



Betalactam antibiotics affect human dendritic cells maturation through MAPK/NF- κ B systems. Role in allergic reactions to drugs☆



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ABSTRACT

The mechanisms leading to drug allergy in predisposed patients, especially those related to T-cell-mediated drug hypersensitivity, are not well understood. A key event in allergic reactions to drugs is the maturation process undergone by dendritic cells (DCs). Although amoxicillin (AX) has been reported to interact and mature DCs from patients with AX-induced delayed-type hypersensitivity, the cell signaling pathways related to AX-mediated DC maturation have not been elucidated. We sought to determine the role of the MAPK and NF- κ B pathways on AX-induced DC maturation and functional status. For that purpose, in monocyte-derived-DCs from AX-delayed allergic patients and tolerant subjects, we analyzed the activation pattern of p38MAPK, JNK, and ERK signaling and the NF- κ B, maturation markers as well as endocytosis and allostimulatory capacities driven by AX-stimulated-DCs. Our data reveal that AX induces an increase in the phosphorylation levels of the three MAPKs and activated NF- κ B in DCs from allergic patients. Moreover, the inhibition of these pathways prevents the up-regulation of surface molecules induced by AX. Additionally, we observed that the allostimulatory capacity and the endocytosis down-regulation in AX-stimulated-DCs from allergic patients depend on JNK and NF- κ B activities. Taken together, our data shed light for the first time on the main signaling pathways involved in DC maturation from AX-delayed allergic patient.

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Introduction

Progress in the understanding of the pathophysiology of drug allergy needs a careful analysis of the early molecular and cellular events

Abbreviations: APC, antigen-presenting cells; AX, amoxicillin; cpm, counts per minute; DCs, dendritic cells; DMSO, dimethylsulfoxide; DNCB, dinitrochlorobenzene; DNFB, 2,4-dinitrofluorobenzene; DPT, drug provocation test; ENDA, European network for drug allergy; ERK, extracellular signal-regulated kinases 1/2; imDCs, immature dendritic cells; JNK, c-Jun N-terminal kinases; LPS, lipopolysaccharide; MAPKs, mitogen-activated protein kinases; MFI, mean fluorescence intensity; MHC, major histocompatibility complex; NF- κ B, nuclear factor kappa B; PBMC, peripheral blood mononuclear cells; p38 MAPK, p38 mitogen-activated protein kinase; TLR, toll like receptors.

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occurring in key immune competent cells. Dendritic cells (DCs) which serve as sentinels of the immune system, are the most potent antigen-presenting cells (APC) and are essential for initiating T-cell responses after undergoing the critical process of maturation (Bousso, 2008). Immature DCs (imDCs) exhibit high phagocytic and antigen processing features, but poor antigen presentation capabilities (Steinman, 2003; Reis, 2006). DCs can be activated by danger signals such as proinflammatory cytokines, viruses, LPS, allergens and haptens (contact sensitizers, drugs), which then initiate their maturation process (Roake et al., 1995; Rodriguez-Pena et al., 2006; Toebak et al., 2009; Gomez et al., 2012). At this point, DCs lose their ability to internalize antigens, with subsequent up-regulation of membrane surface MHCII and other costimulatory molecules in order to gain the capacity to present antigens to naïve T-cells (Bousso, 2008).

Different studies have shown the role of DCs in the response to low-molecular-weight compounds, such as nickel or dinitrochlorobenzene (DNCB), indicating they are able by itself to produce DC maturation, which then induces a specific T-cell response (Enk and Katz, 1992; Arrighi et al., 2001; Ade et al., 2007). The capacity of other haptens, such as drugs, to induce DC maturation has been shown for betalactam (BL) antibiotic (Rodriguez-Pena et al., 2006; Sebastian et al., 2012),

heparins (Lopez et al., 2009), sulfamethoxazole (Sanderson et al., 2007; Sebastian et al., 2012) and radio contrast media (Antunez et al., 2011). Moreover, recently it has been demonstrated that the presence of different TLR ligands could be critical for the induction of the innate and adaptive immune responses. The inclusion of these ligands is important for the evaluation of allergic reactions to delayed-type hypersensitivity (DTH) to BL such as amoxicillin (AX) (Sanchez-Quintero et al., 2013), one of the most common antibiotics reported in drug allergy. However, there are no details about the signaling pathways involved in their driven DC maturation process.

Mitogen-activated protein kinases (MAPKs) are one of the most classic signal transduction pathways in physiological processes and have been reported to be involved in the DC maturation (Nakahara et al., 2006). There are three main MAPK pathways, p38-mitogen-activated protein kinase (p38 MAPK), Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase 1/2 (ERK) demonstrated in both monocyte-derived DCs (Arrighi et al., 2001) and human CD34⁺-derived DCs (Boisleve et al., 2005). In this sense, the involvement of MAPK and NF- κ B in DC maturation induced by TNF- α , LPS and immunotherapy agents such as OK-432 has been described, indicating their influence in the phenotype and functional aspects of these cells (Aiba et al., 2003; Boisleve et al., 2005; Pan et al., 2009). Most studies with haptens in DC maturation have focused on the role of contact sensitizers and irritant molecules. Moreover, data show that nickel, DNCB and 2,4-dinitrofluorobenzene (DNFB) induce the phosphorylation of p38 MAPK (Aiba et al., 2003) and, in a recent work, it has been shown that NiSO₄ activates the complementary signaling pathways MAPK, NF- κ B and interferon regulatory factor-1 (IRF-1) (Antonios et al., 2010).

However, the different pathways involved in DC maturation induced by other haptens, such as drugs, have not been sufficiently studied. Moreover, understanding these pathways may explain why some individuals develop allergic reactions after drug intake while others remain tolerant. Bearing in mind that MAPK and NF- κ B are the two major pathways involved in DC maturation induced by danger signals and that the analysis of the different pathways has thus far received little attention, the aim of this study was to analyze the regulation by AX of the three MAPKs and NF- κ B pathways during BL induction of DC maturation *in vitro* from patients who had a DTH after intake of this BL in comparison with subjects who tolerated the drug.

Materials and methods

Ethics statement

The institutional review board “Comisión de Ética y de Investigación del Hospital Regional Universitario Carlos Haya” approved the study (#04/2009). These studies were carried out in accordance with the Declaration of Helsinki. Oral and written informed consent for all the diagnostic procedures was signed by both patients and controls.

