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Histamine Regulates Actin Cytoskeleton in Human Toll-like Receptor 4-activated Monocyte-derived Dendritic Cells Tuning CD4$^+$ T Lymphocyte Response*

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Histamine, a major mediator in allergic diseases, differentially regulates the polarizing ability of dendritic cells after Toll-like receptor (TLR) stimulation, by not completely explained mechanisms. In this study we investigated the effects of histamine on innate immune reaction during the response of human monocyte-derived DCs (mDCs) to different TLR stimuli: LPS, specific for TLR4, and Pam3Cys, specific for heterodimer molecule TLR1/TLR2. We investigated actin remodeling induced by histamine together with mDCs phenotype, cytokine production, and the stimulatory and polarizing ability of Th0. By confocal microscopy and RT-PCR expression of Rac1/CdC42 Rho GTPases, responsible for actin remodeling, we show that histamine selectively modifies actin cytoskeleton organization induced by TLR4, but not TLR2 and this correlates with increased IL4 production and decreased IFNγ by primed T cells. We also demonstrate that histamine-induced cytoskeleton organization is at least in part mediated by down-regulation of small Rho GTPase CdC42 and the protein target PAK1, but not by down-regulation of Rac1. The presence and relative expression of histamine receptors HR1–4 and TLRs were determined as well. Independently of actin remodeling, histamine down-regulates IL12p70 and CXCL10 production in mDCs after TLR2 and TLR4 stimulation. We also observed a trend of IL10 up-regulation that, despite previous reports, did not reach statistical significance.

The histaminergic network is involved in the regulation and modulation of immune reactions, innate and adaptive, during normal or pathological responses. Histamine, a major mediator in allergic diseases, is an endogenous amine synthesized by histidine decarboxylase, which decarboxylates L-histidine expressed in a wide variety of cells. When activated, dendritic cells (DCs)[2] may express histidine decarboxylase and histamine receptors (HRs) (1). Histamine modulates innate immune responses affecting cell maturation, function, and consequent pro- or anti-inflammatory reactions in different cell populations including human monocyte-derived DCs (mDCs) (2). mDCS are professional antigen presenting cells, key elements of immune responses that bridge innate and adaptive immunity supporting Th lymphocyte responses and polarization through antigen presentation and adequate co-stimulatory signals (3, 4). mDCs principal characteristic resides in their ability to integrate environmental signals, as those generated during infectious processes, which lead to changes in cytokine and chemokine microenvironments, actin cytoskeleton re-organization, and strength of T cell receptor stimulation (5, 6). Toll-like receptors (TLRs) are a class of receptors critical in host defense that specifically detect pathogen-associated molecular patterns and their action has been associated with loss of tolerance and pathogenesis of chronic inflammation, autoimmunity, and allergy (7–9). Histamine modulates several of the TLR-induced cellular responses. For instance, the presence of histamine during TLR4 and TLR3 activation of human mDCs leads to a reduced production of IL12p70, a Th1 polarizing cytokine, of CXCL10, a Th1 attracting chemokine, and increased release of IL10, IL8, or IL6 (10–15). In another study it was reported that the presence of histamine during TLR3 stimulation of mDC down-regulates CCL1, TNFα, IL6, and CXCL10, which is accompanied by up-regulation of CXCL1 (16). Furthermore, Frei *et al.* (15) reported that histamine suppressed TLR4-induced TNFα secretion from mDC cell. TLR activation is followed by the ability of mDCs to stimulate and drive polarization of T lymphocyte responses. The presence of histamine during TLR4 stimulation did not alter the ability of mDC to stimulate T lymphocyte proliferative response, nonetheless Th1/Th2 homeostasis was altered with increased IL-4 producing Th2 cells during mixed lymphocyte reaction (10, 11) or reduced Th1 shift during autologous naive T cell stimulation (15). In a specific experimental setting, where mDCs were stimulated with HIV-1 and histamine, T lymphocytes shifted toward FoxP3+ Treg (17). Among the many studies on human mDCs, few reported that histamine may determine F-actin polarization influencing the migratory ability of mDCs (13, 16), and none investigated the specific histamine effects on actin cytoskeleton organization, which is a way to modulate the activity of mDCs, DC:T conjugate formation, and mDCs polarizing ability (18–20).

To better understand the diverse action of histamine on different TLR stimulations we analyzed histamine modulation of human mDCs matured with two different agonists: LPS, spe-

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[2] The abbreviations used are: DC, dendritic cell; mDC, monocyte-derived DC; HR, histamine receptor; TLR, Toll-like receptor; HA, histamine; MLR, mixed lymphocyte reaction; PE, propidium iodide.

cific for TLR4, and Pam3Cys (a synthetic triacylated lipopeptide), specific for heterodimer molecule TLR1/2 (TLR2 later in the text). Both receptors are present on the cell surface and specific for microbial membrane components such as lipids, lipoproteins, and proteins (7). By using confocal microscopy, we describe for the first time that histamine selectively modifies actin cytoskeleton organization induced by TLR4, but not TLR2, and this correlates with increased IL4 and decreased IFNγ production by primed T cells.

We also demonstrate that histamine-induced cytoskeleton organization is at least in part mediated by small Rho GTPase Cdc42, a molecule shared between H1R and the TLR4 signaling cascade, and protein target PAK1. Independently on actin remodeling, histamine down-regulates IL12p70 and CXCL10 production in mDCs after TLR2 and TLR4 stimulation. Despite previous reports, we did not find up-regulation of IL-10.

Experimental Procedures

Cells—mDCs were generated from human monocytes of healthy donors, as previously described (39). Briefly, $CD14^+$ monocytes were positively sorted by magnetic microbeads (Miltenyi Biotec) and cultured for 6 days in medium supplemented with GM-CSF (1000 units/ml, R&D Systems) and IL-4 (1000 units/ml, R&D Systems). At day 6 of culture, immature DCs were activated by stimulation with 1 μg/ml of LPS (Sigma) or 10 μg/ml of Pam3Cys (InvivoGen), in the presence or absence of 10 μM histamine (HA) (Sigma), for 2 or 24 h depending on the assay. $CD4^+$ T cells were negatively selected from peripheral blood mononuclear cells of healthy donors using the T cell isolation kit II from Miltenyi Biotec.

Phenotype Analysis—mDC surface markers were evaluated by flow cytometry with a 6-color CyFlow Space cytometer (Partec). The monoclonal Abs used (all from eBioscience) were directed against the following antigens (the fluorochrome tags are given in parentheses): CD80 (FITC), CD86 (PE), HLA-DR (antigen presenting cell), and CD83 (PE-Cy5.5). Cell vitality was tested by propidium iodide (Molecular Probes) incorporation. The cells were labeled in PBS with 2% FCS for 20 min room temperature in the dark, washed in PBS, and immediately analyzed.

Micropinocytosis Assay—At day 7 of culture, mDCs treated or not with histamine in the last 24 h of culture in the presence or absence of TLR ligands LPS and Pam3Cys were tested for micropinocytosis ability as described by Zinchuk *et al.* (21). Briefly, 2×10^5 cells were suspended in RPMI 1640 with 10% FCS, equilibrated at 37 or 0 °C for 10 min, and pulsed with FITC-conjugated dextran (40,000 Da; Molecular Probes), 1 mg/ml for 45 min at 37 or 0 °C. Cells were washed four times with cold PBS and analyzed by flow cytometry; propidium iodide was used to exclude dead cells. The labeling of cells pulsed at 0 °C was subtracted from that of cells pulsed at 37 °C to obtain a measure of the endocytosed dextran.

Cytokine Determination—IL4, IL6, IL8, IL10, IL12p70, IL17, IFNγ, TNFα, and CXCL10 production was evaluated in cell supernatants by Milliplex assays (Milliplex MAP kit number HCYTOMAG-60K, Merck Millipore), following the manufacturer's protocols. Samples were analyzed by a Bioplex reader (Bio-Rad).

Immunocytochemistry and Confocal Analysis—To analyze F-actin/CD11c and PAK1 distribution, at day 7 of culture mDCs were seeded on poly-L-lysine-coated glass coverslips (3×10^4 cells/coverslip) after 24 or 2 h of TLR stimulation in the presence or absence of histamine. Samples were incubated at 37 °C for 45 min, then fixed with 4% phosphate-buffered paraformaldehyde for 10 min room temperature and permeabilized 30 min with PBS buffer containing 0.05% saponin (ICN Biomedicals) and 2% BSA (Sigma). To analyze F-actin distribution, cells were labeled with FITC-conjugated phalloidin (Sigma), 0.1 μg/ml, together with PE-conjugated mouse monoclonal anti-CD11c (BD Pharmingen) diluted 1:50 in PBS, 0.05% saponin, 2% BSA) for 1 h, at room temperature in the dark.

To determine PAK1 localization and expression, samples were stained with primary monoclonal Ab rabbit anti-human PAK1 (2 μg/ml; U. S. Biological) for 2 h at room temperature. Then samples were washed three times with permeabilization buffer and incubated 1 h at room temperature in the dark with the fluorescent secondary Ab Alexa Fluor 488-conjugated mouse anti-rabbit IgG (Life Technologies), diluted 1:300 in permeabilization buffer.

To determine IL12p70 distribution at mDCs-T cell immune synapses, mDCs (activated with LPS, Pam3Cys in presence or not of histamine) and $CD4^+$ T cells derived from mismatched donors were seeded as described (19). Samples were stained with mouse monoclonal anti-IL12p70 (clone 24945, R&D Systems) and rabbit polyclonal anti-CD3 (Abcam), overnight at 4 °C. As secondary antibodies we used Alexa Fluor 488 donkey anti-rabbit IgG and Alexa Fluor 546 goat anti-mouse IgG (Life Technologies), diluted each 1:400 in permeabilization buffer, 1 h at room temperature in the dark. Coverslips were washed three times with permeabilization buffer and mounted on glass slides with Prolong Diamond mounting medium containing DAPI (Life Technologies).

