [23] was utilized to block TNF- $\alpha$  release in non-sensitized and IgE-sensitized mast cells. Figure 3 shows that 0.2  $\mu$ M Bay 117085 was able to inhibit close to 50% LPS-induced TNF- $\alpha$  secretion observed in non-sensitized mast cells, whereas ten times more (2  $\mu$ M) inhibitor was necessary to prevent TNF- $\alpha$  release in IgE-sensitized BMMCs. Those results were suggestive of an increased IKK activity in IgE-sensitized mast cells.

Sensitization with IgE leads to increased IKK-I $\kappa$ B phosphorylation and NF $\kappa$ B nuclear translocation

In order to test whether IgE-sensitization induces changes in LPS-dependent activation of the NF $\kappa$ B transcription factor signaling pathway, phosphorylation of IKK and I $\kappa$ B was detected in non-sensitized and IgE-sensitized mast cells after LPS challenge. Figure 4a shows that, on IgE-sensitized cells, an increased basal IKK phosphorylation

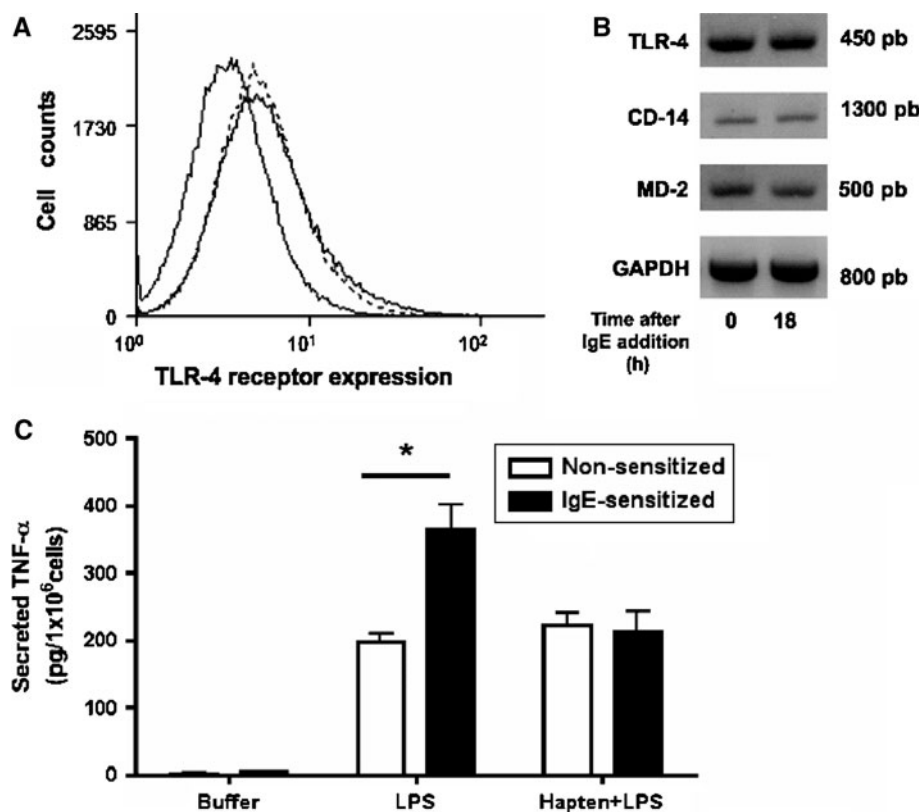


**Fig. 2** IgE-induced sensitization does not change TLR4 receptor expression in plasma membrane or co-receptor mRNA in BMDCs. Sensitization effects are dependent on Fc $\epsilon$ RI crosslinking. **a** Flow cytometry analysis of TLR-4 receptor expression. Mature BMDCs were sensitized or not with IgE and TLR4 expression in plasma membrane was determined by flow cytometry. Continuous low fluorescence line shows the isotype control, continuous high fluorescence line shows TLR-4 receptor expression in non-sensitized mast cells and dotted line shows TLR4 receptor expression in IgE-sensitized mast cells. A representative trace of three independent

