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Extracellular Acidosis Triggers the Maturation of Human Dendritic Cells and the Production of IL-12[1]

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Although the development of an acidic tissue environment or acidosis is a hallmark of inflammatory processes, few studies analyze the effect of extracellular pH on immune cells. We have previously shown that exposure of murine dendritic cells (DCs) to pH 6.5 stimulates macropinocytosis and cross-presentation of extracellular Ags by MHC class I molecules. We report that the transient exposure of human DCs to pH 6.5 markedly increases the expression of HLA-DR, CD40, CD80, CD86, CD83, and CCR7 and improves the T cell priming ability of DCs. Incubation of DCs at pH 6.5 results in the activation of the PI3K/Akt and the MAPK pathways. Using specific inhibitors, we show that the maturation of DCs induced by acidosis was strictly dependent on the activation of p38 MAPK. DC exposure to pH 6.5 also induces a dramatic increase in their production of IL-12, stimulating the synthesis of IFN-γ, but not IL-4, by Ag-specific CD4$^+$ T cells. Interestingly, we find that suboptimal doses of LPS abrogated the ability of pH 6.5 to induce DC maturation, suggesting a cross-talk between the activation pathways triggered by LPS and extracellular protons in DCs. We conclude that extracellular acidosis in peripheral tissues may contribute to the initiation of adaptive immune responses by DCs, favoring the development of Th1 immunity. ***The Journal of Immunology,*** **2007, 179: 1950–1959.**

Dendritic cells (DCs)[3] are highly specialized APCs with a unique ability to activate resting T lymphocytes and initiate primary immune responses as well as to induce peripheral tolerance (1–3). Upon encountering inflammatory stimuli or pathogens in peripheral tissues, DCs become activated and undergo a number of physiological changes leading to their terminal differentiation or maturation. These changes are linked to an enhanced ability to activate T cells and to direct the differentiation of CD4$^+$ T cells into Th1 or Th2 profile (1–4).

The transition from immature, Ag-capturing cells to mature APCs can be triggered by different stimuli. The interaction with pathogen-derived products through TLR is the primary stimulus for DC maturation and the production of IL-12 (4–7). DCs can also be activated by cytokines and inflammatory mediators produced by injured cells such as TNF-α, IL-1α, IL-1β, type I IFNs, NO, and PGE_2, or by signals from other innate cells such as NK, NKT, and $\gamma\delta$ T cells (4, 7–11). Finally, activated CD4$^+$ T cells as well as immune complexes can also function as potent activators of DCs (4, 12–15). Thus, DCs mature in response to most individual components related to innate and adaptive immune responses, including the infectious agents themselves (through TLRs), the inflammatory response (cytokines, inflammatory mediators, stress signals, and others) and the antigenic T and B cell responses.

The development of acidic environments (or extracellular acidosis) is yet another hallmark of inflammatory processes of different etiology. Interstitial acidification (pH 5.5–7.0) is commonly associated with the development of inflammatory reactions against pathogens in peripheral tissues (16–21). Not only are inflammatory reactions against pathogens associated with the development of acidic environments, but autoimmune processes such as those in rheumatoid arthritis and asthma are also associated with the development of acidic microenvironments in injured tissues. The pH of synovial fluid of compromised joints in patients with rheumatoid arthritis is acidic (pH 6.7–7.4), and acidosis appears to correlate with both synovial fluid leukocytosis and joint destruction (22–24). In contrast, observations made in the lower airway of patients with acute asthma showed that the values of pH found in airway vapor condensate samples from asthmatic patients were over 1:2 log orders lower than in control subjects (25, 26). Acidosis also develops in the tumor microenvironment, a terrain poorly irrigated by chaotic vasculature and thus frequently associated to hypoxia and dependency on glycolysis. In fact, studies performed over the past 50 years found values of extracellular pH ranging from 5.8 to 7.4, both in human and rodent malignant tissues (27–30). Surprisingly, although acidosis represents a characteristic trait of inflamed tissue, little is known about the influence of extracellular pH on the function of immune cells, particularly DCs.

We have previously reported that extracellular acidosis improves endocytosis and cross-presentation of extracellular Ags by murine DCs (31). Moreover, we also reported that DCs pulsed

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[3] Abbreviations used in this paper: DC, dendritic cell; TT, tetanus toxoid; fluo-3-AM, fluo-3-acetoxymethyl ester; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration.

with Ag under acidic values of pH show an improved efficacy to induce both specific $CD8^+$ CTLs and specific Ab responses in vivo (31).

In this study we show that transient exposure to acidic conditions, similar to those encountered in vivo, triggers not only the phenotypic maturation of human DCs, but also high levels of IL-12 production, suggesting that extracellular protons may function as chemical stress for DCs, favoring the development of Th1 adaptive immunity.

Materials and Methods

Reagents

LPS from *Escherichia coli* and recombinant human GM-CSF were obtained from Sigma-Aldrich. Recombinant human IL-4 was from PeproTech or R&D Systems. Ficoll-Hypaque and Percoll were from Pharmacia. The ERK inhibitor PD98059 was purchased from Biomol. The PI3K inhibitor wortmannin and the p38 MAPK inhibitor SB202190 were from Calbiochem. Tetanus toxoid (TT) was from the Institute Dr. Carlos G. Malbrán (Buenos Aires, Argentina).

Preparation of human DCs

PBMC were isolated from healthy volunteers by standard density gradient centrifugation on Ficoll-Hypaque. Monocytes were purified by centrifugation on a discontinuous Percoll gradient with modifications of a previously described method (32). Briefly, PBMC were suspended in Ca^{2+} and Mg^{2+}-free Tyrode solution supplemented with 0.2% EDTA and incubated during 30 min at 37°C. During this incubation, the osmolarity of the medium was gradually increased from 290 to 360 Osmol/L by addition of 9% NaCl. Three different Percoll fractions were layered in polypropylene tubes: 50% at the bottom followed by 46% and 40%. PBMC (5.10×10^6/ml) were layered at the top, and they were centrifuged at $400 \times g$ for 20 min at 4°C. Monocytes were recovered at the 50–46% interface. The purity was checked by FACS analysis using anti-CD14 mAb and was found to be >85%. To obtain DCs, monocytes were cultured in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, and 0.1 mM nonessential amino acids (complete medium; all from Invitrogen Life Technologies) at 1.5×10^6 cells/ml with 10 ng/ml IL-4 and 10 ng/ml GM-CSF, as described by Sallusto and Lanzavecchia (33). On day 7, cells were analyzed by FACS.

Culture conditions

Extracellular acidification was achieved by suspending cell pellets in complete medium previously adjusted to the desired pH values or by the addition of a precalculated volume of isotonic hydrogen chloride solution. Similar results were observed using both methods. Cell cultures were maintained at 37°C in a humidified atmosphere either with 5% CO_2, for cells suspended in medium at pH 7.3, or with 7% CO_2, for cells suspended in medium at pH 6.5. Hydrogen chloride solutions were free of endotoxin (<0.5 EU/ml) as determined using a *Limulus* Amebocyte Lysate test (BioWhittaker).