Confocal images were acquired in a Leica TCS SP5 microscope (Leica Microsystems, Mannheim, Germany) equipped with a He/Ne/Ar laser source, using a Leica Plan Apo ×63/1.40 NA oil immersion objectives. A series of optical sections (1,024 × 1,024 pixels each; pixel size 200 × 200 nm) were taken at intervals of 0.35 μm. Confocal images were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD). Colocalization between F-actin and CD11c was visualized by using the ImageJ Colocalization finder plug-in (21) and quantified by determining the mean Pearson's coefficient (r) with ImageJ Jacop plug-in ($r = 1$: complete colocalization; $r = 0$: absence of colocalization) in 5 randomly selected fields for each slide. PAK1 expression was analyzed with ImageJ software by measuring the green mean fluorescence intensity (gray value) in cells seleted by freehand in 5 randomly chosen fields for each slide.

Mixed Lymphocyte Reaction (MLR)—mDCs stimulated with LPS or Pam3Cys in the presence or absence of histamine in the last 24 h of culture were tested in a MLR. After stimulation, mDCs were washed twice in PBS and co-cultured in 96-well U bottom plates (Nunc) at a density of 1×10^4 cells/well with 1×10^5 allogeneic $CD4^+$ T cells/well, for 5 days in RPMI 1640 medium with 10% FCS. Cells were plated in quadrupli-

cate. At day 5, the proliferative response was measured by [^{3}H]thymidine (1 mCi/ml; PerkinElmer Life Sciences) in the incorporation test. [^{3}H]Thymidine was added for the last 8 h of culture. Plates were harvested (Tomtec Mach III, Wallac) on glass fiber filters (PerkinElmer), and [^{3}H]thymidine uptake was measured by liquid scintillation in a Microbeta 1450 Trimux β-counter (Wallac) and expressed as counts per min (cpm).

T Cell Polarization Test—Upon 24 h of incubation with LPS or Pam3Cys in the presence or absence of histamine, mDCs were washed twice in PBS and co-cultured (50×10^4 cells/well) with autologous T cells (2×10^5 cells/well) in 96-well flat bottom plates coated with anti-CD3 (eBioscience, 1 μg/ml). Not stimulated mDCs, treated or not with histamine for 24 h, were also analyzed. Cells were plated in quadruplicate. Upon 4 days of co-culture, supernatants were harvested and analyzed for IL4, IFNγ, and IL17 concentrations by Bioplex, as described above. At the same time, T cell proliferation was evaluated by a [^{3}H]thymidine (1 mCi/ml; PerkinElmer) incorporation test, as described above.

RNA Extraction and Quantitative Real-time RT-PCR—Total RNA was extracted using Qiazol (Qiagen). Concentration and purity were assessed by NanoDrop (ThermoScientific). RNA was reverse transcribed using a Quantitect reverse transcription kit (Qiagen) according to the manufacturer's protocol. Real-time PCR was performed with an ABI Prism 7900HT Sequence Detection System (Applied Biosystems), according to the manufacturer's instructions. All PCR amplifications were performed by MicroAmp optical 96-well reaction plate with TaqMan Universal Master Mix and with Assay-on-Demand primer sets (Life Technologies). Each assay was carried out in duplicate and included a no-template sample as negative control. Hypoxanthine-guanine phosphoribosyltransferase was used as a housekeeping gene for normalization. The expression of histamine H1, H2, H3, and H4 was evaluated on freshly isolated CD14$^+$ monocytes, in differentiating mDCs at days 3 and 6 of culture and at day 7 after 24 h of stimulation with LPS or Pam3Cys in the presence or absence of histamine. The expression of TLR2 and TLR4 was evaluated at day 7 of culture in mDCs incubated for 2 h with LPS or Pam3Cys in the presence or absence of histamine and in non-stimulated ones; the same timing was used for Rac1 and CdC42 evaluation.

Protein Extraction and Cdc42 Pulldown Assay—Full-differentiated DCs were stimulated with LPS and LPS + histamine for 30 min. Proteins were extracted from 10×10^6 cells in each condition. Cdc42 was tested by means of a Cdc42 activation assay kit (Cell Biolabs) following the manufacturer's instructions. Detection of precipitated Cdc42 was performed by a 15% acrylamide gel Western blot (PVDF membrane) followed by mouse monoclonal anti-human Cdc42 antibody and secondary antibody goat anti-mouse IgG/HRP, revealed in chemiluminescence (Clarity Western ECL blotting substrate Bio-Rad). Densitometric analysis was performed by Quantity One Software (Bio-Rad).

Statistics—Results were expressed as mean ± S.E. Statistical analysis was performed using one-way analysis of variance or Mann-Whitney test when adequate (GraphPad Prism 5 software). Statistical significance was for p values < 0.05. Pearson's correlation test was utilized for colocalization.

Results

Expression of HRs in mDC during Differentiation and Activation—Histamine effects on human mDCs are mediated principally by H1 and H2 receptors (25). We first evaluated mRNA expression of HRs by real-time PCR in mDCs of 5 healthy donors, at various times during culture. As reported in previous studies, relative mRNA expression is modulated during culture and during TLR stimulation (12); in our cells mRNAs coding for H1, H2, and H4 receptors were detectable at all times during culture (Fig. 1*A*). In resting cells (day 6) histamine did not significantly affect H1, H2, and H4 receptors expression (Fig. 1*A*, all panels *gray columns*). When resting mDCs were stimulated with the TLR2 agonist Pam3Cys, histamine significantly augmented the H2R relative expression ratio compared with TLR2 stimulation alone (Fig. 1*B*). H1R and H4R, although relative expression of messenger molecules was different, were not modulated by concomitant stimulation of TLR2 and histamine compared with TLR stimulation alone. In all experiments concomitant LPS and histamine application did not affect the expression of HRs compared with TLR4 stimulation alone.

Expression of TLRs during mDCs Histamine Stimulation—A question that we posed was if histamine could modulate TLRs expression during innate stimulation of mDCs, therefore influencing the outcome of the function and immune response of mDC. To test this, we determined by real-time PCR the relative expression of TLRs during LPS and Pam3Cys stimulations in the presence or not of histamine. We found that histamine did not significantly modify the expression of tested TLRs, although a trend toward increased TLR expression was present (Fig. 2).

Effect of Histamine on TLR Agonist-induced mDCs Cytoskeleton Rearrangements—To evaluate the effect of histamine and TLR4 or TLR2 activation on actin cytoskeleton organization, we immunostained mDCs to detect actin and CD11c, an integrin present in the membrane of mDC, and analyzed their distribution in different experimental conditions (Fig. 3). To investigate the organization of the mDCs cytoskeleton, we quantified the colocalized immunofluorescent signals, expressed as Pearson's coefficient r (see "Experimental Procedures"). After LPS or Pam3Cys stimulation, r values were 0.437 ± 0.015 and 0.696 ± 0.027, respectively ($p = 0.02$). Interestingly, we found that the presence of histamine during TLR4 stimulation induced a cytoskeleton remodeling when compared with TLR4 stimulation alone (TLR4 ± histamine, $r = 0.715 \pm 0{,}026$; $p = 0.016$ compared with TLR4 alone; Fig. 3), which is different from the results obtained with TLR2 stimulation (TLR2 ± histamine $r = 0.730 \pm 0{,}018$, p = n.s. compared with TLR2; Fig. 3). Hence, histamine induces a redistribution of actin during TLR4 stimulation that changes the organization of mDCs cytoskeleton.

Histamine Effects on Cytoskeleton-regulating Molecules: Cdc42 and PAK1—To investigate the way histamine may interfere with the LPS downstream pathway in shaping actin cytoskeleton organization, we analyzed by RT-PCR the relative expression of two Rho GTPse involved in cytoskeleton organi-