determinations using cells from different cultures is shown. **b** RT-PCR of TLR4, CD-14 and MD-2 mRNA expression. Two million mast cells were sensitized or not with IgE and total RNA was isolated to perform RT-PCR analysis. **c** Effect of monovalent hapten on LPS-induced TNF- $\alpha$  secretion in sensitized or not sensitized cells. Two million mast cells were sensitized or not with IgE in the presence of DNP-glycine (1 mM). After the sensitization step, LPS was added and TNF- $\alpha$  secretion was determined 4 h later. Values represent mean  $\pm$  SEM of at least three experiments. \* $p < 0.05$

is observed when compared with non-sensitized BMDCs. Accordingly, a higher and sustained phosphorylation of I $\kappa$ B is detected in IgE-sensitized cells after LPS addition. In order to detect the effects of IgE-sensitization on LPS-dependent NF $\kappa$ B nuclear translocation, an EMSA was performed in non-sensitized and IgE-sensitized BMDCs after LPS treatment (Fig. 4b). An increased formation of the p65-containing complex was detected in non-stimulated IgE-sensitized cells and no further increase was observed after LPS stimulation.

Sensitization with monomeric IgE does not modify endotoxin tolerance development in mast cells

In order to analyze the effect of IgE sensitization on the development of endotoxin tolerance in mast cells, an experiment to measure the time necessary for endotoxin tolerance establishment in BMDCs was designed. Cells were treated with LPS and, at different times, cells were

washed, resuspended in fresh media and stimulated with a second addition of LPS. TNF- $\alpha$  release was measured 4 h after the second challenge. Figure 5 shows that non-sensitized and IgE-sensitized mast cells started to develop tolerance to a second addition of LPS between 2 and 4 h after the first stimulus. Almost total inhibition of TNF- $\alpha$  secretion in response to the second LPS challenge was obtained 12–24 h after the first stimulation. Although the time to develop tolerance was not affected by IgE-sensitization, it was possible to observe higher residual synthesis of TNF- $\alpha$  after the second stimulus in IgE-sensitized cells.

## Discussion

Mast cells are important in type I hypersensitivity reactions and also in protective innate responses against Gram-negative bacteria in mice [1]. Mast cell sensitization with

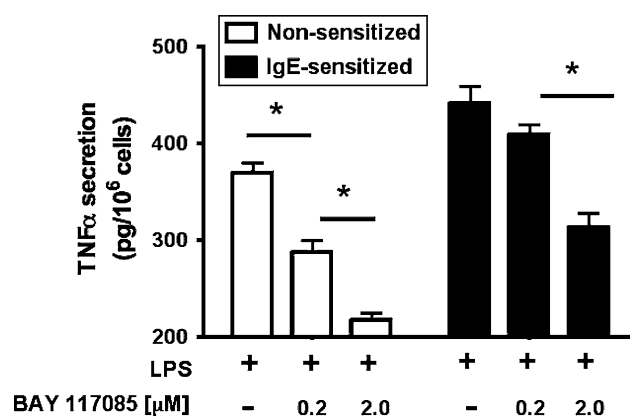


**Fig. 3** IgE-dependent sensitization modifies the effect of BAY 117085 on LPS-induced TNF- $\alpha$  secretion in BMMCs. Ten million BMMCs were sensitized or not with IgE as described in “Materials and methods”. After sensitization, cells were washed and divided into aliquots to be treated or not with different concentrations of BAY 117085 30 min before LPS addition. TNF- $\alpha$  secretion was determined 4 h after LPS challenge. Values represent mean  $\pm$  SEM of at least three experiments. \* $p$  < 0.05

monomeric IgE is a condition observed in atopic individuals and has been understood as a process that improves later IgE/antigen-triggered pro-inflammatory mediator secretion [8, 24]. In the present work, we analyzed the influence of IgE sensitization on the capacity of mast cells to respond to a well-known TLR4 ligand, such as LPS. We found that IgE-dependent sensitization importantly increases early TNF- $\alpha$  secretion after LPS addition in BMMCs.