Flow cytometry

FITC-conjugated mAbs directed to CD1a, CD80, CD86, CD40, HLA-DR, CD83, and CCR7 were from BD Pharmingen. PE-conjugated mAb directed to IL-12 p40/p70, GM-CSF, IL-10, and IFN-γ were also used (BD Pharmingen). Intracellular staining of IL-12, IL-10, GM-CSF, and IFN-γ was performed in cells permeabilized with saponin. In all cases isotype-matched control mAbs were used, and a gate (R1) was defined in the analysis to exclude all nonviable cells and debris, based on size and propidium iodine staining. Analysis was performed using a FACS flow cytometer and CellQuest software (BD Biosciences). The results are expressed as mean fluorescence intensity.

Quantitation of cellular apoptosis and viability by fluorescence microscopy

Quantitation was performed as previously described (34) using the fluorescent DNA binding dyes acridine orange (100 μg/ml) to determine the percentage of cells that had undergone apoptosis and ethidium bromide (100 μg/ml) to differentiate between viable and nonviable cells. With this method, nonapoptotic cell nuclei show "structure"; variations in fluorescence intensity that reflect the distribution of euchromatin and heterochromatin. By contrast, apoptotic nuclei exhibit highly condensed chromatin that is uniformly stained by acridine orange. In fact, the entire apoptotic nucleus is present as bright spherical beads. To assess the percentage of cells showing morphologic features of apoptosis, at least 200 cells were scored in each experiment.

Production of IL-12p70 by DCs evaluated by ELISA

DCs (2.5×10^6/ml) were cultured for 90 min at pH 6.5 or 7.3 followed by 24 h at pH 7.3. Supernatants were harvested and analyzed for the presence of IL-12p70 by ELISA (R&D Systems), following the manufacturer's recommendations.

Production of IFN-γ by T cells

Production of IFN-γ was performed as previously described (35). DCs (2.5×10^6/ml) were cultured for 90 min at pH 6.5 or 7.3 followed by 24 h at pH 7.3, and cell supernatants were harvested. PBMC (2.5×10^6/ml) were cultured for 96 h with immobilized anti-CD3 in the presence of DCs supernatants (50% v/v). Then, PBMC were treated with PMA (10 ng/ml) plus ionomycin (5 μg/ml) and cells were cultured for an additional period of 24 h. Brefeldin A (10 μg/ml) was added during the last 6 h of culture to inhibit the release of IFN-γ. Immunofluorescence staining was performed using anti-IFN-γ PE, anti-CD8 FITC, and anti-CD3 PercP (BD Pharmingen).

MLR analysis

DCs were cultured for 90 min at pH 6.5. Then, the culture medium was neutralized by the addition of a precalculated volume of isotonic sodium hydroxide, and the cells were cultured for an additional period of 48 h at pH 7.3. Control DCs were cultured for 48 h at pH 7.3. In all cases, DCs were irradiated (3000 rad from a ^{137}Cs source) and 2.5×10^4 DCs were cultured alone or in the presence of 2.5 or 5.0×10^5 responding cells from freshly isolated allogeneic PBMC, for 5 days in U-bottom 96-well plates (Costar). Thymidine incorporation was measured on day 5 by a 16-h pulse with [^{3}H]thymidine (1 μCi/well, specific activity, 5 Ci/mM; DuPont).

Endocytosis of FITC-OVA

DCs were suspended in complete medium adjusted to pH 7.3 or 6.5. FITC-OVA was added at final concentrations of 100 μg/ml, and cells were incubated for 30 min at 37°C under 5% or 7% CO_2, for cultures performed at pH 7.3 or 6.5, respectively. The cells were then washed three times with cold PBS containing 1% FCS and 0.01% NaN_3 and were analyzed on a FACS (BD Biosciences). The fluorescence background was determined by incubating cells with FITC-OVA at 4°C. In some experiments we used the dye trypan blue to quench extracellular fluorescence, as described (36, 37). In these experiments, endocytosis assays were performed as previously indicated, but acquisition of samples was conducted in the presence of 200 μg/ml trypan blue. The efficacy of trypan blue to quench extracellular fluorescence was controlled in experiments in which DCs were stained with FITC-mAb directed to cell surface Ags (30 min at 4°C). Fluorescence intensity was diminished by >90% when the acquisition of the samples was performed in the presence of trypan blue.

Western blotting

Abs directed to phospho-JNK (T183/Y185, mouse monoclonal) were obtained from Santa Cruz Biotechnology. Abs directed against phospho-ERK1 and phospho-ERK2 (T202/Y204, mouse monoclonal), phospho-p38 MAPK (T180/Y182, rabbit polyclonal), and phospho-Akt (S473, rabbit polyclonal) were from Cell Signaling Technology. HRP-conjugated anti-mouse or anti-rabbit IgG was from Santa Cruz Biotechnology. DCs suspended in complete medium (1.5×10^6 cells/300 μl) were prewarmed for 30 min at 37°C. The acidification of cells suspended in medium at pH 7.3 was accomplished by the addition of a precalculated volume of isotonic hydrogen chloride solution. Cells were incubated at pH 7.3 or 6.5 for different times (0–30 min) at 37°C. The reactions were stopped at the times indicated by adding cold saline, and the samples were then centrifuged. Cell pellets were resuspended in loading buffer (60 mM Tris (pH 6.8), 2.3% SDS, 10% glycerol, 0.01% bromphenol blue, and 5% 2-ME), boiled at 96°C for 5 min, and stored at −80°C. Samples were then separated by SDS-PAGE (10 or 12%), transferred to polyvinylidene difluoride membranes (Sigma-Aldrich), and then blocked with 5% bovine albumin in PBS containing 0.05% Tween 20. Membranes were then blotted with Abs against phospho-Akt, phospho-ERK, phospho-p38, or phospho-JNK, followed by HRP-conjugated anti-mouse or anti-rabbit IgG. Specific bands were developed by ECL (Amersham Biosciences). Membranes were stripped and reproved with a murine mAb against human β-actin (Cell Signaling Technology).