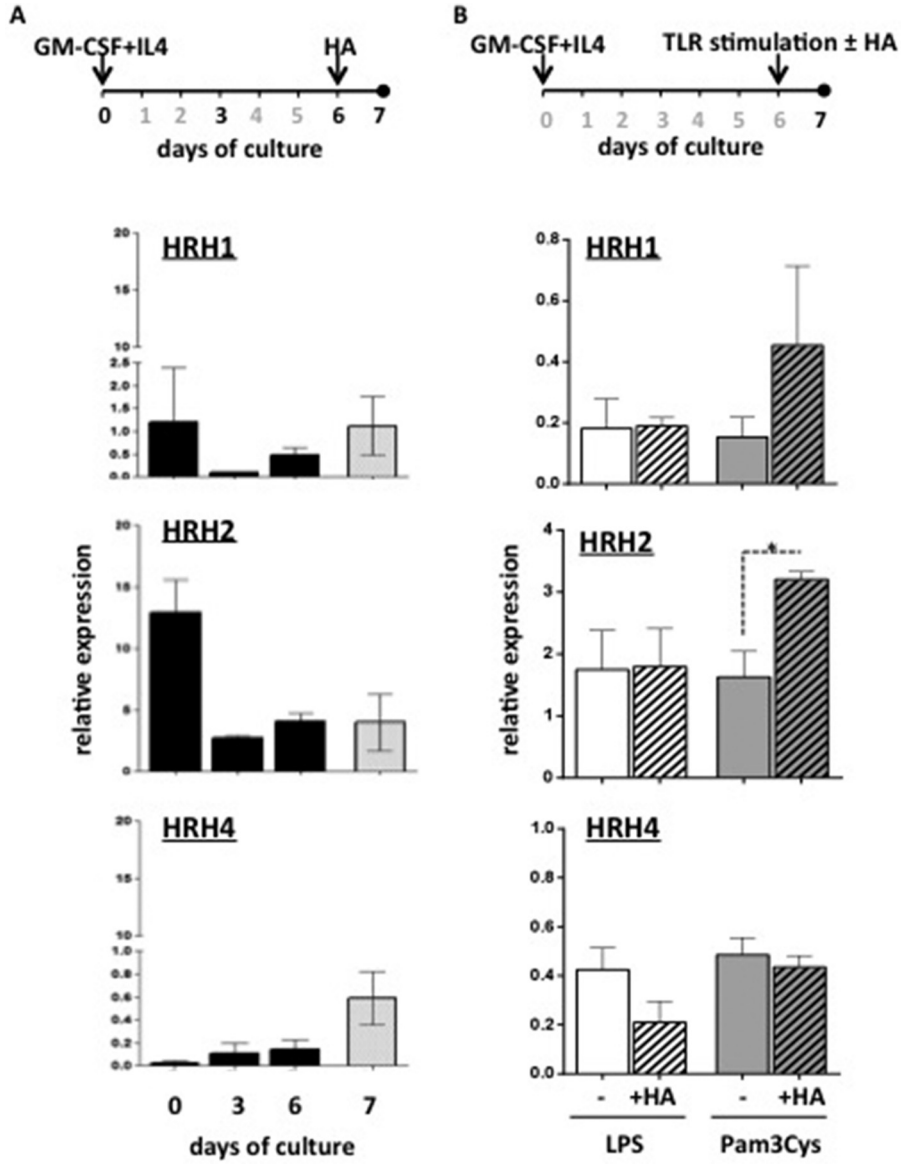

FIGURE 1. **Expression of HR during *in vitro* mDCs differentiation.** *A,* the relative expression of H1R, H2R, H3R, and H4R was evaluated by real-time PCR on $CD14^+$ monocytes at days 0, 3, and 6 of culture in differentiating medium (RPMI 10% FCS supplemented with GM-CSF and IL-4), and at day 7, after 24 h of stimulation with HA (10 μM). Graphs report the mean value of HR relative expression ± S.E. of 5 experiments using cells from 5 different donors. Hypoxanthine-guanine phosphoribosyltransferase was used as housekeeping gene. The H3R was never detectable at any time point. *B,* HR expression at day 7 of cultured mDCs stimulated with TLR ligands with or without HA for 24 h. HRH3 expression was not detectable. *, $p < 0.05$.

zation: Rac1 and Cdc42 and by confocal microscopy the expression of their common protein target PAK1. LPS maturation of mDCs is accompanied by a higher expression of PAK1 and Cdc42 compared with Pam3Cys matured cells (Fig. 4, *A* and *B*) and a comparable amount of Rac1 (Fig. 4*C*). Histamine reduces PAK1 and Cdc42 expression in LPS-stimulated cells, with no effect on Rac1 (Fig. 4, *A*–*C*).

To gain better insight in the possible role of Cdc42 in modulating actin cytoskeleton organization, we investigated the level of Cdc42 activation in our samples. We found that activation is increased after LPS stimulation and diminished in TLR4-stimulated cells in the presence of histamine (Fig. 4*D*). We may conclude that histamine modulation of LPS-induced cytoskeleton organization is at least in part mediated by modulation of Cdc42 and PAK1 expression.

Effect of Histamine on mDCs Function: CKs Production and T Cell Polarization—When TLRs stimulation was performed in the presence of histamine, mDCs changed their cytokine profile, measured in cell supernatants. TLR4 and TLR2 stimulation induced a comparable production of IL12p70 and TNFα; whereas Pam3Cys-stimulated mDC produced significantly less CXCL10, IL10, and IL6 than LPS-stimulated mDC (Fig. 5*A*). We also found that independently of what TLR maturation signal was used, histamine reduced the production of IL12p70 and CXCL10. Histamine reduced TNFα production only in TLR4-stimulated cells (Fig. 5*B*). On the other hand, histamine did not significantly modify TLRs stimulation-induced production of IL10, IL6, and IL8 (Fig. 5*B*). We further tested mDCs by performing a T lymphocyte polarization and priming experiment. In the presence of histamine, T lymphocytes alone or primed

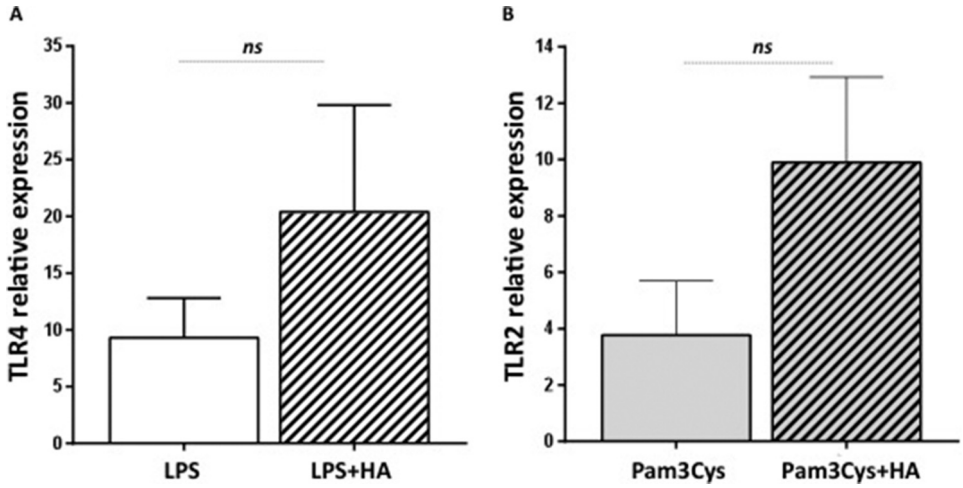

FIGURE 2. **Effect of LPS, Pam3Cys, and histamine on TLR expression.** mDCs expression of TLR4 (*A*) and TLR2 (*B*) was evaluated by real-time PCR after 2 h stimulation with TLRs ligands in the presence or absence of HA in 8 donors. Data are reported as relative expression with respect to the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (mean ± S.E. of five independent experiments). *ns*, not significant.

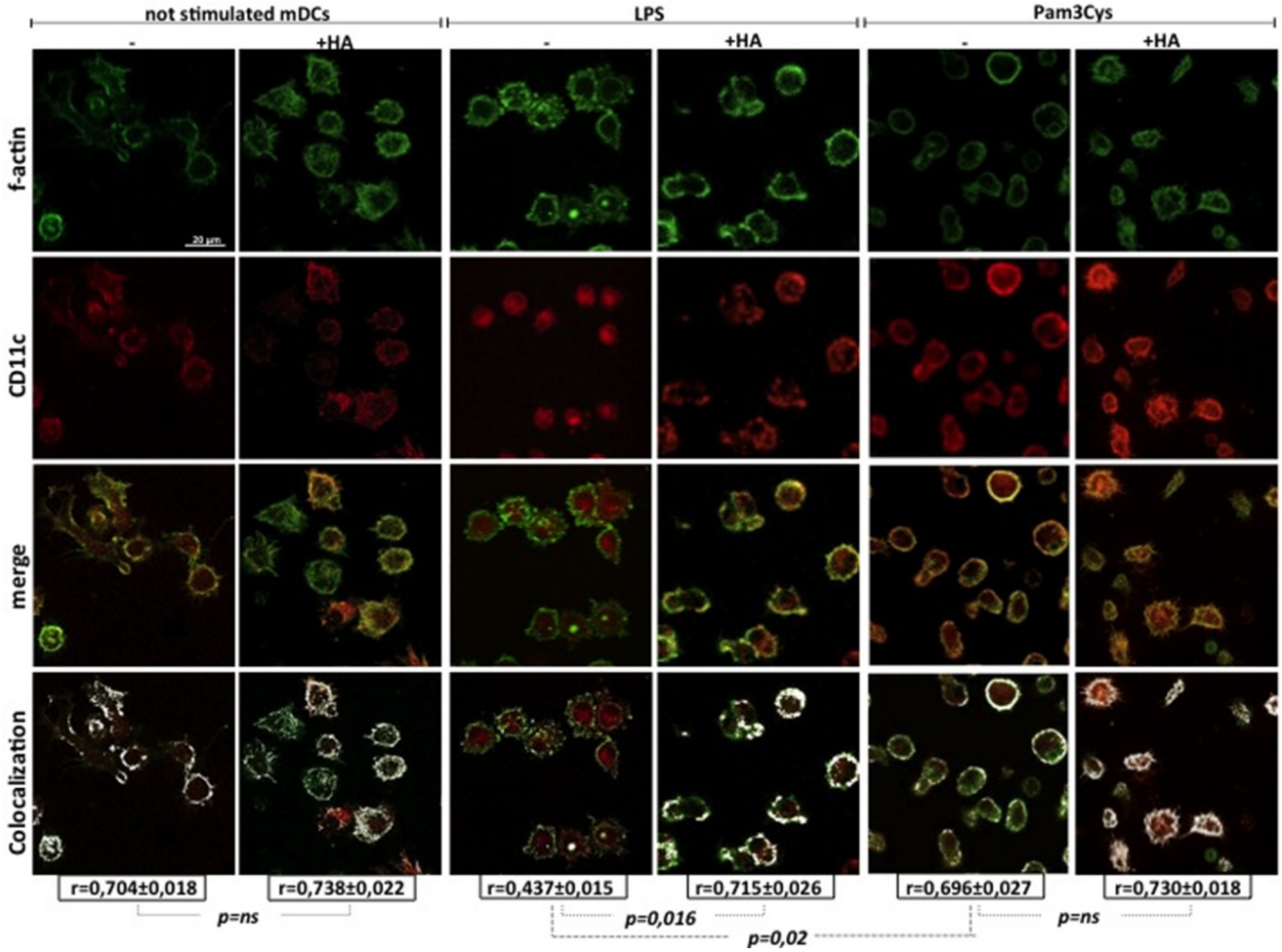

FIGURE 3. **Actin cytoskeleton distribution in mDCs treated with HA during TLR stimulation.** At day 7 of culture, after 24 h of stimulation with either LPS or Pam3Cys in the presence (+) or absence (−) of HA, DCs were analyzed by confocal microscopy for F-actin (*green*) and CD11c (*red*) cell distribution. Colocalization between F-actin and CD11c was visualized in *white* by using the ImageJ colocalization finder plug-in and quantified by Pearson's coefficient (*r*), calculated with ImageJ Jacop plug-in ($r = 1$: complete colocalization; $r = 0$: absence of colocalization). The pictures are from one experiment representative of three independent observations; Pearson's coefficient values (*r*) reported below the colocalization images are the mean ± S.E. of all of three experiments.