Patients and controls

The study involved 14 patients, 12 women and 2 men (mean age, 49.6 $\pm$ 11.7 years old) with a delayed response (exanthematic reactions, erythematous macules or papules, often symmetric and typically polymorphous) to AX and a positive delayed intracutaneous test to AX, according to the general ENDA guidelines (Barbaud et al., 2001; Romano et al., 2004). Exclusion criteria were lower than 18 years old, being treated with immunomodulatory drugs or having any other immunological disease apart from allergy.

Clinical characteristics are presented in Table 1. All patients were skin-test positive to AX and negative to benzylpenicillin derivatives (benzylpenicilloyl-poly-L-lysine and minor determinant mixture). The mean time interval between the reaction and the allergologic-work-up with sample obtention was 154.8 $\pm$ 113.8 months.

Table 1
Clinical characteristics of the patients.

Patient	Sex	Age	Reaction	Drug involved	Time interval (months)	Skin test		
						PPL	MDM	AX
P1	F	63	Exanthema	AX	220	—	—	+
P2	F	54	Exanthema	AX	195	—	—	+
P3	F	42	Exanthema	AX	156	—	—	+
P4	F	59	Exanthema	AX	55	—	—	+
P5	F	51	Exanthema	AX	264	—	—	+
P6	F	45	Exanthema	AX	90	—	—	+
P7	F	56	Exanthema	AX	264	—	—	+
P8	F	18	Exanthema	AX	144	—	—	+
P9	F	51	Exanthema	AX	12	—	—	+
P10	M	41	Exanthema	AX	12	—	—	+
P11	F	59	Exanthema	AX	312	—	—	+
P12	M	47	Exanthema	AX	23	—	—	+
P13	F	54	Exanthema	AX	168	—	—	+
P14	F	55	Exanthema	AX	252	—	—	+

AX: amoxicillin; PPL: benzylpenicillin-poly-L-lysine; MDM: minor determinant mixture.

Tolerant subjects (controls) comprised 15 sex- and age-matched subjects with skin test negative to AX and confirmed tolerance to any penicillin included AX.

Sample collection and cell culture

Samples from patients and controls were processed following current procedures and frozen immediately after their reception and kindly provided by the BBSSPA, unit Regional University Hospital of Málaga.

Peripheral blood mononuclear cells (PBMC) were isolated from 40 ml of blood from both patients and controls by Ficoll-HyPaque density gradient (GE Healthcare, Buckinghamshire, UK). In the former group PBMC were obtained more than three months after reactions subside. Monocytes were purified from PBMC by positive selection with anti-CD14 magnetic microbeads following the manufacturer's protocol (Miltenyi Biotec, Bergisch Gladbach, Germany), showing a purity of more than 95%, as assessed by flow cytometry (data not shown). CD14⁺ cell fractions were then incubated with anti-human CD3 conjugated microbeads to isolate T lymphocytes, which were frozen for further use.

Monocytes (CD14⁺ cells) were cultured in complete medium, composed of RPMI 1640 medium (BioWhittaker, Pittsburgh, PA) supplemented with 2 mM L-glutamine (BioWhittaker), 5 mg/ml gentamycin (Normon, Madrid, Spain) and either fetal bovine serum (FBS) for the generation of monocyte-derived DCs or 10% autologous serum for all subsequent experiments. When analyzing MAPK phosphorylation and NF- κ B activation, imDCs were cultured in complete medium without serum.

Generation of monocyte-derived DCs

Monocyte-derived DCs were generated from CD14⁺ monocytes as described (Sallusto and Lanzavecchia, 1994) by culturing them in complete medium with FBS but adding 200 ng/ml GM-CSF and 100 ng/ml IL-4 (both from R&D Systems Inc, Minneapolis, MN) at 5% CO₂ at 37 °C as described (Lopez et al., 2009).

Activation assays of imDCs

On day 5, imDCs were treated with AX (Clamoxyl®, Smithkline-Beecham, Australia) at 250 μ g/ml, which showed to be the optimal concentration for DC maturation and proved to be non-cytotoxic in previous studies (Rodríguez-Pena et al., 2006; Sanchez-Quintero et al., 2013). Cell viability was assayed using the trypan blue exclusion method, this concentration should not lead to a viability of less than 75%, in order to demonstrate that AX does not affect cell viability or maturation of DCs in both patients and controls. We used LPS of *Escherichia coli* 0127:B8 (Sigma-Aldrich, Saint Louis, MO) at 1 μ g/ml or 50 μ g/ml