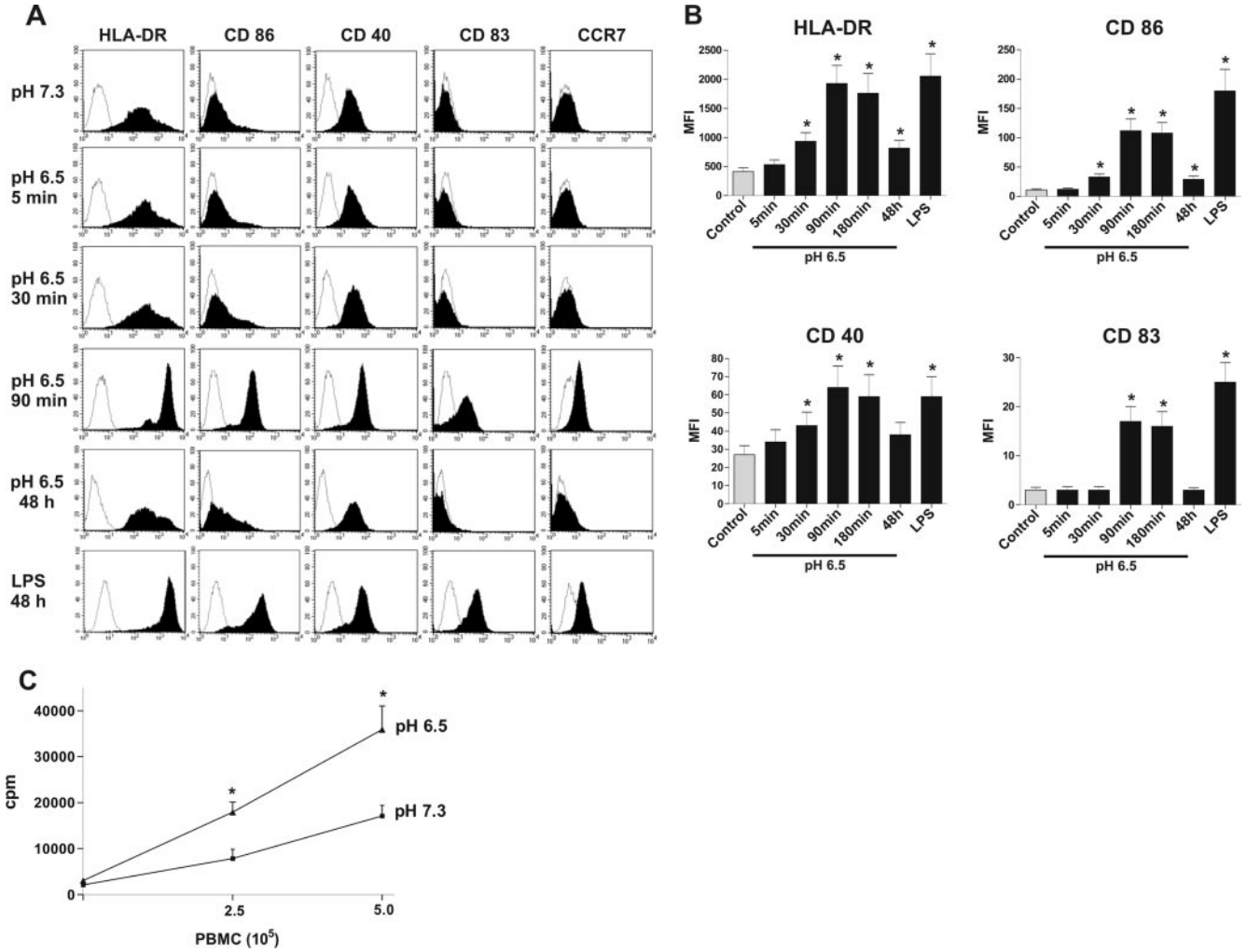

FIGURE 1. Transient exposure to pH 6.5 triggers the phenotypic maturation of human DCs. *A* and *B*, DCs (2.5×10^6/ml) were incubated for 5, 30, 90, and 180 min at pH 6.5, the culture medium was then neutralized and the cells were cultured for an additional period of 48 h at pH 7.3. Control cells were cultured for 48 h at pH 7.3. The phenotypes of DCs cultured continuously for 48 h at pH 6.5 and DCs cultured for 48 h at pH 7.3 in the presence of LPS (100 ng/ml) are also shown. *A*, Histograms from a representative experiment are shown. *B*, Results are expressed as mean fluorescence intensity (MFI) values and represent the arithmetic mean ± SEM of seven experiments. ∗, $p < 0.05$ for pH 6.5 vs 7.3 (control) or LPS vs control. *C*, DCs were cultured for 90 min at pH 6.5, then the culture medium was neutralized and the cells were cultured for an additional period of 48 h at pH 7.3. Control DCs were cultured for 48 h at pH 7.3. MLR was performed as described in *Materials and Methods* using two different DC to allogeneic PBMC ratios (1:10 and 1:20). Results are expressed as cpm and represent the arithmetic mean ± SEM of five experiments. ∗, $p < 0.05$ for pH 6.5 vs 7.3 (control).

Calcium measurements

Changes in intracellular-free calcium concentrations ($[Ca^{2+}]_i$) were measured using fluo-3-acetoxymethyl ester (fluo-3-AM) as previously described (38). DCs suspended at a concentration of 5×10^6 cells/ml in complete medium were incubated with 4 μM fluo-3-AM for 30 min at 30°C. Then, cells were washed three times with RPMI 1640 medium and suspended at 2.5×10^6 cells/ml in RPMI 1640 supplemented with 5% FCS. Aliquots of 50-μl each were then added to 450 μl of 37°C RPMI 1640 medium containing 5% FCS (pH 7.3). The prewarmed sample was immediately loaded onto the flow cytometer, and fluorescence was recorded for ~25 s. Then, the medium was acidified by the addition of a predetermined volume of isotonic solution of hydrogen chloride to adjust its pH to 6.5, and the fluorescence was recorded during an additional 100 s. A gate based on forward and side light scatter was used to exclude debris, whereas a time-based gate was used to divide the original data file and separate cells according to the time at which their fluorescence in an FL1 detector was measured. Measurements of fluorescence in samples suspended at pH 7.3 were used to establish a marker at a FL1 fluorescence channel number greater than measurements exhibited by at least 97% of these resting cells. This marker was then used to determine the relative percentage of activated cells. Cells that raised their $[Ca^{2+}]_i$ to higher levels than that shown by 97% of resting cells in response to pH 6.5 were considered to be activated.

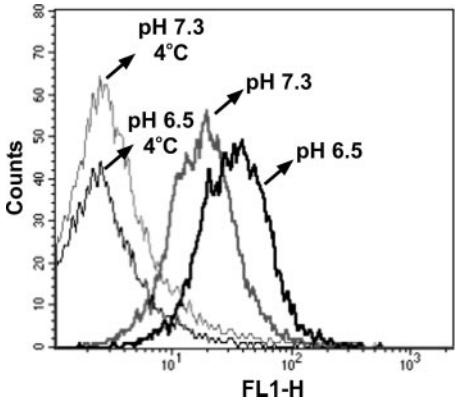

FIGURE 2. Exposure to pH 6.5 enhances endocytosis of FITC-OVA by human DCs. DCs (2.5×10^6/ml) were incubated for 30 min at 37°C with FITC-OVA (100 μg/ml) at pH 7.3 or 6.5, and the amount of ligand accumulated was measured by flow cytometry. The uptake of FITC-OVA by DCs after incubation for 30 min at 4°C is also shown. Histograms from one representative experiment ($n = 9$) are shown.

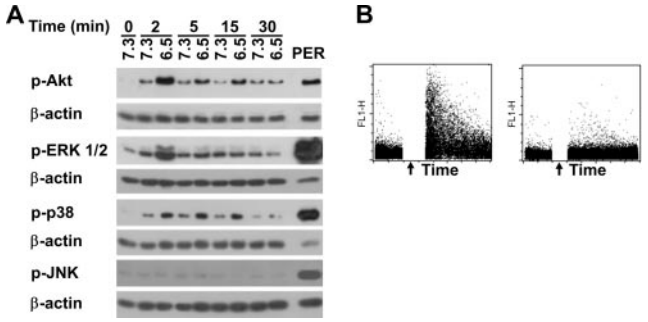

FIGURE 3. Exposure to pH 6.5 activates PI3K, and the MAPKs ERK1/2 and p38 in human DCs. *A*, DCs (1.5×10^6 cells per 300 μl of complete medium) were prewarmed for 30 min at 37°C, and acidification was accomplished by the addition of a precalculated volume of isotonic hydrogen chloride solution. Cells were incubated at pH 7.3 or 6.5 for different times at 37°C, and the samples were then analyzed by Western blotting as described in *Materials and Methods*. Pervanadate-treated DCs (with 0.1 mM orthovanadate plus 0.3 mM H_2O_2 for 10 min at 37°C) were used as positive controls. Western blots are representative of four to five experiments. *B*, Involvement of PI3K in the induction of calcium transients triggered by extracellular acidification. Dot plots showing fluo-3-AM fluorescence vs time from a single representative experiment ($n = 6$). DCs (2.5×10^6/ml) were cultured for 20 min at 37°C in complete medium at pH 7.3 in the absence (*left*) or presence (*right*) of 50 nM wortmannin. Arrow indicates the addition of an isotonic hydrogen chloride solution to each sample tube to adjust the extracellular pH to 6.5.