with non-TLR activated mDCs did not significantly change the production of IL4, IFNγ, nor IL17 (Fig. 6*A*). However, mDCs activated with LPS and histamine induced autologous $CD4^+$ naive T lymphocytes to produce significantly more IL4 and less IFNγ compared with LPS-activated T lymphocytes. This effect was not present in mDCs activated with Pam3Cys that induced

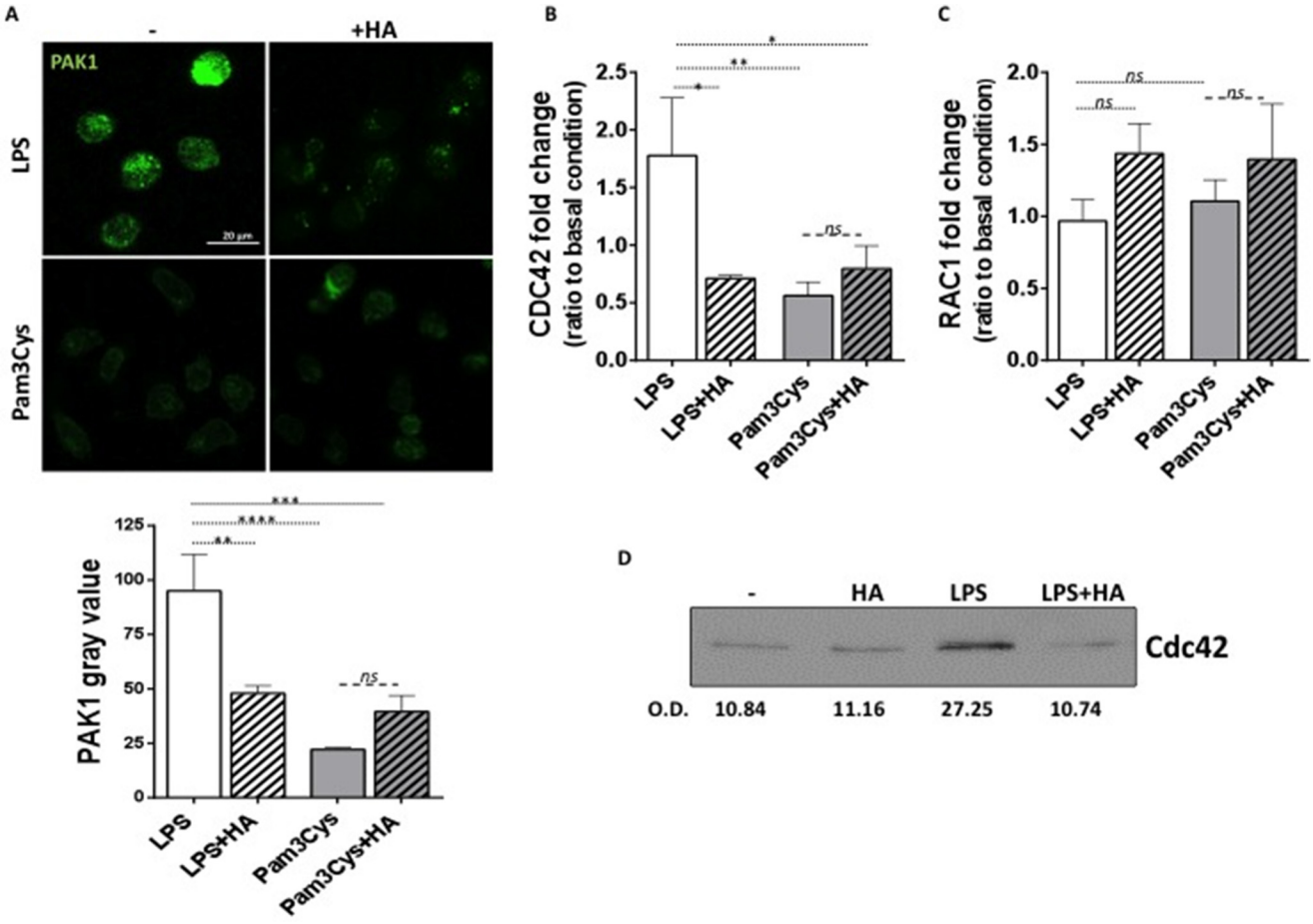

FIGURE 4. **Effect of histamine on the expression of cytoskeleton-regulating molecules.** mDCs stimulated for 2 h with LPS or Pam3Cys in the presence or absence of HA were analyzed for PAK1 expression by confocal microscopy (*A*) and for Cdc42 and RAC1 expression by real-time PCR (*B*). *A,* PAK1 expression (*green*) was quantified as mean fluorescence intensity (mean of green fluorescence measured in five randomly selected fields in three independent experiments) and reported as *gray* value ± S.E. **, $p = 0.009$; ***, $p = 0.0002$; ****, $p < 0.0001$; *ns*, not significant. *B,* Cdc42 and RAC1 expression in TLR-stimulated mDCs in the presence or absence of HA, normalized with respect to CDC42 and RAC1 expression in non-stimulated mDCs (ratio to basal condition). Hypoxanthine-guanine phosphoribosyltransferase was used as housekeeping gene. Mean ± S.E. of three independent experiments are shown. *, $p < 0.05$; **, $p = 0.006$; *ns*, not significant. *D,* Western blot of active precipitated Cdc42, 10×10^6 cells for each point: first row, DCs; second row, 2 DCs + HA; third row, DCs + LPS; fourth row, DCs + LPS + HA, cells were stimulated for 30 min; optical density of each band is reported. One experiment representative of two independent observations is shown.

a comparable production of IL4 and IFNγ, independently of histamine (Fig. 6*A*). In all samples IL17 was not modified although highly variable (Fig. 6*A*). T lymphocytes proliferative response was not changed by the presence of histamine (Fig. 6*B*). Of note, TLR2 and TLR4 stimulations induced a complete maturation of mDCs that was not modified by histamine as shown by a co-stimulation molecule expression (Fig. 7, *A* and *B*), micropinocytosis ability, and MLR (Fig. 7, *C* and *D*). We then investigated DC-T cell immune synapses to understand if histamine-induced actin cytoskeleton organization determines a different IL12p70 distribution at cell-cell contact zone altering cell microenvironment, a process functionally responsible of T cell polarization. Indeed, in the presence of histamine, LPS-activated mDCs showed the Th1 polarizing cytokine IL12 dispersed in the cytoplasm, whereas in control samples (LPS mDCs) it was concentrated and partially colocalized with CD3 molecules at the T cell mDCs contact zone (Fig. 8).

Histamine Receptor Antagonist Effects on Actin Cytoskeleton—Finally, we confirmed the involvement of H2R in mediating actin cytoskeleton modifications induced by histamine during mDCs LPS stimulation (22). In Fig. 9 we show by confocal microscopy that the H2R antagonist zolantidine prevented the cytoskeleton modifications induced by histamine and LPS. This was not observed in the presence of the H1R antagonist pyrilamine or the H4R antagonist JNJ7777120s.

Discussion

Histamine differentially regulates the polarizing ability of mDC consequent to TLRs stimulation. Agrawal *et al.* (23) showed that different TLR stimulations instruct DCs to induce distinct Th responses via differential CKs production. More recently, Deifl *et al.* (24) showed that dendritic cells derived from allergic patients and stimulated by different TLR agonists cause different Th0 naive cell responses due to different activation of three signals: antigen presentation, co-stimulatory molecules, and CKs milieu.