TNF- $\alpha$  is a cytokine that is secreted under low- to medium-intensity stimulation of mast cells [25]. This potent pro-inflammatory mediator is not, however, pre-

stored in secretory granules in the mast cell model we used (BMMCs) [26]. We were able to confirm this since no important TNF- $\alpha$  release was observed before 4 h of LPS stimulation in non-sensitized mast cells. The early release of TNF- $\alpha$  observed in IgE-sensitized cells suggests that TNF- $\alpha$  is pre-synthesized and stored in a TLR4-sensitive reservoir in BMMCs. To date, no detailed knowledge exists about the molecular mechanism of coupling TLR4 stimulation to TNF- $\alpha$  secretion in mast cells, although it is known that in macrophages newly synthesized TNF- $\alpha$  is initially accumulated in the Golgi compartment [27]. Pro-TNF- $\alpha$  containing vesicles are then transported from the trans-Golgi network to the recycling endosomes and subsequently to the cell surface through two distinct membrane fusion processes: the first one is mediated by t-SNARE proteins, comprising syntaxin 6, syntaxin 7 and vit1B in a trans-Golgi TNF- $\alpha$  carrier vesicle and the v-SNARE VAMP3 in a recycling endosome. The second fusion process involves the VAMP3-containing recycling endosome and the t-SNARE complex at the plasma membrane comprising syntaxin 4 and SNAP-23 [28, 29]. Distinct modifications to mast cell physiology have been associated with the IgE-sensitization process; changes in membrane localization of SNARE proteins have been reported after 6 weeks of BMMC culture in the presence of IgE [30]. However, no detailed analysis of IgE-dependent changes in the secretory machinery of mast cells, which could modify early secretion of pro-inflammatory mediators after TLR4 receptor activation, has been performed.

Some molecular mechanisms of IgE-dependent sensitization to later stimulations with IgE/antigen have been previously described. Utilizing BMMCs, it has been shown

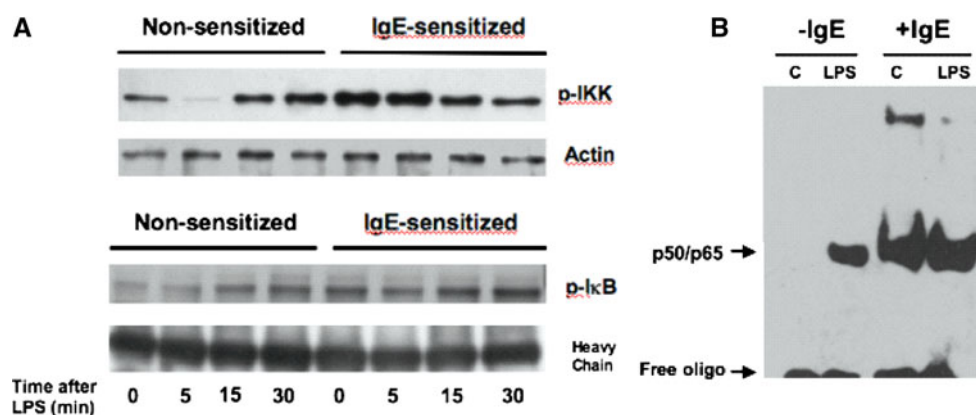


**Fig. 4** IgE-sensitized mast cells show increased IKK-I $\kappa$ B phosphorylation and NF $\kappa$ B nuclear translocation. **a** IKK and I $\kappa$ B phosphorylation in sensitized and non-sensitized BMMCs. Non-sensitized and IgE-sensitized mast cells were stimulated with 500 ng/ml LPS for different times and cell lysates were obtained as described in “Materials and methods”. IKK phosphorylation was detected by western blot; actin was included as a loading control. Phospho-I $\kappa$ B was analyzed by immunoprecipitation (IP) and the heavy chain of the