Ag-specific T cell responses

Ag-specific proliferation of T $CD4^+$ lymphocytes was examined using cells from TT-vaccinated volunteers. DCs were obtained from monocytes cultured with IL-4 plus GM-CSF for 7 days, as described. Autologous T $CD4^+$ cells were isolated from PBMC by immunomagnetic negative selection using a mix of mAbs directed to anti-HLA class II, CD19, CD16, CD11b, CD8, and CD14 and magnetic beads coated with anti-mouse IgG Abs (Dynal Biotech), according to the manufacturer's instructions. DCs were cultured with TT (5 μg/ml) for 90 min at pH 7.3 or 6.5. Then, cells were washed and cultured for 48 h at pH 7.3. LPS-treated DCs were prepared by incubating DCs with TT (5 μg/ml) and LPS (100 ng/ml) for 48 h at pH 7.3. In all cases, DCs were washed and irradiated (3000 rad from a ^{137}Cs source), and 2.0×10^4 DCs were cultured in the presence of autologous T $CD4^+$ cells at different DC to T cell ratios (1:5, 1:10, and 1:20), for 5 days in U-bottom 96-well plates (Costar). Thymidine incorporation was measured in triplicate on day 5 by a 18-h pulse with [^{3}H]thymidine (1 μCi/well, specific activity, 5 Ci/mM; DuPont). Ag-specific stimulation of IFN-γ and IL-4 production by T $CD4^+$ cells was assessed by incubating 2.0×10^5 DCs pulsed with TT (5 μg/ml) at pH 7.3, pH 6.5, or in the presence of LPS (100 ng/ml), as described, with 1.0×10^6 autologous T $CD4^+$ cells. After 5 days of culture, T cells were restimulated for 18 h with 2.0×10^5 autologous DCs pulsed with TT (5 μg/ml) at pH 7.3, pH 6.5, or in the presence of LPS, respectively. Brefeldin A (10 μg/ml) was added during the last 6 h of culture to inhibit the release of cytokines. The production of IFN-γ and IL-4 was assessed by immunofluorescence staining using anti-CD3 PercP, and anti-IFN-γ PE or anti-IL-4 PE Abs.

Statistical analysis

Student's paired *t* test was used to determine the significance of differences between mean values, and $p < 0.05$ was determined to indicate statistical significance.

Results

Transient exposure to acidic conditions triggers the phenotypic maturation of human DCs

Extracellular acidosis is a hallmark of inflammatory sites in peripheral tissues (16–20) in which immature DCs sample Ags for subsequent presentation in lymphoid tissues (1). We therefore studied whether the transient exposure to pH 6.5 induced DC maturation. DCs were incubated for 5, 30, 90, and 180 min at pH 6.5 and the phenotype was analyzed immediately or after 48 h of culture at pH 7.3. No changes in the phenotype were observed immediately after exposure of DCs for 5–180 min to pH 6.5 (data not shown). By contrast, a time-dependent change in the phenotype of DCs was observed when DCs were transiently exposed to pH 6.5 and then incubated at neutral pH for 2 days. As shown in Fig. 1, *A* and *B*, exposure of DCs for 5 min at pH 6.5 did not induce any change in the phenotype of DCs, whereas exposure for 30 min at

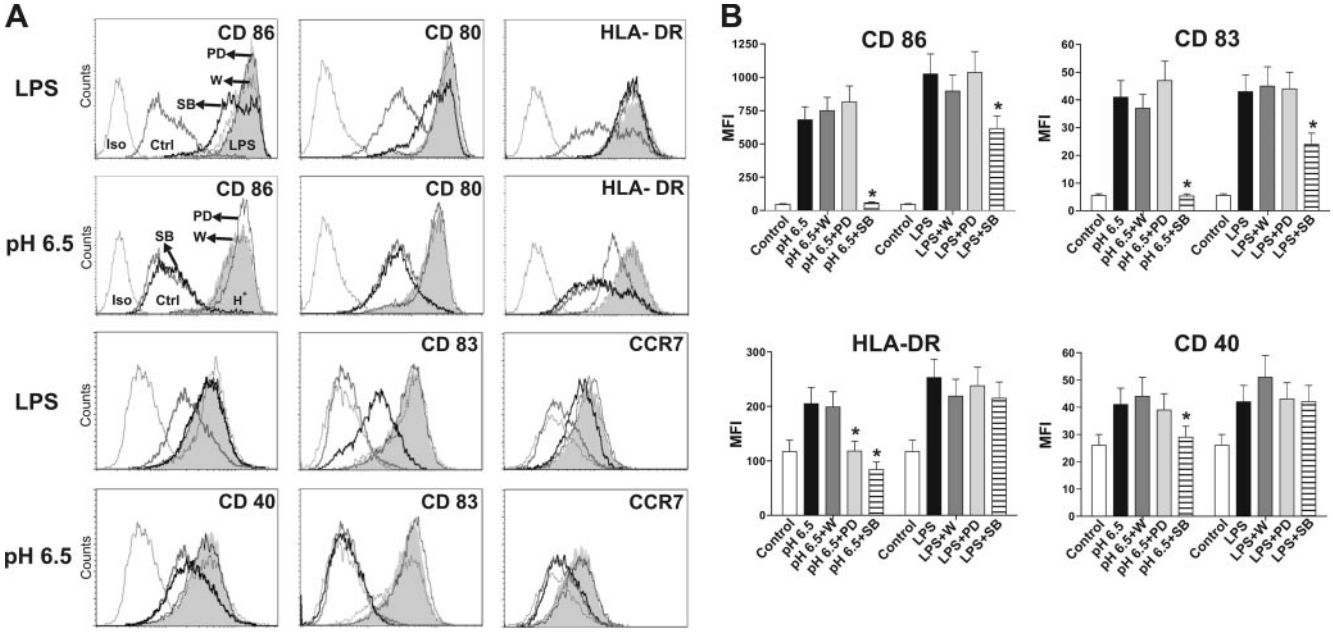

FIGURE 4. Phenotypic maturation of DCs induced by pH 6.5 is dependent on the activation of p38 MAPK. DCs (2.5×10^6/ml) were incubated for 20 min at 37°C and pH 7.3 without inhibitors or in the presence of 50 nM wortmannin (W), 50 μM PD98059 (PD), or 50 μM SB202190. Then, the cells were exposed to pH 6.5 or LPS (100 ng/ml, pH 7.3) for 90 min at 37°C. After this time, DCs were washed and cultured for an additional period of 48 h at 37°C and pH 7.3. *A*, Histograms from a representative experiment are shown. *B*, Results are expressed as mean fluorescence intensity (MFI) and represent the arithmetic mean ± SEM of five experiments. *, $p < 0.05$ for DCs pretreated or not with inhibitors and then exposed to pH 6.5 or LPS.