In the present work we show that histamine differently shapes the TLR4 and TLR2 responses of mDC, and we propose that this is at least in part mediated by a fourth signal: rearrangement of the cytoskeleton. In our experiments,

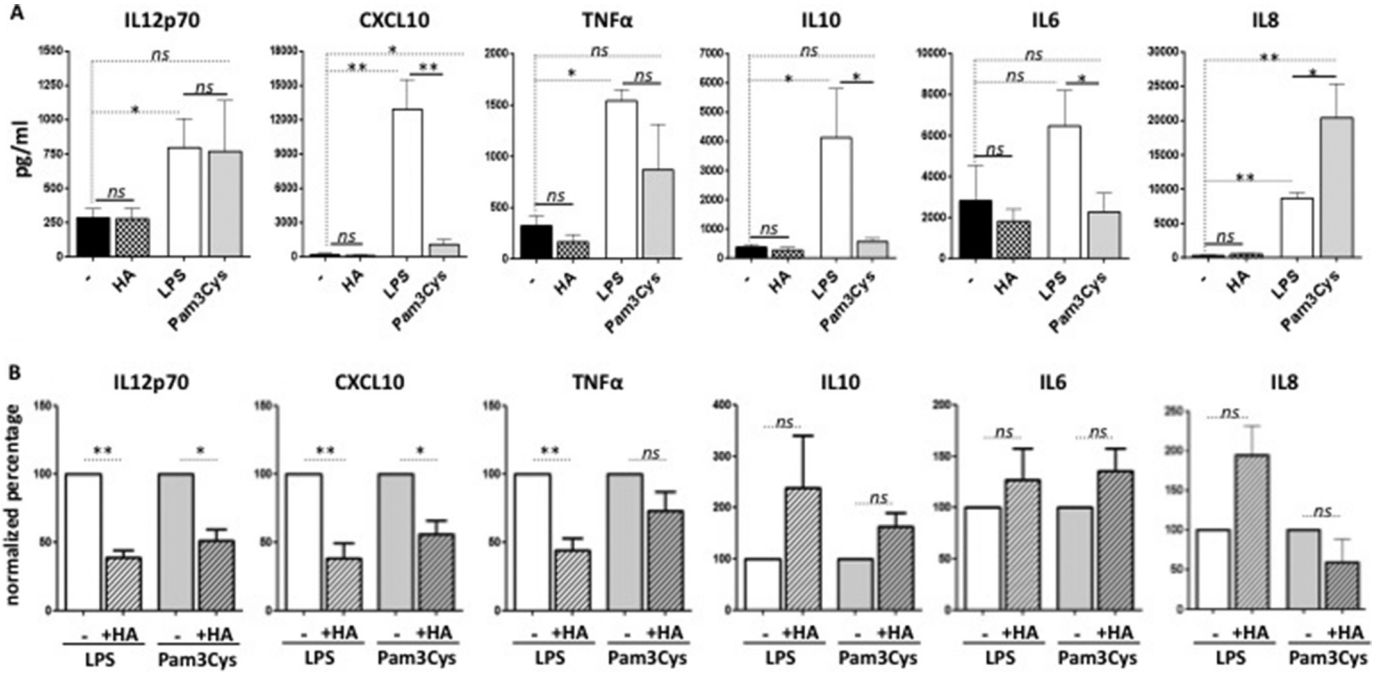

FIGURE 5. **Effect of histamine on mDC cytokine and chemokine production.** *A,* cytokine and chemokine production (pg/ml) determined at day 7 of culture on supernatants of mDCs under basal conditions (−), after 24 h of exposure to HA, or upon 24 h of stimulation with TLR2/4 ligands (LPS; Pam3Cys). Mean ± S.E. of five independent experiments are shown. *B,* effect of HA on cytokine and chemokine production induced by LPS or Pam3Cys stimulation. Data are expressed as mean ± S.E. of five independent experiments, normalized with respect to cytokine production (pg/ml) measured in LPS- or Pam3Cys-stimulated cells reported in *A*. *, $p < 0.05$; **, $p < 0.01$; *ns,* not significant.

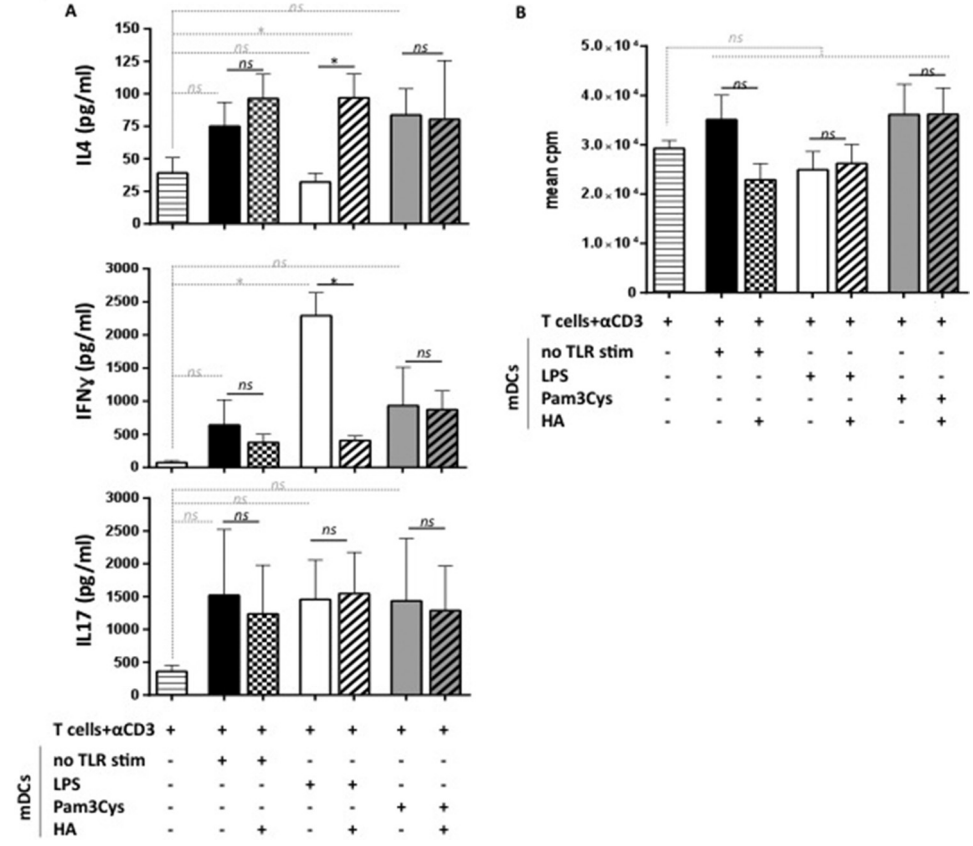

FIGURE 6. **Effect of histamine on the capability of mDC to polarize T cells.** Upon 24 h of stimulation with LPS or Pam3Cys in the presence or absence of HA, mDCs were co-cultured with autologous T cells in anti-CD3-coated plates (T cells + αCD3). As controls, we used non-stimulated (no TLR stim) mDCs, exposed or not to HA in the last 24 h of culture. *A,* IL4, IFNγ, and IL17 were measured by Bioplex on cell supernatants after 4 days of co-culture. Mean (pg/ml) ± S.E. of five experiments with cells from five different donors are shown. *B,* after 4 days of co-culture as described above, T cell proliferation was evaluated by [^{3}H]thymidine incorporation and expressed as mean cpm (counts per min); mean values of cpm ± S.E. of the same five experiments reported in *A*. *, $p < 0.05$; *ns*, not significant.