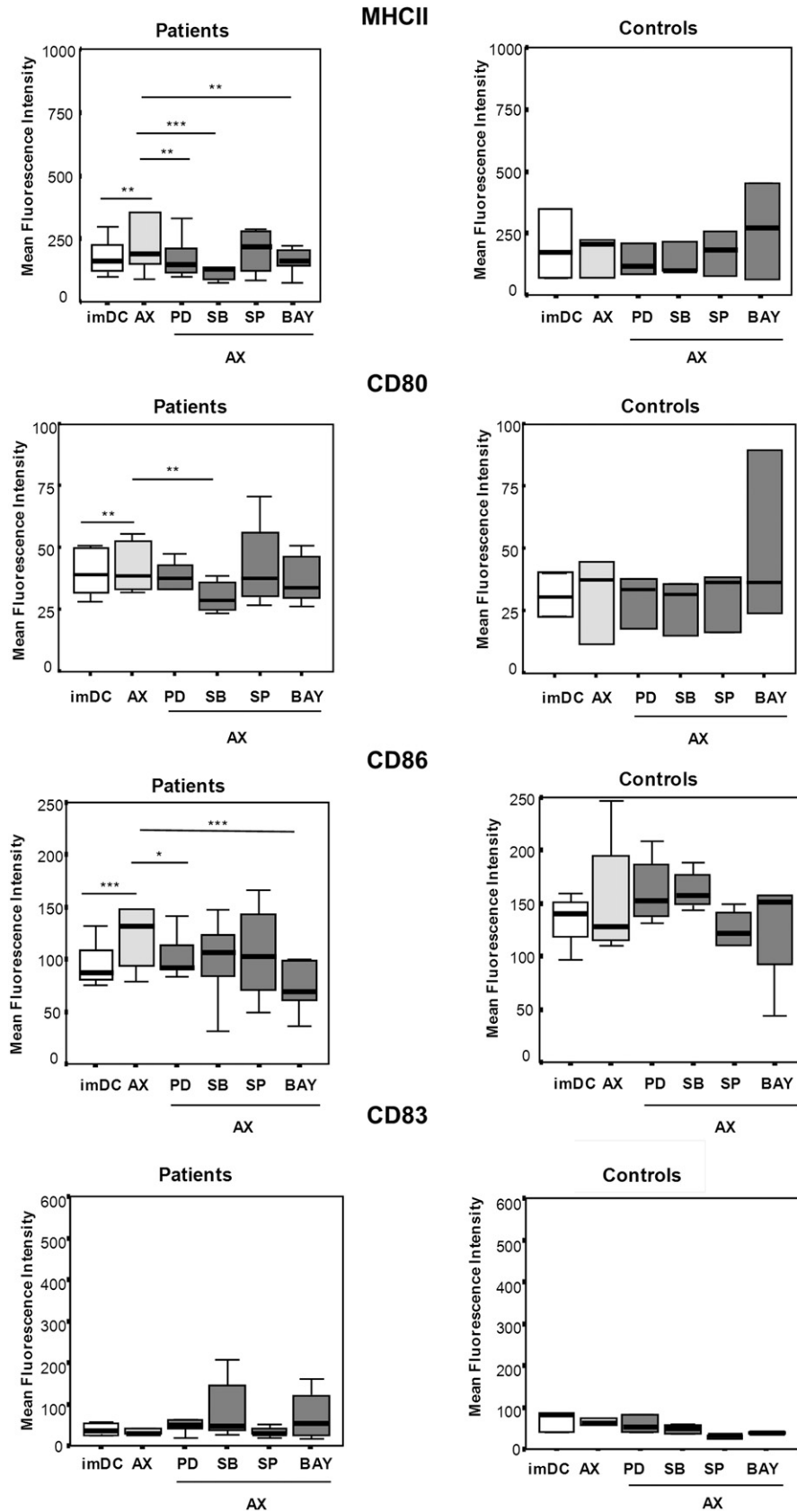


Fig. 1. Expression of different maturation markers in imDCs. Box-plots represent differential expression of maturation markers (MFI for MHCII, CD80 and CD86 and percentage of cells for CD83) in imDCs from patients ($n = 14$) and controls ($n = 15$) in the presence or absence of AX. Number of events acquired = 3000. Prior incubations with specific inhibitors were designated as PD for PD98059, SB for SB203580, SP for SP600125, and BAY for BAY11-7085. Differences with a non-parametric test for related samples (Wilcoxon test) were considered to be significant if $P < 0.05$; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

and TNF α (from R&D Systems Inc, Minneapolis MN) at 100 U/ml or 500 U/ml as positive controls, depending on the incubation time. The activation of three different MAPKs as well as NF- κ B was analyzed in both

AX- and LPS-sensitized DCs at different time intervals (0, 5, 10, 15, 30, 45, 60, 75 and 90 min). In some experiments, imDCs were pre-treated for 1 h with SB203580 (20 μ M), PD98059 (40 μ M), SP600125 (20 μ M)

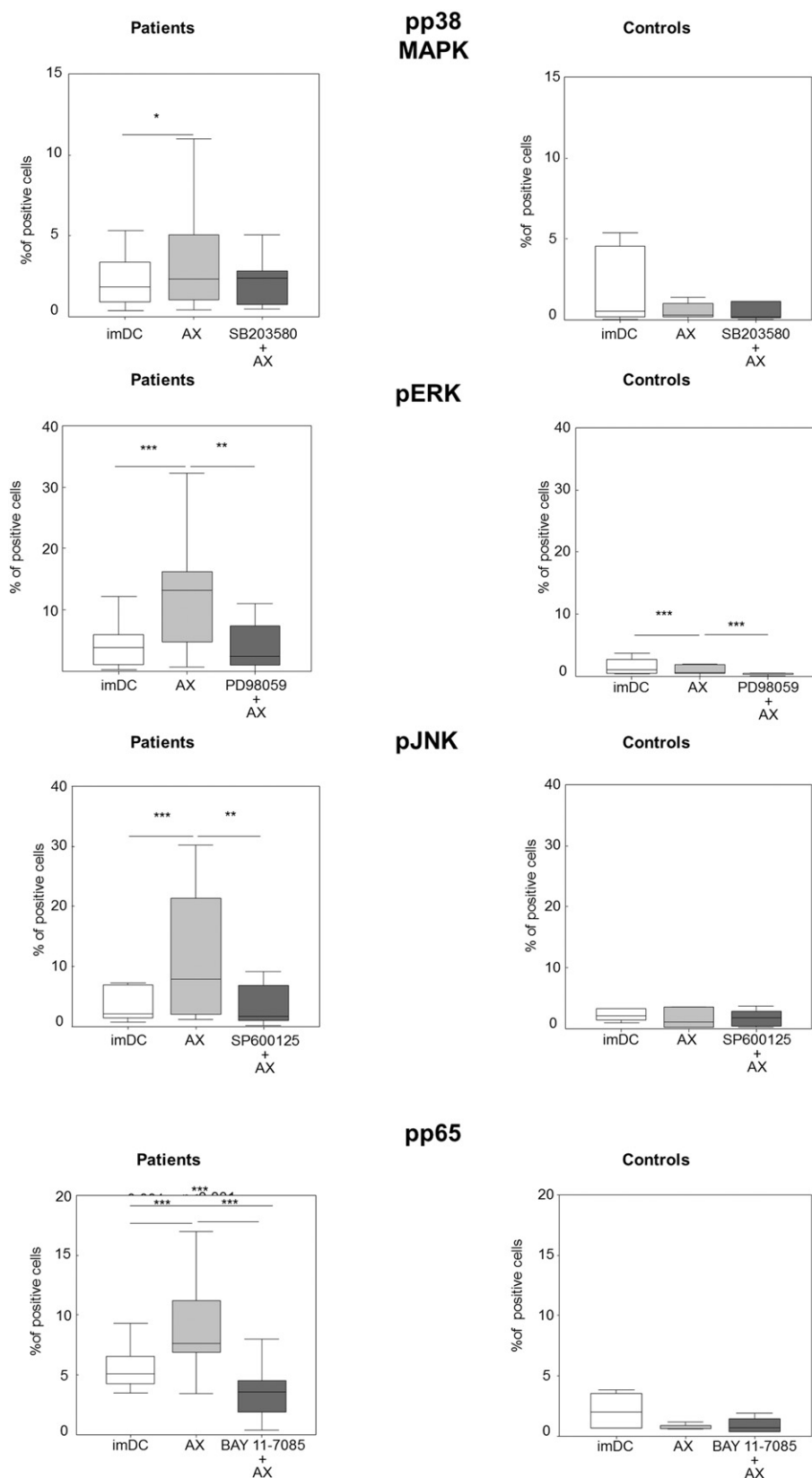


Fig. 2. MAPK and NF- κ B activation in AX-stimulated imDCs. Box-plots represent the median of the percentage of activated MAPK and NF- κ B cells in AX-stimulated DCs from patients ($n = 14$) (left panels) and controls ($n = 15$) (right panels). Number of events acquired = 3000. Prior incubations with specific inhibitors were designated as PD for PD98059, SB for SB203580, SP for SP600125, and BAY for BAY11-7085. Differences with a non-parametric test for related samples were considered to be significant if $P < 0.05$; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