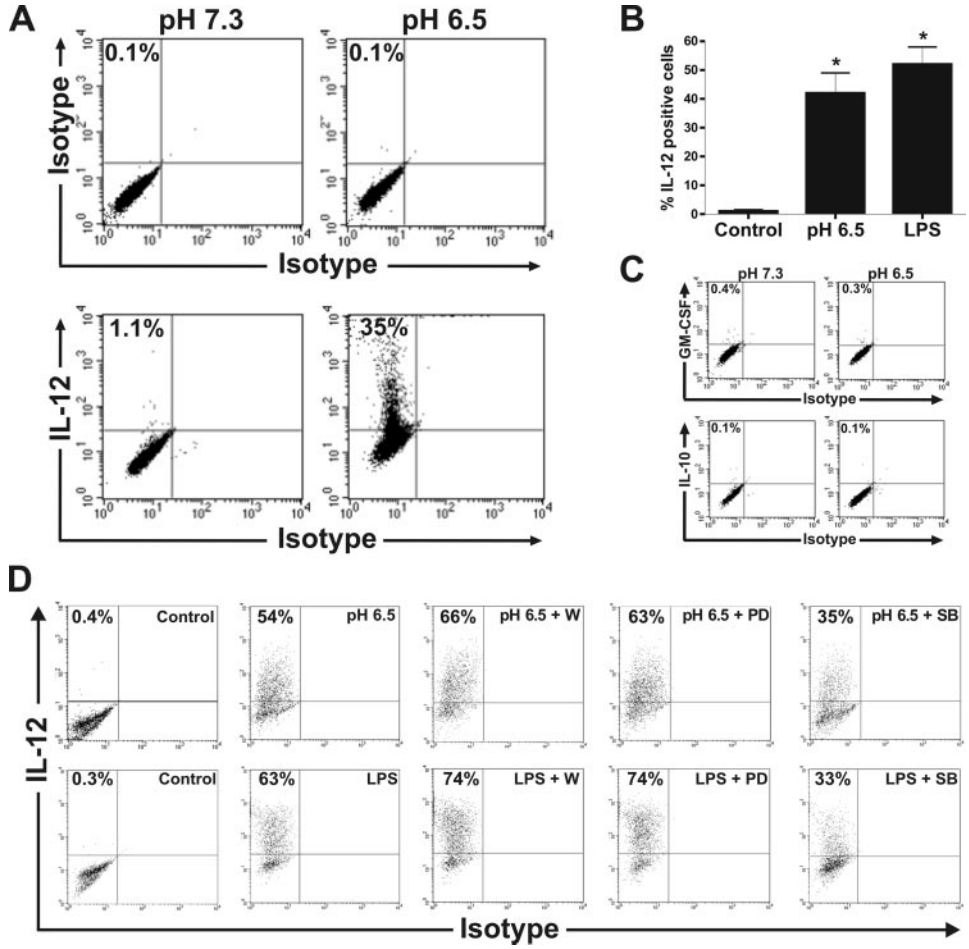

FIGURE 5. Exposure to pH 6.5 triggers the production of IL-12 by human DCs. *A* and *B*, DCs (2.5×10^6/ml) were exposed to pH 6.5 for 90 min at 37°C. Then, the culture medium was neutralized and the cells were cultured for an additional period of 24 h at 37°C. Control cells were cultured for 25.5 h at pH 7.3, whereas LPS-treated DCs were cultured with LPS (100 ng/ml) at pH 7.3 for the same period. In all cases, brefeldin A (10 μg/ml) was added during the last 6 h of culture to inhibit the release of IL-12. *A*, Dot plots from a representative experiment are shown. *B*, The percentage of IL-12-positive cells is shown and represents the arithmetic mean ± SEM of seven experiments. *, $p < 0.05$ for pH 6.5 or LPS vs pH 7.3 (control). The percentages shown in *C* and *D* represent cells positive for 6M-CSF or IL-10 (*C*) or IL-12 (*D*). *C*, DCs were cultured at pH 6.5 or 7.3, as described, and the production of IL-10 and GM-CSF was evaluated by flow cytometry. Dot plots from one representative experiment are shown ($n = 3$). *D*, DCs (2.5×10^6/ml) were incubated for 20 min at 37°C and pH 7.3 without inhibitors or in the presence of 50 nM wortmannin (W), 50 μM PD98059 (PD), or 50 μM SB202190. Then, the cells were exposed to pH 6.5 for 90 min at 37°C. After washing, cells were cultured for an additional period of 24 h at 37°C and pH 7.3. Control cells were cultured for 25.5 h at pH 7.3, whereas LPS-treated DCs were cultured with LPS (100 ng/ml) for the same period at pH 7.3. In all cases, brefeldin A (10 μg/ml) was added during the last 6 h of culture to inhibit the release of IL-12. Dot plots from a representative experiment ($n = 6$) are shown.

pH 6.5 caused the up-regulation of HLA-DR, CD86, and CD40 but not CD83 or CCR7. Culture of DCs for 90 or 180 min at pH 6.5 effectively triggered the phenotypic maturation of DCs, as indicated by the marked increase in the expression of HLA-DR, CD86, CD40, CD83, and CCR7. In contrast, DCs continuously cultured for 48 h at pH 6.5 showed only a moderate increase in the expression of HLA-DR and CD86, while the expression of CD40, CD83, and CCR7 remained unchanged, suggesting that a period of recuperation at pH 7.3 is needed to allow the full maturation of DCs in response to acidosis. As expected, LPS induced the phenotypic maturation of DCs, as indicated by the increase in the expression of all the markers analyzed (Fig. 1, *A* and *B*). In all the experiments DC viability was evaluated by fluorescence microscopy using the fluorescent DNA binding dye acridine orange. Cell viability was in all cases >85%.

To rule out that the maturation of DCs induced by acidic conditions was due to contaminating LPS in the hydrogen chloride solutions, we performed another set of experiments using culture medium adjusted to pH 6.5 by the addition of isotonic hydrogen chloride solution and then neutralized by the addition of isotonic sodium hydroxide. When DCs were cultured in this medium for 48 h, there were no changes in the phenotype of DCs compared with control cells (data not shown).

As expected, phenotypic maturation of DCs correlated with T cell-priming capacity, as indicated by the ability of DCs transiently exposed for 90 min at pH 6.5 and then cultured for 48 h at pH 7.3 to stimulate higher MLR responses than control DCs (Fig. 1*C*).

DC maturation is dependent on changes at the transcriptional level and takes several hours to become evident (1, 2). However, DC maturation stimuli can also trigger immediate responses. For example, although maturation of DCs is associated to the down-regulation of endocytic capacity, maturation stimuli such as microbial products actually lead to the enhancement of endocytosis when it was evaluated shortly after the addition of the stimulus (39). This response seems to play an important role "in vivo" because it enables immature activated DCs to sample their environment by endocytosis (1, 2). To analyze whether acidic conditions increase the uptake of Ags by human DCs we used FITC-OVA, a