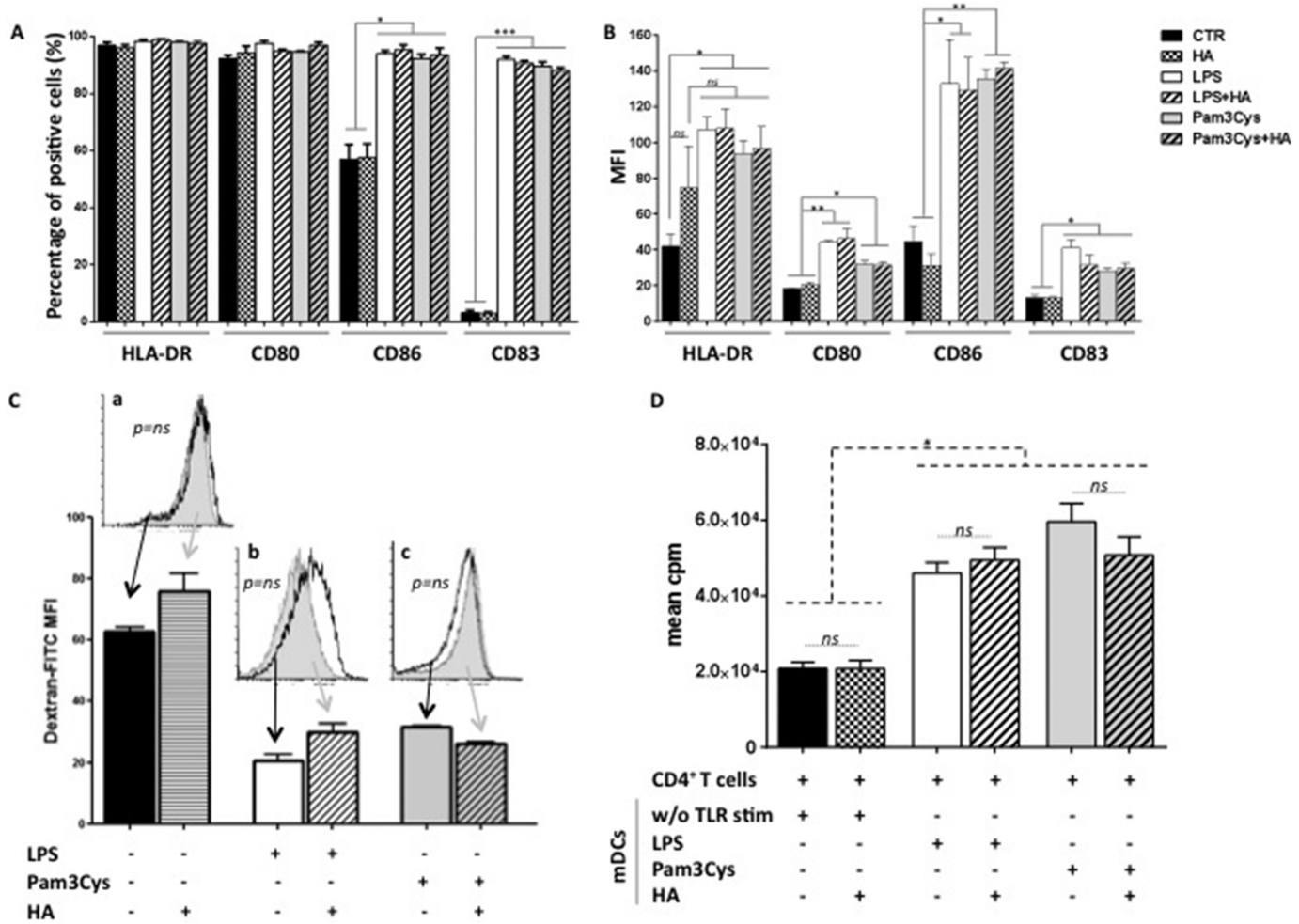

FIGURE 7. **Phenotypic and functional characterization of mDCs treated with histamine in the presence or absence of TLR stimuli.** *A,* percentage of cells expressing the mDC characteristic markers HLA-DR, CD80, CD86, CD83; and *B,* the mean fluorescence intensity (*MFI*) of each marker (mean ± S.E. of 8 experiments, using mDCs from 8 different donors). *CTR*, unstimulated and untreated mDCs; *HA*, mDCs treated with HA in the absence of TLR stimulation; *LPS*, LPS-stimulated mDCs; *LPS + HA*, mDCs treated with HA in the presence of LPS; *Pam3Cys*, Pam3Cys-stimulated mDCs; *Pam3Cys + HA*, mDCs treated with HA in the presence of Pam3Cys. *C,* micropinocytosis ability of mDCs with various combinations of TLR4, TLR2 stimulation, and HA. Micropinocytosis was evaluated by flow cytometry following dextran-FITC uptake; *FITC MFI* indicates the mean amount of dextran-FITC endocytosed (mean ± S.E. of three experiments). *Insets*, overlay plots (one experiment representative of three): HA-treated mDCs, *gray histogram plots*; untreated empty histogram plots, in the absence of TLR stimulation (*a*), in the presence of LPS stimulation (*b*), and in the presence of Pam3Cys stimulation (*c*). *D,* allostimulatory ability of mDCs treated or not with HA in the presence or absence of TLR stimulation was evaluated by a MLR, where mDCs were co-cultured with allogeneic $CD4^+$ T cells at the ratio of 1:10 for 5 days. T cell proliferative response was assessed by [^{3}H]thymidine incorporation and expressed as counts per min (cpm). Graph reports the mean cpm ± S.E. of 5 experiments done with coupled mDCs and T cells from 5 different donors.

mature TLR4 and TLR2 mDCs were comparable in terms of phenotypes: a comparable level of co-stimulatory and MHCII molecules, the same reduction of endocytosis upon stimulation, and an equal T cell proliferative response to mDCs. IL8 is elevated in both samples and increased in Pam3Cys-stimulated cells compared with LPS. Histamine, added to fully differentiated mDCs did not change these phenotypes.

TLRs activation determines, to various extents, maturation of mDCs accompanied by actin cytoskeleton remodeling with consequent functional effects: cytokine production, efficient immune synapse formation, and T cell priming and polarization. When we investigated the actin distribution in mDCs, we observed that TLR4 and TLR2 activation determined different re-organization of actin cytoskeleton. Among the many intracellular responses that follow TLR activation, actin re-organization is a key factor for the maturation state of mDC, inducing a migratory phenotype *versus* the antigen presenting ability. Cytoskeleton re-organization is also responsible for T cell polarization and activation (18). Idzko *et al.* (25) showed that histamine stimulation of immature mDCs modified actin that in turn influenced the migratory ability and endocytosis. In the present work we observed that histamine affected the mature cytoskeleton of mDCs when cells were stimulated with the TLR4 agonist LPS, whereas it did not modify TLR2-induced cytoskeleton organization. Mature TLR4 cells presented low colocalization of actin and CD11c, two molecules that precisely describe mDCs morphology (26), compared with mature TLR2 cells. Histamine does not seem to affect TLR2-induced cytoskeleton organization. In the complex signaling cascade that determines actin cytoskeleton organization, Rac1, CdC42, and the target protein PAK1 play an important role (27, 28). We found that histamine is responsible for the differential expression and activation of CdC42 and PAK1 in TLR4, but not in TLR2-stimulated cells.

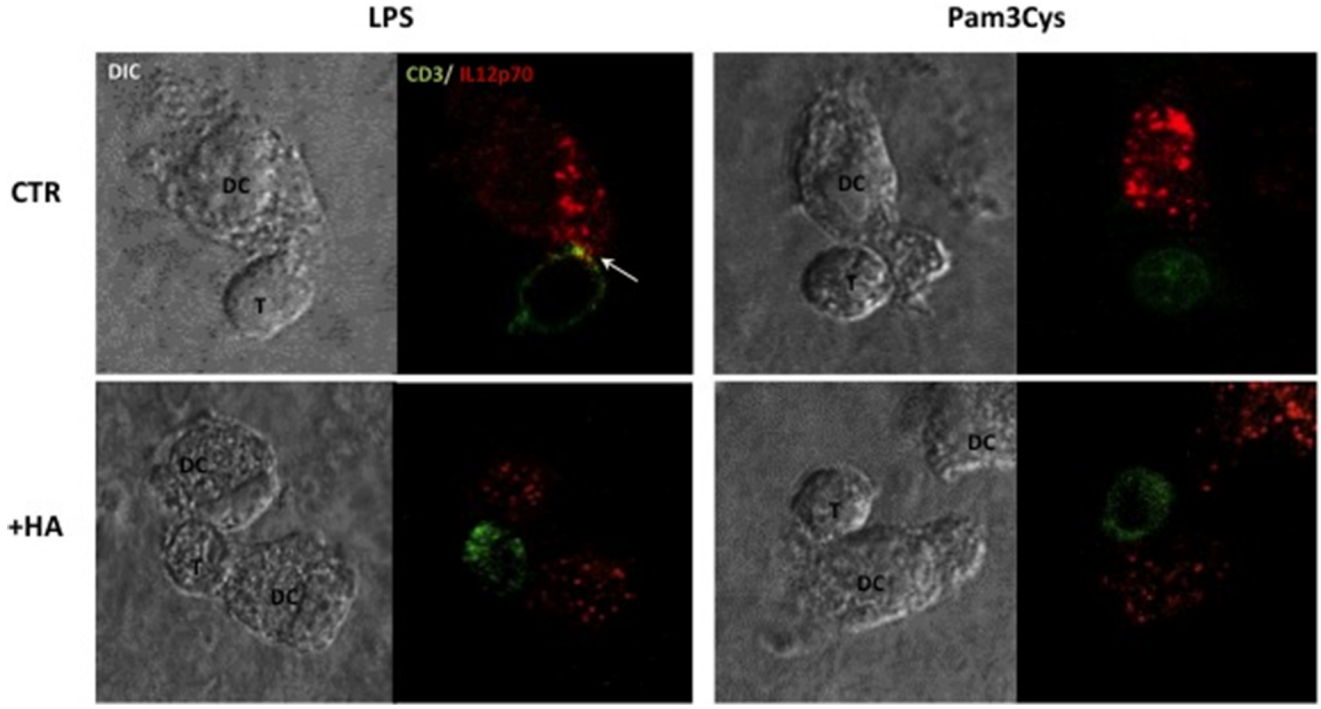

FIGURE 8. **IL12p70 distribution at the immune synapse.** Confocal microscopy images of IL12p70 (*red*) and CD3 (*green*) at the immune synapse between mDCs (*DC*) and $CD4^+$ T cells (*T*), and relative DIC images. *Upper row*: LPS and Pam3Cys activated mDCs (*CTR*). *Lower row*: LPS and Pam3Cys activated mDCs in presence of histamine (+HA). *Arrow,* colocalization (*yellow*) between IL12p70 and CD3.

When we looked at functional effects of histamine on mDCs we observed that mDCs stimulated with TLR4 and TLR2 agonists changed their cytokines profile in the following way: Th1 polarizing cytokine IL12p70 and TNFα were equally produced by LPS and Pam3Cys, whereas IL10, an anti-inflammatory cytokine, IL6 and the Th1 chemoattracting CXCL10 were produced at a significantly higher level by LPS compared with Pam3Cys-stimulated cells. In agreement with other authors' results, histamine modulates this profile and reduces both TLRs induced IL12p70 and CXCL10 production (15, 29, 30). On the other hand, we did not confirm previous data showing that histamine determines TNFα reduction in TLR2-activated cells, nor that histamine increases IL10 by TLR4- and TLR2-stimulated mDCs (11, 13, 15, 29). We attribute the lack of a clear effect on the induction of IL10 by histamine to a high variability between subjects (see Fig. 5*B*). Presumably, and opposite to the effects of histamine on TLRs-induced IL12p70 and CXCL10 production, modulation of IL10 is a sensitive subject. In our hands, the production of TLR-induced IL6 and IL8 was never affected by histamine. When we tested $CD4^+$ naive T lymphocytes for cytokine production in different priming conditions, we found that when primed with histamine-treated, mature TLR4 mDCs (but not TLR2-matured cells), T lymphocytes increased IL4 and decreased IFNγ production, compared with mDC-TLR4 stimulation alone. Therefore, histamine appears to polarize mDC cells into Th2-promoting DC in a manner not linked to the maturation state of DC, as described in other experimental designs (10).

