Acidosis Improves Uptake of Antigens and MHC Class I-Restricted Presentation by Dendritic Cells[1]

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It is widely appreciated that inflammatory responses in peripheral tissues are usually associated to the development of acidic microenvironments. Despite this, there are few studies aimed to analyze the effect of extracellular pH on immune cell functions. We analyzed the impact of acidosis on the behavior of dendritic cells (DCs) derived from murine bone marrow. We found that extracellular acidosis (pH 6.5) markedly stimulated the uptake of FITC-OVA, FITC-dextran, and HRP by DCs. In fact, to reach similar levels of endocytosis, DCs cultured at pH 7.3 required concentrations of Ag in the extracellular medium almost 10-fold higher compared with DCs cultured at pH 6.5. Not only the endocytic capacity of DCs was up-regulated by extracellular acidosis, but also the expression of CD11c, MHC class II, CD40, and CD86 as well as the acquisition of extracellular Ags by DCs for MHC class I-restricted presentation. Importantly, DCs pulsed with Ag under acidosis showed an improved efficacy to induce both specific $CD8^+$ CTLs and specific Ab responses in vivo. Our results suggest that extracellular acidosis improves the Ag-presenting capacity of DCs. ***The Journal of Immunology*****, 2004, 172: 3196–3204.**

Extracellular acidosis is a condition frequently associated with a variety of pathological situations. There is a long line of observations, consisting especially of measurements of titratable acidity, which indicate that the inflammatory area is markedly acidic whether the initial irritant be a chemical substance, an allergic reaction, a microbial toxin, or the proliferation of a microorganism (1–7). Measurements of pH achieved in peritoneal fluid during or after abdominal surgery in infected patients, as well as in situ in peripheral tissues during the development of inflammatory response against many pathogens, have shown extracellular pH values as low as 5.5–7.0 (1, 2, 5, 6). Similar observations were made in the lower airway of patients with acute asthma. Indeed, the values of pH found in airway vapor condensate samples from asthmatic patients were over two log orders lower than in control subjects (7). Moreover, exhaustive researches performed over the past 50 years in solid tumors such as brain tumors and brain metastases, malignant melanomas, sarcomas, breast cancer, squamous cell carcinomas, and adenocarcinomas have shown that tumor microenvironments are usually more acidic than the normal ones, with values of extracellular pH ranging from 5.8 to 7.4, both in human and rodent malignant tissues (8–12). Of note, taking this into account, many studies have been performed in past years to exploit the relative acidity of tumor vs normal tissue for the treatment of cancer (13–15).

Considering the widespread distribution of acidic microenvironments, it is surprising that to date there are relatively few studies focused on the effect of extracellular acidosis on the behavior of immunocompetent cells (reviewed in Ref. 16). Studies performed by us in human neutrophils have shown that extracellular acidosis induces a transient increase in intracellular Ca^{2+} concentration over the resting levels, a shape change response, the up-regulation of the expression of the β_2 integrin CD18, and a delay in the rate of spontaneous apoptosis (17). Observations made in stimulated macrophages indicated that extracellular acidification results in the inhibition of superoxide anion production, Fc-mediated phagocytosis, and TNF-α release (18), whereas the production of NO was found to have increased (19). Ratner (20) investigated lymphocyte motility stimulated by IL-2 in neutral and acidified three-dimensional extracellular matrix, and found increased motility at pH 6.5 compared with pH 7.1. Severin et al. (21) showed diminished cytotoxic activity of human lymphokine-activated killer cell activity at acidic pH, whereas Loeffler et al. (22) found a similar depression of murine NK cell activity with lowered pH. They also demonstrated that acidic pH inhibits IL-2-stimulated lymphocyte proliferation (23). These results support the notion that extracellular acidosis exerts enhancing or suppressing effects on immune response depending on the function analyzed.