or BAY 11-7085 (10 μ M) in DMSO (Sigma-Aldrich), used as pharmacological inhibitors of the p38 MAPK pathway (Lee et al., 2000), ERK pathway (Alessi et al., 1995), JNK phosphorylation and dependent signaling pathways (Bennett et al., 2001; Anand et al., 2013; Kanaji et al., 2013) and NF- κ B activation (Nasu et al., 2007), respectively. For this study

we used LPS as a positive control in DCs from allergic patients and controls. Optimal concentrations were selected for each inhibitor studied based in the previous work, for p38 MAPK pathway (Antonios et al., 2009), ERK pathway (Agaugue et al., 2006), JNK phosphorylation (Nakahara et al., 2004; Antonios et al., 2009) and NF- κ B activation

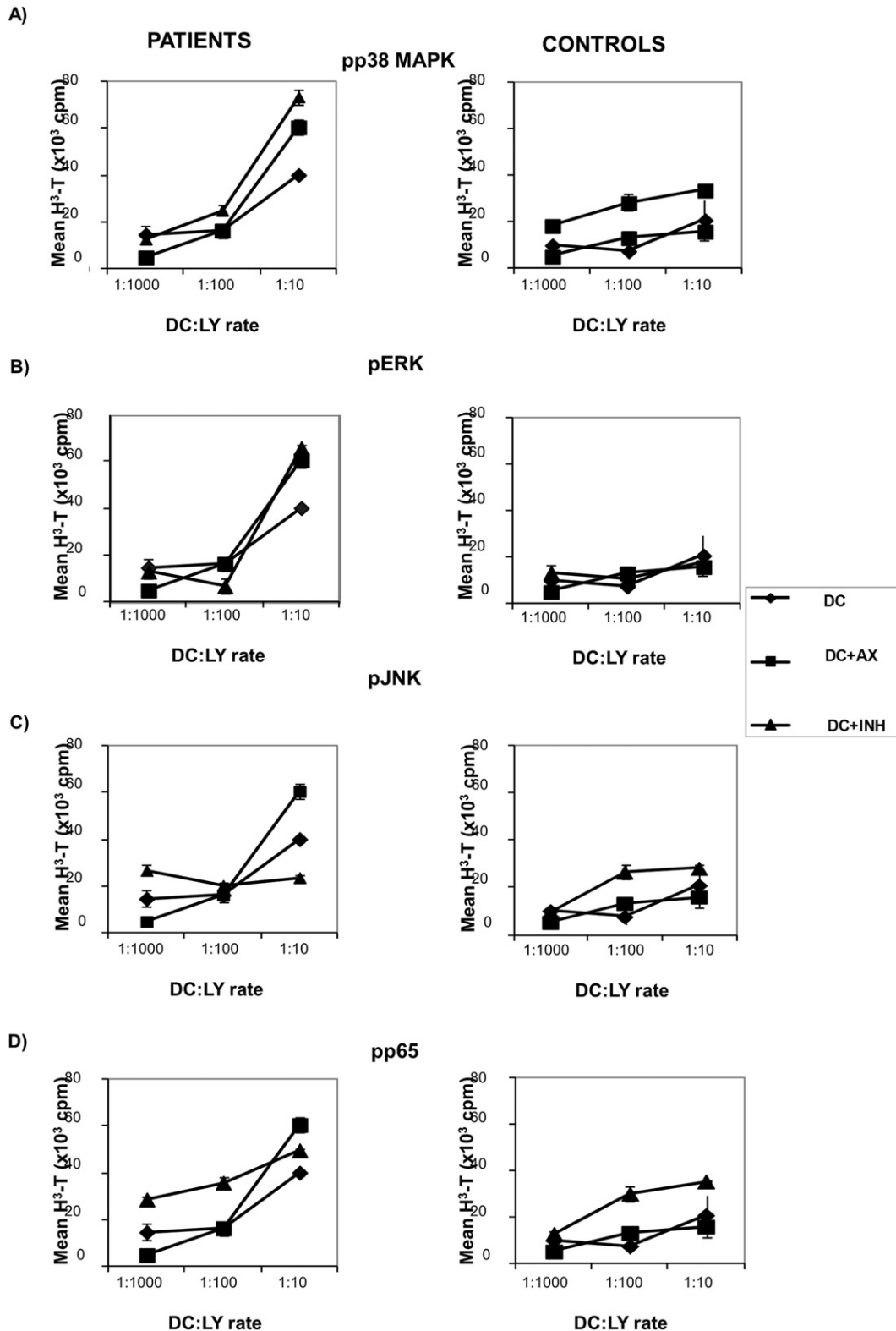
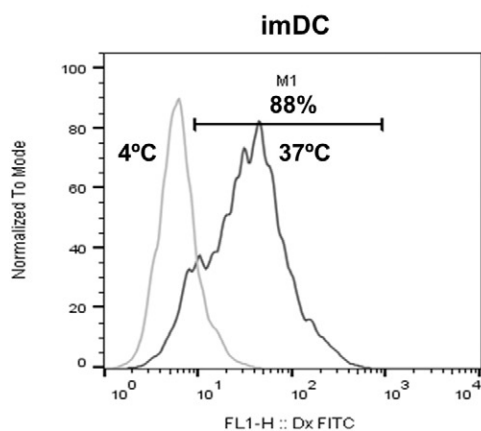


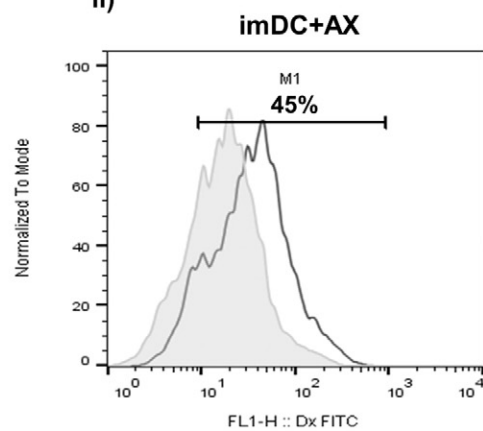
Fig. 3. Allostimulation in AX-stimulated DCs. Analysis of the allostimulatory capacity of AX-stimulated DCs incubated with lymphocytes at different rates, with and without MAPK/NF- κ B inhibitors, in allergic patients ($n = 3$) and controls ($n = 3$). Prior incubations with different MAPK and NF- κ B inhibitors A) SB203580 for p38 MAPK pathway; B) PD98059 for ERK pathway; C) SP600125 for JNK pathway and D) and BAY11-7085 for NF- κ B. These results are presented as the mean values of three independent experiments from patients and controls.