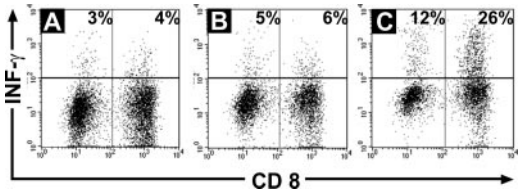

FIGURE 6. Exposure of DCs to pH 6.5 favors the production of IFN-γ by both $CD4^+$ and $CD8^+$ T cells. DCs (2.5×10^6/ml) were exposed to pH 6.5 for 90 min at 37°C. Then, the culture medium was neutralized and the cells were cultured for an additional period of 24 h at 37°C and pH 7.3. Control DCs were cultured for 25.5 h at pH 7.3. Freshly isolated PBMC (2.5×10^6/ml) were cultured for 96 h on immobilized anti-CD3 in the absence (*A*) or presence of supernatants (50% v/v) harvested from control DCs (*B*) or pH 6.5-treated DCs (*C*). Then, PBMC were treated with PMA (10 ng/ml) plus ionomycin (5 μg/ml) and they were cultured for an additional period of 24 h. Brefeldin A (10 μg/ml) was added during the last 6 h of culture to inhibit the release of IFN-γ. Immunofluorescence staining was performed using anti-CD3 PerCP, anti-CD8 FITC, and anti-IFN-γ PE. A gate for $CD3^+$ cells was defined and the proportion of $CD8^+$ and $CD8^-$ cells stained with anti-IFN-γ PE was established in the gate of $CD3^+$ cells. A representative experiment ($n = 5$) is shown.

marker taken up exclusively by fluid phase endocytosis. In agreement with our previous observations in murine DCs (31), our results (Fig. 2) showed that pH 6.5 stimulated endocytosis by human DCs. Stimulation of endocytosis by pH 6.5 was also observed when the samples were acquired in the presence of trypan blue, a dye able to quench extracellular fluorescence (36, 37); the rate of increase in endocytosis at pH 6.5 compared with pH 7.3 was 212 ± 38% (mean ± SEM) for $n = 6$ experiments ($p < 0.05$), supporting that the fluorescent marker was actually internalized.

Extracellular acidosis stimulates ERK1/2, p38 MAPK, and PI3K in human DCs

The activation of MAPK appears to play a major role in DC function. It has been shown that LPS as well as CD40 cross-linking induces the activation of the three MAPKs, ERK1/2, p38, and JNK (40–42). To gain insight into the signaling pathways triggered by acidosis we first analyzed the activation of MAPK. Western blots of lysates from DCs cultured for different periods at pH 7.3 or 6.5 were probed with Abs against phosphorylated MAPK. The results obtained (Fig. 3*A*) showed that acidosis triggers the phosphorylation of ERK1/2 and p38 but not JNK. Interestingly, these results contrast with our previous observations in human neutrophils showing that acidosis induced phosphorylation of ERK, but not p38 and JNK (43), indicating that the signaling pathways triggered by extracellular acidosis differ among different cell types. Fig. 3*A* also shows that exposure to acidosis results in the phosphorylation of Akt, a major target of the PI3K. The activation of PI3K usually leads to the stimulation of phospholipase C and the generation of inositol 1,4,5-trisphosphate, which induces cytosolic Ca^{2+} transients (44). Our results showed that pH 6.5 actually stimulates Ca^{2+} transients in human DCs. Fig. 3*B* illustrates the pattern of $[Ca^{2+}]_i$ changes induced after extracellular acidification. A fraction of DCs exhibit a transient increase in $[Ca^{2+}]_i$, followed by a gradual decrease to the resting $[Ca^{2+}]_i$ levels, ~35–45 s after acidification. The induction of Ca^{2+} transients was completely prevented by the inhibitor of PI3K wortmannin (50 nM), supporting results that it was dependent on PI3K activity.

p38 MAPK plays a critical role in the phenotypic maturation of DCs induced by extracellular acidosis

To study the roles of MAPK and PI3K/Akt signaling pathways in DC maturation induced by acidosis, immature DCs were treated with the inhibitor of ERK1/2 pathway PD98059 (50 μM), the inhibitor of p38 MAPK SB203580 (50 μM), and the specific blocker of PI3K wortmannin (50 nM). These inhibitors were used at concentrations able to almost completely suppress the phosphorylation of ERK1/2, p38 MAPK, and Akt induced by acidosis revealed by Western blot analysis (data not shown). These concentrations are similar to those used in previous studies to define the signaling pathways involved in the maturation of DCs triggered by LPS,

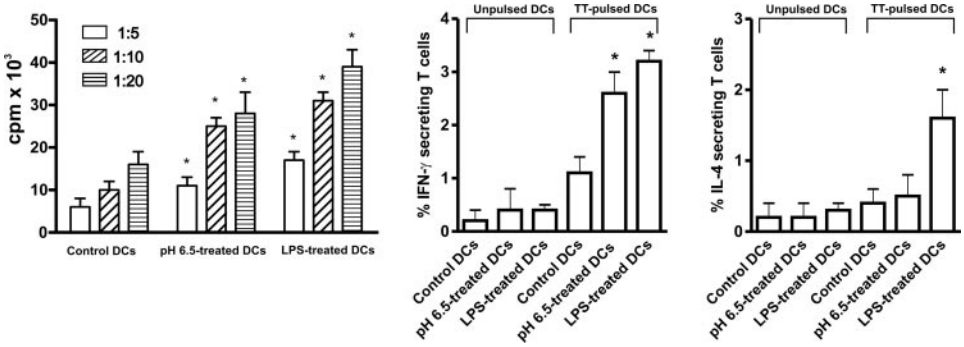

FIGURE 7. Exposure of DCs to pH 6.5 promotes Ag-specific proliferation and production of IFN-γ by $CD4^+$ T cells. DCs and T $CD4^+$ lymphocytes were obtained from TT-vaccinated volunteers, as described in *Materials and Methods*. DCs were cultured with or without TT (5 μg/ml) for 90 min at pH 6.5 (pH-treated DCs) or pH 7.3 (controls). Cells were then washed and cultured for an additional period of 48 h at pH 7.3. LPS-treated DCs were prepared by incubating DCs with TT (5 μg/ml) and LPS (100 ng/ml) for 48 h at pH 7.3. In all cases, DCs were washed and irradiated (3000 rad from a ^{137}Cs source). *A*, Proliferation assays were conducted by incubating 2.0×10^4 TT-pulsed DCs with autologous $CD4^+$ T cells at different DC to T cell ratios (1:5, 1:10, and 1:20) for 5 days in U-bottom 96-well plates (Costar). Thymidine incorporation was measured in triplicate on day 5 by a 18-h pulse with [^{3}H]thymidine (1 μCi/well, specific activity, 5 Ci/mM; DuPont). Results are expressed as the arithmetic mean ± SEM of five experiments. *, $p < 0.05$ for pH 6.5 or LPS-treated DCs vs control DCs. The proliferative response mediated by unpulsed DCs (control, pH 6.5-treated, or LPS-treated DCs) cultured with autologous $CD4^+$ T cells was in all cases lower than 3500 cpm (data not shown). *B* and *C*, Ag-specific stimulation of IFN-γ and IL-4 production by $CD4^+$ T cells was evaluated by incubating 2.0×10^5 DCs pulsed with TT (5 μg/ml) at pH 7.3, pH 6.5, or in the presence of LPS (100 ng/ml), as earlier described, with 1.0×10^6 autologous $CD4^+$ T cells for 5 days. Then, T cells were restimulated for 18 h with 2.0×10^5 autologous DCs pulsed with TT (5 μg/ml) at pH 7.3, pH 6.5, or in the presence of LPS, respectively. Brefeldin A (10 μg/ml) was added during the last 6 h of culture. Production of IFN-γ and IL-4 was assessed by intracellular staining and flow cytometry using anti-CD3 PercP, and anti-IFN-γ PE, or anti-IL-4 PE Abs. Results are expressed as the percentage of IFN-γ- or IL-4-secreting T cells, and represent the arithmetic mean ± SEM of five to six experiments. *, $p < 0.05$ for pH 6.5 and/or LPS-treated DCs vs control DCs.