antibody used for IP was used as a loading control. One representative image of three independent experiments is shown. **b** Nuclear localization of NF $\kappa$ B. Ten million sensitized and non-sensitized BMMCs were stimulated or not with 500 ng/ml LPS for 20 min. Nuclear extracts and EMSA assays were performed as described in “Materials and methods” and a representative image of three independent experiments is shown

that monomeric IgE is able to increase Fc $\epsilon$ RI presence in plasma membrane [3, 4], and also induces the activation of early protein kinases such as Lyn, Syk, Btk, Akt and MAPKs [31, 32]. In accordance with the existence of a basal pre-activated state of mast cells induced by IgE-dependent sensitization, we observed an increased TNF- $\alpha$  secretion early after LPS challenge; however, this was not related to augmented TLR4 receptor expression in plasma membrane. This result indicates that the reported IgE-induced mechanism of up-regulation of membrane Fc $\epsilon$ RI is not shared by the TLR4 receptor.

The described effects of monomeric IgE on mast cells have been proven to be differentially dependent on Fc $\epsilon$ RI crosslinking. Whereas increase of IgE receptor on plasma membrane is insensitive to monovalent hapten addition [33], IgE-induced receptor internalization, degranulation, survival and IL-6 secretion are dependent on Fc $\epsilon$ RI crosslinking and on Lyn, Syk and Btk activities [33]. Our results show that increased TNF- $\alpha$  secretion after LPS challenge depends on IgE-induced Fc $\epsilon$ RI crosslinking, suggesting that intracellular kinases could play a role in the Fc $\epsilon$ RI-TLR4 cross-sensitization process.

TLR4-dependent TNF- $\alpha$  mRNA synthesis has been demonstrated to depend on the activation of the NF $\kappa$ B pathway [22] and an increase in the activity of this signaling cascade after IgE sensitization was suggested by the concentrations of BAY 117085 needed for inhibition of LPS-induced TNF- $\alpha$  production in IgE-sensitized versus non-sensitized mast cells. Notably, inhibition of TNF- $\alpha$  secretion by BAY 117085 was not complete, indicating that other pathways independent of IKK activation can importantly contribute to TNF- $\alpha$  production in LPS-activated mast cells. It has been reported that TNF- $\alpha$  gene promoter has consensus sequences for other transcription factors, such as AP-1, NFAT, and Jun/CREB/ATF2 [34]. Participation of transcription factors downstream of MAPK activation in TNF- $\alpha$  gene expression is possible since MAPK can be activated after TRAF6 ubiquitination in the TLR4 signal transduction system, in a signaling pathway independent of IKK [35].

In accordance with augmented IKK activity, I $\kappa$ B phosphorylation and NF $\kappa$ B nuclear translocation after LPS challenge was detected in IgE-sensitized cells. Distinct pathways could be responsible for the increased activity of NF $\kappa$ B. PI3K plays an important positive role in the observed effects of IgE on mast cell survival and NF $\kappa$ B activation, whereas SHIP is a negative regulator of the pathway [5]. Future experiments analyzing PIP3 production after IgE addition to mast cells will shed more light on the role of the PI3K on the increased effects of LPS. Our observation of an increased NF $\kappa$ B presence in nuclei of 18 h-sensitized mast cells indicates that low-intensity stimulation of Fc $\epsilon$ RI with monomeric IgE produces