A)

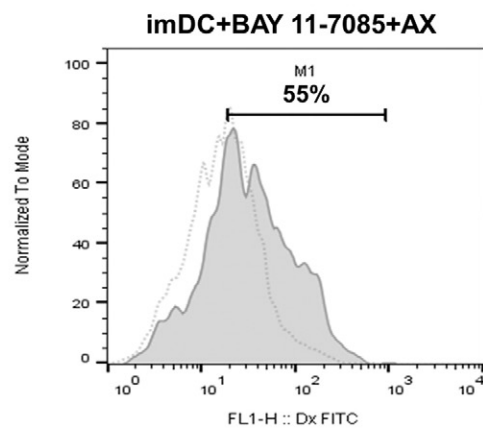
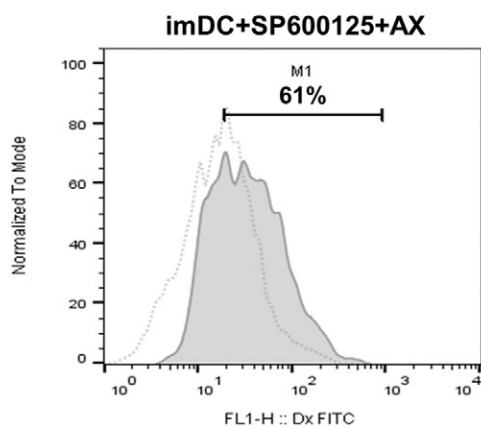
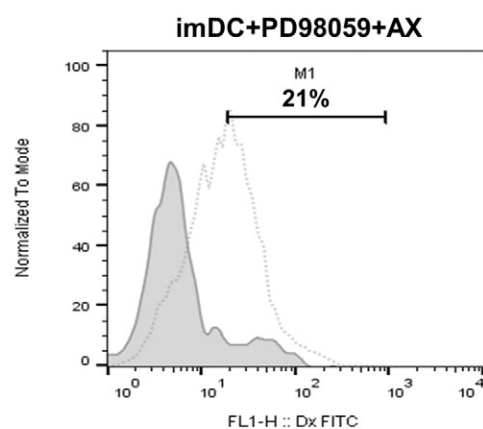
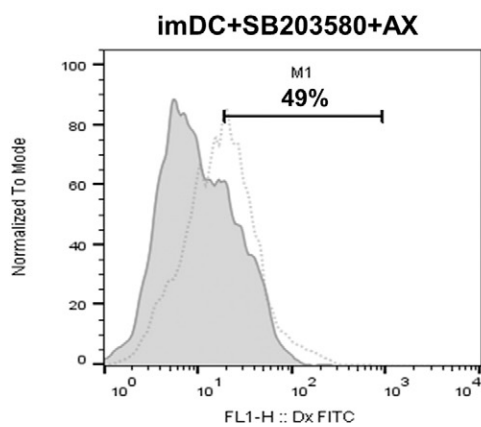
i)



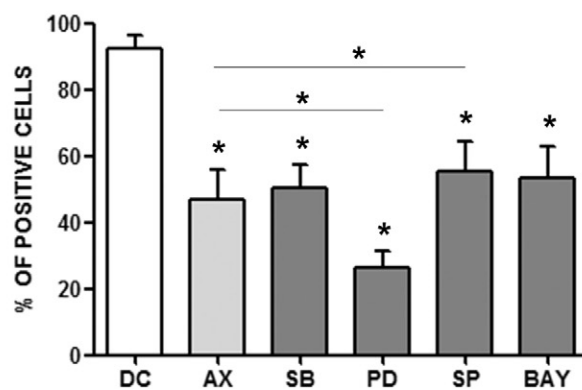
ii)



iii)



B)



(Ahmad et al., 2014) and following the manufacturer's instructions. Cells activated in the absence of inhibitors received an equivalent amount of vehicle (DMSO). The final concentration of DMSO was 0.05% in all conditions. Cell viability was assessed by the trypan blue exclusion method, showing that any of these inhibitors affects cell viability or maturation of DCs, which agrees with previous reports (Ade et al., 2007).

Phosphorylated MAPK and NF- κ B analysis by flow cytometry

A sensitive phosphospecific flow cytometry method (Schulz et al., 2007) was performed using the BD™ Phosflow starter kit (Becton-Dickinson Biosciences, San Jose, CA), as described (Chang et al., 2006; Al-Shanti and Stewart, 2008). In brief, prior to cell stimulation, imDCs were incubated in complete medium without serum for 2 h and then at different time intervals with specific stimulators and/or inhibitors depending on the pathway studied as phosphorylated isoforms. For LPS, cells were stimulated for 5–10 min, whereas for AX incubation times were 60 min for pp38 MAPK and 75 min for pERK, pJNK and NF- κ B pp65 subunit. Immediately afterwards, cells were fixed, permeabilized and incubated with PE-anti-phospho-p38-MAPK (T180/Y182) [26], PE-anti-phospho-ERK1/2 (T202/Y204) (Chang et al., 2006; Al-Shanti and Stewart, 2008), anti-JNK (pT183/py185) (Fleming et al., 2000), and PE-anti-phospho-NF- κ B p65 (pS529) subunit, for each stimulator (Viatour et al., 2005). A second incubation step was needed for anti-JNK (pT183/py185) with PE-anti-F(ab')₂-IgG. Cells were then washed and data acquisition was done in a FACSCalibur flow cytometer (Becton-Dickinson Biosciences). Results were analyzed with CellQuest software (Becton-Dickinson Biosciences) and FlowJo software (TreeStar, Ashland, OR) and expressed as percentage of positive cells from total cells and the mean fluorescence intensity (MFI).

Maturation assays of imDCs

The imDCs were seeded at 1×10^5 cells/ml in 48-well plates (Nunc AS, Roskilde, Denmark) in complete medium with autologous serum and incubated with 250 μ g/ml AX or 1 μ g/ml LPS for 72 h, in the presence or absence of pharmacological inhibitors (which were added 1 h before stimulation). Cells were phenotyped and used for endocytosis and proliferation analysis.