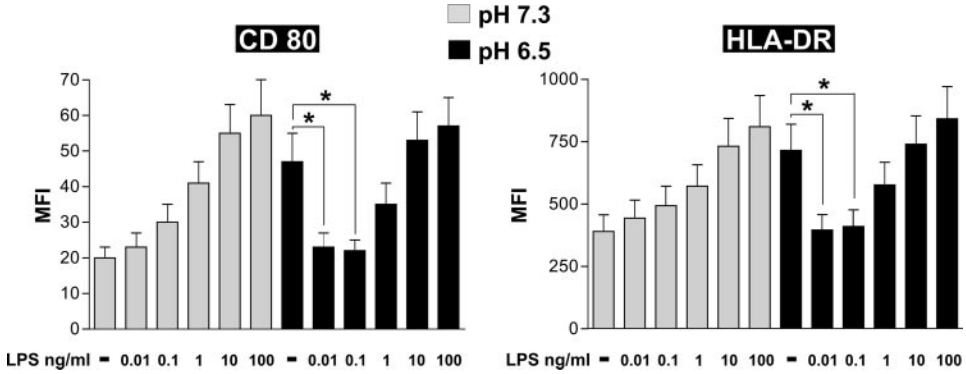

FIGURE 8. Suboptimal doses of LPS impair the maturation of DCs induced by pH 6.5. DCs (2.5×10^6/ml) were incubated for 1–2 min with LPS (0.1–100 ng/ml) at pH 7.3 and then exposed, or not, to pH 6.5 for 90 min. The culture medium was then neutralized and the cells were in all cases cultured for an additional period of 48 h at pH 7.3. Results are expressed as mean fluorescence intensity (MFI) values and represent the arithmetic mean ± SEM of four to five experiments. *, $p < 0.05$.

TNF-α or CD40L (40–42). DCs were cultured in the absence or presence of the inhibitors for 20 min at pH 7.3 and 37°C. Then, the cell suspensions were acidified to pH 6.5 by the addition of a precalculated volume of an isotonic hydrogen chloride solution. An equal volume of saline was added to control cells. After 90 min of culture, the cells were washed, resuspended at pH 7.3 and incubated for 48 h at 37°C in the absence of the inhibitors. Parallel experiments were performed using LPS as the maturation stimulus. Changes in the phenotype of DCs were analyzed by flow cytometry (Fig. 4). Maturation of DCs induced by acidosis was strongly dependent on the activation of p38 MAPK because SB203580 completely suppressed the increase in the expression of all the markers analyzed. Wortmannin, the inhibitor of the PI3K/Akt pathway did not mediate any effect, whereas the specific blocker of the ERK1/2 pathway PD98059 only partially inhibited the up-regulation of HLA-DR. Consistent with previous reports (40–42), we observed that SB203580, but not PD98059 nor wortmannin, inhibited the up-regulation of CD86, CD80, CD83, and CCR7 in LPS-treated DCs.

Extracellular acidosis triggers the synthesis of IL-12 by human DCs stimulating the production of IFN-γ by T CD4$^+$ cells

The maturation of DCs leads to increased expression of MHC class II and costimulatory activity and, in some instances, production of Th-polarizing cytokines (4, 5, 7). We then examined whether the maturation of DCs triggered by acidosis was associated to the stimulation of the production of IL-12, which plays a central role in the generation of Th1 cells (5, 7). Human DCs were incubated for 90 min at pH 6.5, the culture medium was then neutralized and the production of IL-12 was evaluated after 24 h of culture at pH 7.3 by intracellular staining and flow cytometry. Fig. 5, *A* and *B*, shows that transient exposure to pH 6.5 markedly stimulated the production of IL-12 by DCs. Similar results were observed when the production of IL-12 was analyzed by ELISA in the supernatants of DCs (2.5×10^6/ml) cultured for 90 min at pH 6.5 or 7.3 followed by 24 h at pH 7.3: the concentration of IL-12 was 187 ± 19 vs 11 ± 4 pg/ml, for pH 6.5 vs 7.3 ($n = 6$ experiments, $p < 0.05$). Exposure of DCs to pH 6.5 did not stimulate the production of IL-10 nor GM-CSF (Fig. 5*C*).

Consistent with the central role of p38 MAPK in the phenotypic maturation of DCs induced by acidosis (Fig. 4), Fig. 5*D* shows that the production of IL-12 stimulated by pH 6.5 was inhibited by the specific blocker of p38 MAPK SB203580. The percentage of inhibition was 53 ± 13, (mean ± E.S.) ($n = 5$ experiments, $p < 0.05$), whereas it was slightly, but not significantly, increased by the inhibitors of PI3K/Akt and ERK1/2 pathways. Similar results were observed using LPS as stimulus.

To gain information about the ability of acidosis to modulate the production of cytokines by DCs directing the differentiation profile of T cells, DCs were incubated for 90 min at pH 6.5 and then cultured for 24 h at pH 7.3. Cell supernatants were collected and their ability to modulate the production of cytokines by T cells stimulated by immobilized anti-CD3 plus PMA was assessed. As shown in Fig. 6 supernatants from DCs treated with pH 6.5 (Fig. 6*C*) but not from control DCs (Fig. 6*B*) markedly enhanced the proportion of IFN-γ-producing cells in both, CD4$^+$ and CD8$^+$ T cell subsets. No production of IL-4 was detected (data not shown), suggesting that transient exposure of DCs to acidic conditions promotes the development of Th1 immunity. This hypothesis is also supported by the analysis of Ag-specific T cell responses. These assays were performed using cells from TT-vaccinated volunteers. DCs were obtained from monocytes cultured with IL-4 plus GM-CSF, as described, whereas autologous CD4$^+$ T cells were purified from PBMC by immunomagnetic negative selection. DCs were pulsed with TT for 90 min at pH 7.3 (controls), pH 6.5, or in the presence of LPS, as described in *Materials and Methods*, and then were cultured with purified autologous CD4$^+$ T cells. The results obtained (Fig. 7) showed that pH 6.5-treated DCs displayed a higher ability to stimulate the proliferation of Ag-specific T CD4$^+$ cells compared with control DCs. More interestingly, pH 6.5-treated DCs also induced a higher increase in the percentage of specific T CD4$^+$ cells positive for IFN-γ, but not for IL-4 production (Fig. 7). By contrast, LPS-treated DCs induced a significant increase in the proportion of both IL-4- and IFN-γ-secreting CD4$^+$ T cells.

LPS abrogates pH 6.5-induced maturation of DCs

We then analyzed whether suboptimal doses of LPS could exert additive or synergistic effects on the maturation of DCs induced by pH 6.5. Experiments were performed using different concentrations of LPS (0.1–100 ng/ml). DCs were treated for 1–2 min with LPS and then were exposed to pH 6.5 for 90 min at 37°C. Cells were then washed and cultured for 48 h at pH 7.3, and the phenotype (expression of CD80 and HLA-DR) was analyzed by flow cytometry. The results obtained are shown in Fig. 8. Suboptimal doses of LPS did not improve the maturation of DCs induced by pH 6.5 but rather, they completely abrogated the ability of pH 6.5 to induce the phenotypic maturation of DC.