Taken together our data suggest that LPS-induced cytoskeleton re-arrangement determines the polarizing ability of mDC and this leads to variable local release and concentration of IL12p70, altering the immune synapse microenvironment and therefore Th1/Th2 polarization A possible explanation is that during innate immune response, TLRs stimulation affects DC function and that different TLRs may determine different cell phenotypes and functions (32, 33). Our data suggest that histamine acts on different activation states of DCs with different pathways involved, *e.g.* TLR2 signaling is strictly MyD88 dependent, whereas TLR4 is not (6). In our hypothesis the different histamine effects on TLR2 and TLR4 activated DCs may lead to T cell skewed response.

Histamine exerts its diverse effects on mDCs activating H1, H2, or H4 receptors. Here, we confirm the expression of mDCs H1R, H2R, and H4R during *in vitro* culture and maturation with or without histamine as reported by other authors (10, 11). In our experimental setting we found that histamine *per se* did not change the expression of any of these receptors. However, the application of histamine increased H2R expression in TLR2- compared with LPS-stimulated or non-stimulated cells. In the past, it has been reported that histamine modulates actin polarization and chemotaxis in immature mDCs mainly through H1R and H2R cytokine production exclusively through H2R and H3R (1); more recently H4R has been shown to be involved in both pathways (16), here we confirm the involvement of H2R in actin cytoskeleton modulation (Fig. 9). Probably, the signaling responsible for histamine modulation during TLRs stimulation is more complex, here we did not find expression of H3R, in agreement with most reports (14, 34). Finally, to assess the possibility that histamine regulates TLR4 and TLR2 expression we analyzed mRNA for both receptors and showed for the first time that in the mDC cultures, histamine did not significantly modulate TLR2 and TLR4 transcripts, as reported in fibroblast cultures (31), although a trend toward increased expression was observed.

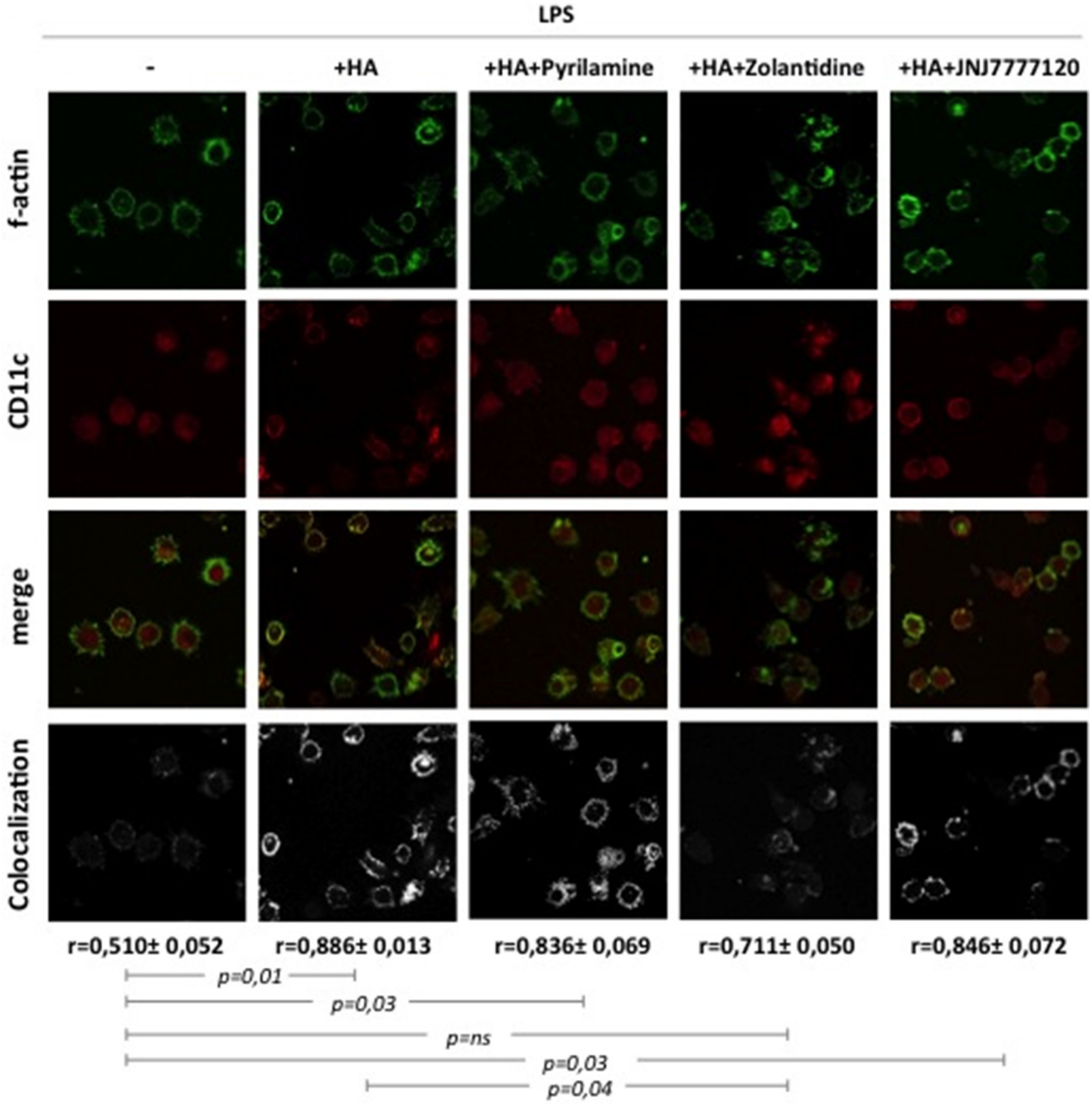

FIGURE 9. **Effect of histamine receptor antagonists on actin cytoskeleton distribution in mDCs treated with histamine during LPS stimulation.** At day 6 of culture, mDCs were stimulated 24 h with LPS alone (−) or simultaneously treated with the following drugs: histamine (+*HA10*; μM), histamine together with the H1R antagonist pyrilamine, 1 μM (+*HA* + *Pyrilamine*), or with the H2R antagonist zolantidine, 10 μM (+*HA* + *Zolantidine*), or with the H4R antagonist JNJ7777120, 10 μM (+*HA* + *JNJ7777120*). DCs were analyzed by confocal microscopy for F-actin (*green*) and CD11c (*red*) cell distribution. Colocalization between F-actin and CD11c was visualized in *white* by using the ImageJ colocalization finder plug-in and quantified by Pearson's coefficient (*r*), calculated with ImageJ Jacop plug-in ($r = 1$: complete colocalization; $r = 0$: absence of colocalization). The pictures are from one experiment representative of three independent observations; Pearson's coefficient values (*r*) reported below the colocalization images are the mean ± S.E. of all of three experiments.

In a translational perspective we may consider that *in vivo* DCs are often in close proximity to mast cells and therefore the microenvironment may be rich in histamine during microbe stimulation of innate immune receptors and inflammation (34–37); if so the reaction of TLR4 and TLR2 mDCs to infections or allergen will determine diverse T subpopulation responses. This TLRs/histamine interaction could participate to pathological allergic processes, as loss of tolerance (38) and exacerbation of asthma (39). Indeed, histamine influences responses against parasitic or bacterial infections in several animal models (15), and the mode of action that we propose adds a relevant piece of knowledge on the complex mechanism behind it. In the future it would be of interest to study these mechanisms in pathological situations as allergic subjects or autoimmune diseases, to evaluate the relevance of actin cytoskeleton as a possible target for chronic inflammation therapy.

Author Contributions—A. A. and D. N. designed, performed, and analyzed the experiments showed in Figs. 3, 4, and 7; A. A. and E. B. designed, performed, and analyzed the experiments showed in Figs. 5–7; B. P. and C. M. designed, performed, and analyzed the experiments showed in Figs. 1 and 2; B. P. and E. M. critically revised the article for important intellectual content; C. B. conceived and coordinated the study and wrote the paper. All authors reviewed the results and approoved the final version of the manuscript.