Phenotype analysis of DCs

A total of 2×10^4 DCs from different conditions were incubated with PE-anti-CD83, PE-anti-CD80, PE-anti-CD86 (Immunotech, Marseille, France) and FITC-anti-HLA-DR (MHCII), PerCP-anti-CD14 and isotype controls (Becton-Dickinson Biosciences), and characterized by flow cytometry (Torres et al., 2006).

FITC-dextran endocytosis

A total of 2×10^4 DCs, obtained after being cultured with the conditions described at *Maturation assays of imDCs* section were incubated with FITC-dextran (40,000 Da) (Sigma-Aldrich) at 500 μ g/ml for 1 h at 37 °C. Non-specific-binding negative controls were performed under the same conditions but at 4 °C (Sallusto et al., 1995). The fluorescence from the internalized dextran was analyzed by flow cytometry as described above. All data were compared to non-stimulated imDCs and DMSO (0.05%) at 37 °C.

Mixed leukocyte reaction

The analysis of allogenic proliferation induced by DCs non-stimulated and stimulated with AX and LPS for 72 h, in the presence or absence of each inhibitor (added 1 h prior treatment) was done by culturing 1.5×10^5 of T lymphocytes and 150, 1500 or 15,000 of stimulated and non-stimulated DCs. After 5 days, 1 μ Ci of ³H-thymidine (Moravek Biochemicals, Ca USA) was added and the cells were incubated for 18 h and then harvested onto glass fiber filters (Skatron AS, Tranby, Norway). Cell-associated radioactivity was assessed in a beta scintillation counter (Minaxi β , Packard Instrument Company, Laguna Hills, CA) and results were expressed as the mean counts per minute (cpm) from triplicate cultures.

Statistical analysis

Comparison of the results with the different treatments for the quantitative variables was done with non-parametric analysis, the Mann–Whitney test for non related samples and the Wilcoxon test for related samples. All reported *P* values represent two-tailed tests, with values < 0.05 considered statistically significant.

Results

Inhibition of MAPK and NF- κ B pathways prevented the up-regulation of MHCII and costimulatory molecules induced by AX in DCs from allergic patients

The expression of MHCII, CD80, CD86 and CD83 in imDCs in both allergic patients and healthy controls was assessed at basal conditions, showing no significant differences (data not shown).

AX-stimulated imDCs from patients showed significant increases in the MFI of MHCII ($p = 0.007$), CD80 ($p = 0.002$) and CD86 ($p < 0.001$) compared with non-stimulated imDCs. Even though no significant differences were found in the MFI of CD83 (Fig. 1), we did find differences when the percentage of cells expressing CD83 was analyzed ($p < 0.001$) (data not shown). In controls, there was no significant changes in any of the markers studied (Fig. 1).

Fig. 1 shows our results analyzing the role of the MAPK and NF- κ B systems in the regulation of the expression of surface markers induced by AX in imDCs. Each single inhibitor does not affect maturation marker expression in imDCs (data not shown). The blockade of p38 MAPK, JNK and NF- κ B pathways inhibited the up-regulation on different maturation markers induced by AX in patients (Fig. 1). Thus, MHCII expression decreased significantly when p38 MAPK and NF- κ B pathways were blocked ($p < 0.001$ for both), CD80 when was p38 MAPK pathway ($p = 0.006$), and CD86 related when NF- κ B pathway ($p < 0.001$). Furthermore, PD98059, an inhibitor of the ERK pathway, induced a decrease in the surface expression of MHCII, CD83 and CD86 molecules ($p = 0.007$, $p < 0.001$ and $p = 0.030$, respectively) (Fig. 1). None of these changes were observed in AX controls.

AX-induced phosphorylation of the three MAPK members and NF- κ B activation in imDCs from allergic patients

We analyzed phosphorylated MAPK (p38 MAPK, JNK and ERK) and active NF- κ B (phosphorylated NF- κ B p65 subunit) in imDCs before and after treatment with AX by flow cytometry. We used this methodology, instead of western-blot, due to the limited number of DCs, and

Fig. 4. Study of the endocytic capacity of imDCs with different stimuli. (A) Representative histograms of one independent experiment of the FITC-dextran-intake analysis of AX-stimulated DCs from allergic patients incubated without or with different specific inhibitors. (i) Changes in the endocytic capacity in imDCs at 37 °C as positive control (bold line) and at 4 °C as negative control (thin line); (ii) imDCs (thin lines) and AX-stimulated DCs (filled lines) both at 37 °C; (iii) AX-stimulated DCs (thin lines) and AX-stimulated DCs with different MAPK and NF- κ B inhibitors (filled lines), all at 37 °C. (B). Histogram represent the mean $\pm$ SD of three independent experiments of the FITC-dextran-intake analysis of AX-stimulated DCs from allergic patients ($n = 3$) incubated without or with different specific inhibitors. Number of events acquired = 3000. Differences with a non-parametric test for related samples were considered to be significant if $P < 0.05$; * $P < 0.05$.

because it has proved to be useful. We analyzed the MAPK and NF- κ B phosphorylation in DCs from patients and controls treated with AX in the absence or presence of inhibitors. We did not observe any effect of each single inhibitor in the imDCs activation pathways (data not shown). AX-stimulated imDCs from allergic patients showed an increased phosphorylation of the three MAPKs (p38 MAPK, JNK and ERK) and NF- κ B p65 subunit ($p = 0.014$, $p < 0.001$, $p < 0.001$ and $p < 0.001$, respectively) (Fig. 2). No differences were detected when imDCs were also preincubated with SB203580, SP600125 or PD98059, which inhibit p38 MAPK, JNK and ERK pathways, respectively. However, a statistically significant decrease was observed after preincubation with BAY11-7085, the inhibitor of the NF- κ B pathway ($p = 0.017$). Comparisons between treatments with AX and with AX plus inhibitors showed a significant decrease in the phosphorylation levels for JNK, ERK and NF- κ B ($p = 0.001$, $p = 0.002$ and $p < 0.001$, respectively) but not for p38 MAPK (Fig. 2). In controls, no significant activation for any of the three MAPKs or NF- κ B analyzed was observed, nor when the stimulation was performed in the presence of inhibitors (Fig. 2).

Up-regulation of the allostimulatory capacity and down-regulation of endocytosis in AX-stimulated DCs

We applied inhibitors for each particular signaling pathway in order to assess their role in the allostimulatory capacity of AX-stimulated DCs. Data showed that only the presence of SP600125 (JNK pathway inhibitor) and BAY11-7085 (NF- κ B pathway inhibitor) was able to impair the lymphocyte proliferative responses driven by AX-stimulated DCs (Fig. 3A). Interestingly, the allostimulation primed by AX-stimulated DCs was not related with the p38 MAPK pathway, as indicated the results with the inhibitor SB203580 (Fig. 3A). However, the inhibition of the ERK pathway (with PD98059) produced a slight increase in the allostimulatory capacity compared with the one after treatment with AX alone (Fig. 3A). Finally, when the experiments were carried out in AX-stimulated DCs from controls, we did not detect changes in the proliferative responses at any of the conditions analyzed (Fig. 3B).