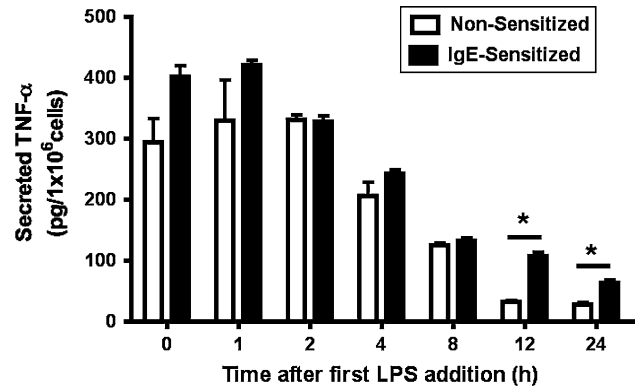


**Fig. 5** IgE-dependent sensitization does not modify the establishment of endotoxin tolerance in BMMCs. Ten million sensitized and non-sensitized BMMCs were treated with LPS (500 ng/ml). At distinct times after LPS addition, cells were washed once and plated in new fresh media. A second LPS stimulation was then performed and TNF- $\alpha$  was detected 4 h later. Values represent mean  $\pm$  SEM of at least three experiments. \* $p$  < 0.05

changes that result in long-term negative regulation or rapid desensitization.

Tolerance to LPS has been widely described as a physiological response of the cells to a prolonged stimulation with endotoxin. Molecular mechanism of endotoxin tolerance has been described mostly in macrophages, where the expression of a number of proteins prevents a second response to LPS [14]. Described changes produced by the first stimulation with LPS range from the expression of early negative regulators of the TLR4 signaling pathway to long-term effects on downregulation of transcription factors [14]. In mast cells, an increased expression of SHIP and the secretion of the immunomodulatory cytokine TGF- $\beta$  have been related to endotoxin tolerance [15]. Despite the changes we observed in early TNF- $\alpha$  secretion after LPS addition, and the increased NF $\kappa$ B activity in IgE-sensitized BMMCs, we were not able to detect any change in the time needed for the establishment of the endotoxin tolerance state. Our results show that the signaling pathways mediating LPS tolerance in mast cells are not affected by the IgE-dependent sensitization process, and indicate that signaling cascades leading to endotoxin tolerance are independent of those pre-activated by IgE.

The relationship between Fc $\epsilon$ RI and TLR4 signal transduction systems in BMMCs has been explored recently [36]. After simultaneous stimulation of BMMCs by monomeric IgE and TLR4 ligands, a synergistic augmentation of inflammatory cytokine production was observed and this increase was dependent on MAPK activation [36]. Those studies strongly suggest the existence of common elements shared by the signaling cascades activated by Fc $\epsilon$ RI and TLR4 receptors in mast cells. Our results support that conclusion, because characterizing the

effects of IgE on LPS-elicited mast cell responses in a different condition (where IgE was added long before LPS), we found that IgE was able to modify some parameters of BMMC responsiveness to LPS stimulation. In contrast with the reported synergistic augmentation of cytokine production when IgE and LPS were added at the same time, we found that effects of monomeric IgE were dependent on FcεRI aggregation. This difference can be attributable to experimental conditions, since we incubated BMCMs with low concentrations of IgE (300 ng/ml) for a longer time (18 h) before LPS addition, and it has been suggested that a low-rate FcεRI receptor aggregation could be responsible for low-intensity signaling [37].

Some observations indicate that *in vivo* IgE sensitization of mast cells is able to increase duration and the extent of allergic reactions. Atopic individuals produce large amounts of IgE, priming basophils, eosinophils and mast cells to respond with higher intensity after allergen-dependent FcεRI activation [8, 24]. Our results support the idea that atopy can regulate the outcome of mast-cell-dependent innate immune responses, either activating pre-existing FcεRI and TLR4 common cooperative pathways [36] or also inducing changes on mast cell capability of pro-inflammatory cytokine release after TLR4 receptor triggering.

The results presented here suggest that low-intensity stimulation of FcεRI is able to improve TLR4 receptor signaling, mainly by generating a pre-stored pool of TNF-α and probably inducing the synthesis of new intracellular effectors of the TLR4 signal transduction system. Interestingly, this process seems not to include those molecules related to the capacity to develop endotoxin tolerance, since the inhibition of TNF-α secretion after a second stimulus with LPS was observed independently of the IgE sensitization step. These latter results suggest that proteins leading to tolerance development are not necessarily part of the shared elements in the FcεRI and TLR4 signaling pathways.

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