Discussion

Extracellular acidosis is a hallmark of inflammatory processes. It results from a number of factors: 1) the massive infiltration of leukocytes, mainly neutrophils and macrophages, and the production of protons during the activation of the respiratory burst (45, 46); 2) the accumulation of by-products of microbial metabolism such as short-chain fatty acids produced by bacteria (47, 48); and 3) tissue hypoxia resulting in the stimulation of glycolysis in the ischemic tissue and the subsequent accumulation of lactate

(49–51). Although the inflammatory reactions against pathogens (16–21) and tumor cells (27–31), as well as the effector mechanisms responsible for host injury in autoimmune processes such as rheumatoid arthritis and asthma (22–26), are strongly associated with acidic microenvironments in injured tissues, little is known about the influence of acidosis on the immune response.

We hypothesize that extracellular acidosis is a chemical stressor for immune cells. Supporting this hypothesis, we have previously shown that acidic conditions induce neutrophil activation by a mechanism dependent on the stimulation of PI3K/Akt and ERK pathways (43, 52). Consistent with these results, Owen et al. (53) have recently shown that exposure of human neutrophils to low pH values results in the production of platelet-activating factor, a strong inflammatory stimulus. Interestingly, it has also been shown that extracellular acidosis efficiently activates the alternative pathway of complement (54, 55). Together, these results support the notion that acidosis is able to stimulate two of the major arms of innate immunity.

We report that exposure to pH 6.5 triggers the maturation of DCs. Of note, maturation was observed in DCs incubated at pH 6.5 for 90–180 min and then cultured for 48 h at pH 7.3, but not in DCs cultured for 48 at pH 6.5. It is well established that the maturation of DCs requires "de novo" synthesis of different sets of proteins (56, 57). Because acidosis is able to inhibit protein synthesis in different cell types (58, 59), this may explain why DCs continuously exposed to pH 6.5 fail to progress to a mature phenotype. In any case, it is important to note that a short-term exposure of DCs to acidic conditions in peripheral tissues followed by longer periods at a neutral pH probably reflects what occurs in vivo because the trafficking of DCs from the periphery to draining lymph nodes usually demands several hours and occurs at a neutral pH value (60).

Our results indicate that the activation of p38 MAPK plays a critical role in the phenotypic maturation of DCs and the production of IL-12 triggered by pH 6.5. In fact, the specific blocker of p38 MAPK SB202190 completely prevented the up-regulation of CD80, CD86, CD40, HLA-DR, CD83, and CCR7 induced by acidosis, whereas the production of IL-12 was inhibited by 50%. Previous studies have shown that p38 MAPK plays a major role in the maturation of DCs induced by different stimuli including LPS (40–42). In agreement with these reports, we observed that SB202190 inhibited the production of IL-12 as well as the up-regulation of CD86, CD80, CD83, and CCR7 in LPS-treated DCs.

DCs can be activated by triggering of pattern-recognition receptors or cytokine receptors. Reis e Sousa and colleagues (4–7) showed that TLR signaling results in activated DCs that primed an effective Th1 response, whereas inflammatory cytokines induced DCs that support clonal expansion of $CD4^+$ T cells but fail to promote differentiation of $CD4^+$ T cells into a Th1 or Th2 profile. Our results suggest that extracellular acidosis acts as pathogen-associated molecular patterns because it triggers not only the expression of costimulatory and MHC class II molecules but also the production of IL-12 by DCs, stimulating the production of IFN-γ by Ag-specific $CD4^+$ T cells. Interestingly, our results shown that exposure of DC to low doses of LPS results in a state of refractoriness to subsequent challenge by pH 6.5, suggesting a cross-talk between the activation pathways triggered by LPS and extracellular protons in DCs. Studies are currently underway in our laboratory to elucidate the mechanisms responsible for this tolerogenic effect that may involve changes in the expression or function of proton receptors, inhibition of signaling pathways and/or primary effects on gene transcription required for DC maturation.

How could DCs sense extracellular acidosis? This process of recognition by DCs remains under review. Two hypothesis should be considered. First, as observed for conventional agonists, extracellular protons may activate DCs through the interaction with specific receptors. In fact, recent reports have shown that extracellular protons can be effectively recognized by a subfamily of G protein-coupled receptors (61–63). This subfamily comprises four receptors, G protein-coupled receptor 4 (GPR4), ovarian cancer G protein-coupled receptor 1 (OGR1), T cell death-associated gene 8 (TDAG8), and G2 accumulation (G2A). These receptors were originally characterized by their ability to bind inflammatory lipids such as lysophosphatidylcholine, sphingosylphosphorylcholine, and psychosine; however, recent studies performed in a variety of transfected cell lines have shown that all of them can be activated by extracellular protons inducing a variety of signaling pathways (61–63). Thus, proton-sensing G protein-coupled receptors appear to be unique receptors that recognize both lysolipids and protons as ligands. Of note, we observed that human DCs express mRNA for the four members of the proton-sensing G protein-coupled receptor (A. Ceballos, unpublished observation). However, the role of these receptors is not easy to define because no specific inhibitors or functional blocking Abs are available. Other receptors expressed by DCs could also recognize protons. Basu and Srivastava (64) have recently described that, as nociceptive neurons, DCs also express the vanilloid receptor 1 (VR1). Moreover, experiments performed in DCs from $VR1^{+/+}$ and $VR1^{-/-}$ mice demonstrated that this receptor is able to trigger the maturation of DCs (64). Of note, VR1 is activated not only by noxious temperature, lipid derivates and vanilloid molecules, but also by low extracellular pH (65). Together, these findings support the possibility that acidic conditions trigger the maturation of DCs through the interaction of protons with specific receptors on DCs. However, other mechanisms should also be considered. According to Seong and Matzinger's (66) danger model, which supports that APCs are activated by danger/alarm signals from injured host cells, it is possible to speculate that extracellular acidosis may directly modify the conformation and/or the hydrophobicity of certain enzymes in DCs that in turn may acquire the capacity to trigger their maturation. In this regard, it should be mentioned that extracellular acidic conditions, similar to those used in our study, have shown to be able to induce the activation of both phospholipase A_2 and C. Owen et al. (52) have recently shown that exposure of human neutrophils to acidic pH (range 5.4–6.4) triggers the production of platelet-activating factor, an extremely potent inflammatory agent, which is synthesized through the concerted action of two enzymes, phospholipase A_2 and lysoplatelet-activating factor acetyltransferase (53). Moreover, Smith et al. (67) have shown that changing extracellular pH from 7.4 to 6.1 results in the activation of phospholipase C with the subsequent production of inositol trisphosphates and Ca^{2+} mobilization, in human fibroblasts, endothelial, smooth muscle, and neuroblastoma cells. Studies are in progress in our laboratory to define the influence of extracellular pH on the phospholipid metabolism in DCs because we observed that blocking of phospholipase A_2 exerted a profound inhibitory effect on the production of IL-12 by DCs stimulated by pH 6.5 (D. Martínez, unpublished result).

Studies of the mechanisms involved in the regulation of DC activity are usually restricted to cytokines, bacterial products, and tumor-related compounds. We demonstrate in this study that transient exposure to acidic conditions triggers the maturation of DCs and the production of IL-12. These observations may have important implications to our understanding of the mechanisms leading to the activation of DCs during the course of infectious processes.

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Disclosures

The authors have no financial conflict of interest.

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