We next analyzed whether the decreases in the endocytic capacity of dextran-FITC as a consequence of the maturation of AX-stimulated DCs were affected by the blocking of MAPK/NF- κ B signaling pathways. Our results showed that the dextran uptake was partially increased in the presence of SP600125 and BAY11-7085 (Fig. 4A and B). Thus, the DC endocytic capacities were 56% and 53% after blocking the JNK and NF- κ B pathways, respectively, while DC endocytic capacity of non-stimulated AX-stimulated DCs was 44% (Fig. 4A). PD98059, as expected, resulted in a weak decrease in the DC endocytic capacity stimulated with AX (24%). However, no changes in the endocytic capacity of AX-stimulated DCs were observed in the presence of SB203580 (47%) (Fig. 4A). Compared with imDCs, we observed a significant decrease in the endocytic capacity in AX-stimulated DCs and even after blocking the p38, ERK, JNK and NF- κ B pathways ($p = 0.026$, for each). Moreover, compared to AX-stimulated DCs we observed a significant increase after blocking the JNK pathway and a decrease after blocking the ERK pathway ($p = 0.026$, for each) (Fig. 4B).

Discussion

DCs play a central role in the induction of allergic reactions by undergoing a complex maturation process to become highly efficient APC for naïve T lymphocytes (Steinman, 2003; Bousso, 2008; Novak and Bieber, 2008).

In this context, different signals have shown to be able to drive DC differentiation, including LPS and TNF- α (Roake et al., 1995; Verhasselt et al., 1997). In particular, haptens involved in contact dermatitis were found to induce DC stimulation (Manome et al., 1999; Boislevé et al., 2004; Antonios et al., 2010). Moreover, recently DCs had been proposed for evaluation of the allergenic potential of some drug compounds, such as betalactams and sulfamides (Sebastian et al.,

2012). In this context, we have previously shown that other low-molecular-weight compounds such as AX are able to induce maturation changes on imDCs from patients with allergic reactions to this drug but not in tolerant subjects (Rodríguez-Pena et al., 2006). However, the low expression of costimulatory molecules on DCs, the mixed T-cell phenotype and the low cytokine production by both DC and T cells suggested that AX induces, at least *in vitro*, semimaturation on imDCs, a possibility that is consistent with other studies (Lutz and Schuler, 2002). Thus, it has been proposed that AX could induce cell activation either alone or conjugated with autologous proteins (Landsteiner and Chase, 1942) through their own (as yet unknown) receptors, or receptors for pathogen-associated molecular patterns (like the Toll-like receptors, TLR). This activation, *in vivo*, could be due to concomitant conditions (*i.e.*, virus infections or chronic inflammation identified as ligands for TLR). In fact, it has been demonstrated that the combinations AX plus ligands of TLR2 and TLR4 on one hand and TLR7/8 on the other, produced a significant increase in IL-12p70 during both DC maturation and T-cell proliferation only in allergic patients (Sanchez-Quintero et al., 2013). Furthermore, non-allergic subjects who have had previous contact with this drug could eventually respond to AX but without DC activation or compromising the immunological tolerance.

Therefore, the present report extends our previous observations by providing further mechanistic information about this process from the study of different signal transduction pathways inducing DC maturation, which is a crucial event for the development of drug allergic reactions.

Under these circumstances and irrespective of the mechanisms involved, AX is capable of acting as a danger signal, leading to the activation of stress-associated signal transduction pathways (such as MAPK and NF- κ B) (Matzinger, 2002). Ultimately, AX-stimulated DC up-regulates different proteins on their surface and proceeds to drug presentation *via* the MHCII to the lymphocytes.

Among the best studied signal transduction pathways in relation to DC maturation are the phosphorylation cascade of the MAPK family pathway (p38 MAPK, ERK and JNK) and the activation of the nuclear factor NF- κ B (Boislevé et al., 2005; Yoon et al., 2006; Ade et al., 2007). Previous studies on the involvement of the MAPK/NF- κ B systems in DC maturation marker expression and DC functional properties showed differences depending on the stimulus employed (Nakahara et al., 2006; Ade et al., 2007). In this sense, no studies had addressed the role of the MAPK/NF- κ B signaling pathways in DC maturation induced by BL and the possible differences between DTH patients and tolerant subjects. In this work, we demonstrate that AX induces phosphorylation of the three MAPKs (p38 MAPK, JNK and ERK) and activation of NF- κ B in imDCs exclusively from AX allergic patients. Additionally, the study of the p38 MAPK, JNK and NF- κ B pathways using inhibitors revealed the up-regulation of MHCII and costimulatory molecules by AX. Reciprocally, AX alone induced changes in patients but not in controls.

As far as ERK phosphorylation is concerned, we observed a phenotype characterized by an increase in MHCII and costimulatory molecules, which was not expected. However, this seems not to affect the functional properties of DCs, since after blocking with an ERK inhibitor in the presence of AX, we observed an increase in the allostimulatory capacity and a decrease in the endocytic property of AX-stimulated DCs, indicating that ERK is involved in the decrease of the allostimulatory capacity and in the increase of the endocytic properties leading to inhibition of DC maturation.

In addition, our results show that the DC maturation induced by AX follows a similar pattern to that obtained with other haptens like Ni₂SO₄, with activation of the three relevant groups of MAPK and NF- κ B, as well as phenotypical changes involving MHCII, CD80 and CD86 (Boislevé et al., 2005; Ade et al., 2007; Antonios et al., 2009). However, we observed that although the three MAPKs and NF- κ B signaling are involved, the phenotypical changes in the AX-stimulated DCs induced by ERK and the functional effect induced by p38 MAPK were not so clearly defined. Previous data obtained by others had suggested that the role of