References

1. Jutel, M., Watanabe, T., Akdis, M., Blaser, K., and Akdis, C. A. (2002) Immune regulation by histamine. *Curr. Opin. Immunol.* **14,** 735–740
2. Ferstl, R., Akdis, C. A., and O'Mahony, L. (2012) Histamine regulation of innate and adaptive immunity. *Front. Biosci.* **17,** 40–53
3. Kapsenberg, M. L. (2003) Dendritic-cell control of pathogen-driven T-cell polarization. *Nat. Rev. Immunol.* **3,** 984–993
4. Banchereau, J., and Steinman, R. M. (1998) Dendritic cells and the control of immunity. *Nature* **392,** 245–252
5. Constant, S., Pfeiffer, C., Woodard, A., Pasqualini, T., and Bottomly, K. (1995) Extent of T cell receptor ligation can determine the functional differentiation of naive $CD4^+$ T cells. *J. Exp. Med.* **182,** 1591–1596
6. West, M. A., Wallin, R. P., Matthews, S. P., Svensson, H. G., Zaru, R., Ljunggren, H. G., Prescott, A. R., and Watts, C. (2004) Enhanced dendritic cell antigen capture via Toll-like receptor-induced actin remodeling. *Science* **305,** 1153–1157
7. Kawai, T., and Akira, S. (2010) The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol.* **11,** 373–384
8. Reuter, S., Dehzad, N., Martin, H., Böhm, L., Becker, M., Buhl, R., Stassen, M., and Taube, C. (2012) TLR3 but not TLR7/8 ligand induces allergic sensitization to inhaled allergen. *J. Immunol.* **188,** 5123–5131
9. Kücüksezer, U. C., Palomares, O., Rückert, B., Jartti, T., Puhakka, T., Nandy, A., Gemicioğlu, B., Fahrner, H. B., Jung, A., Deniz, G., Akdis, C. A., and Akdis, M. (2013) Triggering of specific Toll-like receptors and proinflammatory cytokines breaks allergen-specific T-cell tolerance in human tonsils and peripheral blood. *J. Allergy Clin. Immunol.* **131,** 875–885
10. Caron, G., Delneste, Y., Roelandts, E., Duez, C., Bonnefoy, J. Y., Pestel, J., and Jeannin, P. (2001) Histamine polarizes human dendritic cells into Th2 cell-promoting effector dendritic cells. *J. Immunol.* **167,** 3682–3686
11. Mazzoni, A., Young, H. A., Spitzer, J. H., Visintin, A., and Segal, D. M. (2001) Histamine regulates cytokine production in maturing dendritic cells, resulting in altered T cell polarization. *J. Clin. Invest.* **108,** 1865–1873
12. Gutzmer, R., Langer, K., Lisewski, M., Mommert, S., Rieckborn, D., Kapp, A., and Werfel, T. (2002) Expression and function of histamine receptors 1 and 2 on human monocyte-derived dendritic cells. *J. Allergy Clin. Immunol.* **109,** 524–531
13. Gutzmer, R., Diestel, C., Mommert, S., Köther, B., Stark, H., Wittmann, M., and Werfel, T. (2005) Histamine H4 receptor stimulation suppresses IL-12p70 production and mediates chemotaxis in human monocyte-derived dendritic cells. *J. Immunol.* **174,** 5224–5532
14. Simon, T., Gogolák, P., Kis-Tóth, K., Jelinek, I., László, V., and Rajnavölgyi, E. (2012) Histamine modulates multiple functional activities of monocyte-derived dendritic cell subsets via histamine receptor 2. *Int. Immunol.* **24,** 107–116
15. Frei, R., Ferstl, R., Konieczna, P., Ziegler, M., Simon, T., Rugeles, T. M., Mailand, S., Watanabe, T., Lauener, R., Akdis, C. A., and O'Mahony, L. (2013) Histamine receptor 2 modifies dendritic cell responses to microbial ligands. *J. Allergy Clin. Immunol.* **132,** 194–204
16. Glatzer, F., Mommert, S., Köther, B., Gschwandtner, M., Stark, H., Werfel, T., and Gutzmer, R. (2014) Histamine downregulates the Th1-associated chemokine IP-10 in monocytes and myeloid dendritic cells. *Int. Arch. Allergy Immunol.* **163,** 11–19
17. Zhai, R. R., Jiang, A. P., Wang, H. B., Ma, L., Ren, X. X., Jiang, J. F., Wu, L., Wei, J. F., and Wang, J. H. (2013) Histamine enhances HIV-1-induced modulation of dendritic cells to skew naive T cell differentiation toward regulatory T cells. *Virology* **442,** 163–172
18. Benvenuti, F., Hugues, S., Walmsley, M., Ruf, S., Fetler, L., Popoff, M., Tybulewicz, V. L., and Amigorena, S. (2004) Requirement of Rac1 and Rac2 expression by mature dendritic cells for T cell priming. *Science* **305,** 1150–1153
19. Aldinucci, A., Rizzetto, L., Pieri, L., Nosi, D., Romagnoli, P., Biagioli, T., Mazzanti, B., Saccardi, R., Beltrame, L., Massacesi, L., Cavalieri, D., and Ballerini, C. (2010) Inhibition of immune synapse by altered dendritic cell actin distribution: a new pathway of mesenchymal stem cell immune regulation. *J. Immunol.* **185,** 5102–5110
20. Morrison, V. L., James, M. J., Grzes, K., Cook, P., Glass, D. G., Savinko, T., Lek, H. S., Gawden-Bone, C., Watts, C., Millington, O. R., MacDonald, A. S., and Fagerholm, S. C. (2014) Loss of β2-integrin-mediated cytoskeletal linkage reprogrammes dendritic cells to a mature migratory phenotype. *Nat. Commun.* **5,** 5359
21. Zinchuk, V., Zinchuk, O., and Okada, T. (2007) Quantitative colocalization analysis of multicolor confocal immunofluorescence microscopy images: pushing pixels to explore biological phenomena. *Acta Histochem. Cytochem.* **40,** 101–111
22. Thurmond, R. L., Gelfand, E. W., and Dunford, P. J. (2008) The role of histamine H1 and H4 receptors in allergic inflammation: the search for new antihistamines. *Nat. Rev. Drug Discov.* **7,** 41–53
23. Agrawal, S., Agrawal, A., Doughty, B., Gerwitz, A., Blenis, J., Van Dyke, T., and Pulendran, B. (2003) Cutting edge: different Toll-like receptor agonists instruct dendritic cells to induce distinct Th responses via differential modulation of extracellular signal-regulated kinase-mitogen-activated protein kinase and c-Fos. *J. Immunol.* **171,** 4984–4989
24. Deifl, S., Kitzmüller, C., Steinberger, P., Himly, M., Jahn-Schmid, B., Fischer, G. F., Zlabinger, G. J., and Bohle, B. (2014) Differential activation of dendritic cells by toll-like receptors causes diverse differentiation of naive $CD4^+$ T cells from allergic patients. *Allergy* **69,** 1602–1609
25. Idzko, M., la Sala, A., Ferrari, D., Panther, E., Herouy, Y., Dichmann, S., Mockenhaupt, M., Di Virgilio, F., Girolomoni, G., and Norgauer, J. (2002) Expression and function of histamine receptors in human monocyte-derived dendritic cells. *J. Allergy Clin. Immunol.* **109,** 839–846
26. Aldinucci, A., Turco, A., Biagioli, T., Toma, F. M., Bani, D., Guasti, D., Manuelli, C., Rizzetto, L., Cavalieri, D., Massacesi, L., Mello, T., Scaini, D., Bianco, A., Ballerini, L., Prato, M., and Ballerini, C. (2013) Carbon nanotube scaffolds instruct human dendritic cells: modulating immune responses by contacts at the nanoscale. *Nano Lett.* **13,** 6098–6105
27. Hall, A. (1998) Rho GTPases and the actin cytoskeleton. *Science* **279,** 509–514
28. Kong, L., and Ge, B. X. (2008) MyD88-independent activation of a novel actin-Cdc42/Rac pathway is required for Toll-like receptor-stimulated phagocytosis. *Cell Res.* **18,** 745–755
29. Thomas, C. M., Hong, T., van Pijkeren, J. P., Hemarajata, P., Trinh, D. V., Hu, W., Britton, R. A., Kalkum, M., and Versalovic, J. (2012) Histamine derived from probiotic Lactobacillus reuteri suppresses TNF via modulation of PKA and ERK signaling. *PLoS ONE* **7,** e31951
30. O'Mahony, L., Akdis, M., and Akdis, C. A. (2011) Regulation of the immune response and inflammation by histamine and histamine receptors. *J. Allergy Clin. Immunol.* **128,** 1153–1162
31. Jang, S., Park, J. S., Won, Y. H., Yun, S. J., and Kim, S. J. (2012) The expression of Toll-like receptors (TLRs) in cultured human skin fibroblast is modulated by histamine. *Chonnam Med. J.* **48,** 7–14
32. Smolinska, S., Jutel, M., Crameri, R., and O'Mahony, L. (2014) Histamine and gut mucosal immune regulation. *Allergy* **69,** 273–281
33. Farina, C., Theil, D., Semlinger, B., Hohlfeld, R., and Meinl, E. (2004) Distinct responses of monocytes to Toll-like receptor ligands and inflammatory cytokines. *Int. Immunol.* **16,** 799–809
34. Bashir, M. E., Louie, S., Shi, H. N., and Nagler-Anderson, C. (2004) Toll-like receptor 4 signaling by intestinal microbes influences susceptibility to food allergy. *J. Immunol.* **172,** 6978–6987
35. Yang, C., Mo, X., Lv, J., Liu, X., Yuan, M., Dong, M., Li, L., Luo, X., Fan, X., Jin, Z., Liu, Z., and Liu, J. (2012) Lipopolysaccharide enhances FcεRI-mediated mast cell degranulation by increasing Ca^{2+} entry through store-operated Ca^{2+} channels: implications for lipopolysaccharide exacerbating

allergic asthma. *Exp. Physiol.* **97,** 1315–1327
36. Shao, L., Cong, Z., Li, X., Zou, H., Cao, L., and Guo, Y. (2015) Changes in levels of IL-9, IL-17, IFN-γ, dendritic cell numbers and TLR expression in peripheral blood in asthmatic children with *Mycoplasma pneumoniae* infection. *Int. J. Clin. Exp. Pathol.* **8,** 5263–5272
37. Carroll-Portillo, A., Cannon, J. L., te Riet, J., Holmes, A., Kawakami, Y., Kawakami, T., Cambi, A., and Lidke, D. S. (2015) Mast cells and dendritic cells form synapses that facilitate antigen transfer for T cell activation. *J. Cell Biol.* **210,** 851–864
38. Beghdadi, W., Porcherie, A., Schneider, B. S., Dubayle, D., Peronet, R., Huerre, M., Watanabe, T., Ohtsu, H., Louis, J., and Mécheri, S. (2008) Inhibition of histamine-mediated signaling confers significant protection against severe malaria in mouse models of disease. *J. Exp. Med.* **205,** 395–408
39. Metz, M., Doyle, E., Bindslev-Jensen, C., Watanabe, T., Zuberbier, T., and Maurer, M. (2011) Effects of antihistamines on innate immune responses to severe bacterial infection in mice. *Int. Arch. Allergy Immunol.* **155,** 355–360

Histamine Regulates Actin Cytoskeleton in Human Toll-like Receptor 4-activated Monocyte-derived Dendritic Cells Tuning CD4 $^{+}$ T Lymphocyte Response

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