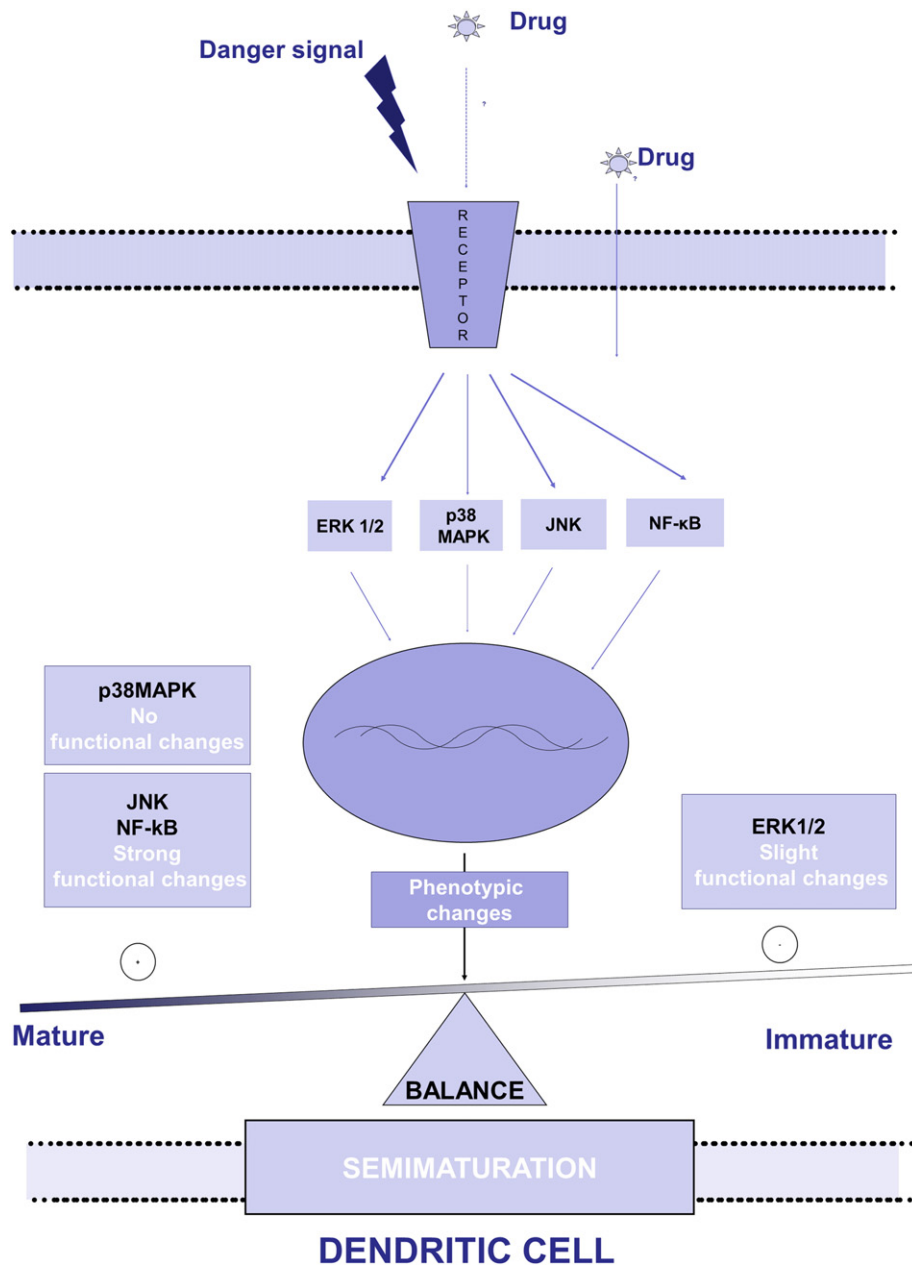


Fig. 5. Signaling pathways involved in DCs after treatment with AX in allergic patients. The scheme represents the pathways involved in AX-stimulated DCs. The drug in the presence of a stimulus that would act as a danger signal active MAPK and NF- κ B pathways leading DC maturation process.

the activation of the three MAPKs is different and affects DC maturation in a complementary manner, with p38 MAPK being the major protein involved in this process (Nakahara et al., 2006). However, our results showed that AX induced a small increase in p38 MAPK activation, whereas ERK, which has an inhibitory effect on DC maturation, was highly increased with AX (Fig. 2). Moreover, the data obtained in the allostimulatory activity showed no participation of p38 MAPK. Thus, taking all together, it is tempting to speculate that the lower DC maturation observed with AX, in this work and in previous studies (Rodríguez-Pena et al., 2006), could be a consequence of the balance of the activities of the three MAPK pathways and the low participation of some of them, especially p38 MAPK (Fig. 5; summary in Table 2). Markedly, given the different degrees of p38 MAPK activation in AX-stimulated DCs among the allergic patients and the effect of NF- κ B and MAPK pharmacological inhibition on DC surface markers, we could also hypothesize that there might be different subsets of DCs that are responsible for a particular responder phenotype in AX allergic patients. This circumstance has been

lately outlined from a broader perspective for allergic reactions and it could have an impact for personalized immunotherapies (Palomares et al., 2011).

The fact that AX induced maturation of imDCs only in allergic patients but not in tolerant subjects might indicate that this process was

Table 2

The effect of different inhibitors of MAPK and NF- κ B signaling pathways on phenotypal markers, allostimulatory and endocytic capacities of AX-treated human dendritic cells from AX-allergic patients.

Inhibitors/ Pathways	Phenotypic study	Functional studies	
		Allostimulation reaction	Endocytic capacity
PD/ERK	--	+	+
SB/p38MAPK	--	++	--
SP/JNK	--	--	++
BAY/NF- κ B	--	--	++

initially produced in the presence of another stimulus, like TLR agonists, that acted as a danger priming signal and which did not occur in controls (Sanchez-Quintero et al., 2013). Indeed, TLR2, 4 and 7/8 that were demonstrated to have a synergistic effect on AX-induced DC maturation, activate ERK1/2, JNK1/2 and p38 MAPK pathways through MyD88 (Brown et al., 2011). This correlates with the activation that we have found in the present work. Moreover, given the small amount of previous study in this area, the study in these allergic patients of coinhibitory receptors involved in the regulatory signaling cascades that modulates NF- κ B activation and cytokine production, and may establish the threshold for T-cell activation and tolerance, such as CD31, could help shed further light on the precise mechanisms involved (Ma et al., 2010).

In summary, in the present study we report a number of novel observations about the cellular and molecular processes involved in DC maturation in patients allergic to BL. We have found that AX by itself is able to activate the MAPK family and NF- κ B that participate in the DC maturation process only in allergic patients. The involvement of JNK and NF- κ B and to a lesser extent the p38 MAPK, could be the cause of an incomplete DC maturation, namely a semimaturational process by AX in these patients. However, further studies in this allergic reaction, are needed to explore the underlying mechanisms involved in the initial steps of DTH to drugs that involve AX–DC interaction.

Conflict of interest

The authors have declared no commercial or financial conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.taap.2015.08.001>.

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