Dendritic cells (DCs)[3] are highly specialized APCs with a unique ability to activate resting T lymphocytes. A current paradigm holds that DCs arise from proliferating precursors found in the bone marrow. They give raise to circulating precursors that home to tissues, in which they reside as immature cells and serve a sentinel function awaiting signals that indicate local infection or inflammation (24–27). Because immature DCs pick up and process Ags in peripheral tissues (25, 27), and considering that the

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[3] Abbreviations used in this paper: DC, dendritic cell; PLC, phospholipase C; PI3K, phosphatidylinositol-3-kinase; MFI, mean fluorescence intensity; NCDC, 2-nitro-4-carboxyphenyl-*N*,*N*-diphenylcarbamate.

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development of many inflammatory processes as well as the growth of different tumors result in the development of acidic microenvironments in peripheral tissues (1–15), in this study we examined the impact of extracellular acidosis on the function of immature DCs. Our observations indicate that extracellular acidosis improves internalization of Ags, increases the expression of cell surface proteins involved in Ag presentation, promotes an efficient MHC class I-restricted presentation of peptides from exogenous Ags, and also increases the ability of Ag-primed DCs to induce both specific $CD8^+$ CTL responses and specific Ab responses in vivo.

Materials and Methods

Reagents

HRP, OVA, dextran (40,000 Da), LPS from *Escherichia coli*, recombinant murine GM-CSF, amiloride, cytochalasin B, wortmannin, 2-nitro-4-carboxyphenyl-*N*,*N*-diphenylcarbamate (NCDC), and ammonium chloride were from Sigma-Aldrich (St. Louis, MO). Dextran and OVA were conjugated with FITC, as described (28). The $OVA_{257–264}$ peptide was kindly provided by Dr S. Amigorena (Institut Curie, Paris, France).

Mice

Experiments were conducted using 2-mo-old virgin female C57BL/6 mice raised at the National Academy of Medicine, Buenos Aires, Argentina. They were housed six per cage and kept at 20 ± 2°C under an automatic 12-h light-dark schedule. Animal care was in accordance with institutional guidelines.

Culture conditions

The standard medium used in this study was bicarbonate-buffered RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, 0.1 mM nonessential amino acids, and 5.5×10^{-5} mercaptoethanol (all from Invitrogen) (complete medium), pH 7.3. It was adjusted to pH 6.5 by addition of isotonic HCl. 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 7% CO_2, for cells suspended in medium at pH 6.5.

Dendritic cells

DCs were obtained as described by Inaba et al. (29) with some minor modifications. Briefly, after removing all muscle tissues from the femurs, the bones were washed twice with PBS, and transferred into a fresh dish with RPMI 1640 medium. Both ends of the bones were cut with scissors in the dish, and then the marrow was flushed out using 2 ml of RPMI 1640 with a syringe and 25-gauge needle. The tissue was suspended, passed through a wire mesh to remove small pieces of bone and debris, and red cells were lysed with ammonium chloride. After washing, cells were suspended at a concentration of 1×10^6 cells/ml in RPMI 1640 medium supplemented with 10% FCS, 5.5×10^{-5} mercaptoethanol (Sigma-Aldrich) and 30% conditioned medium from GM-CSF-producing NIH 3T3 cells, and cultured for 9 days. The cultures were fed every 2 days by gently swirling the plates, aspirating 50% of the medium, and adding back fresh medium supplemented with recombinant murine GM-CSF. At day 9 of the culture, >85% of the harvested cells expressed MHC class II, CD40, CD80, and CD11c, but not Gr-1 (data not shown).

Flow cytometry

Cell staining was conducted using the following FITC-conjugated mAbs: anti-CD11c, anti-CD40, anti-IA^b (MHC class II), anti-H-$2K^b$ (MHC class I), and anti-CD86 (BD PharMingen, San Diego, CA). The expression of the receptors for the Fc portion of IgG types II and III (FcγRII/III) was analyzed using the rat mAb 2.4G2 and FITC-conjugated mouse IgG anti-rat IgG Abs. Analysis was performed using a FACS flow cytometer and CellQuest software (BD Biosciences, San Jose, CA). The results are expressed as the mean fluorescence intensity (MFI).

Endocytosis of HRP

Endocytosis of HRP was performed as previously described (30). Briefly, DCs were suspended in complete medium adjusted to pH 7.3 or 6.5. HRP was added at the final concentration of 1, 10, and 150 μg/ml HRP, and cells were cultured for 30 min at 37°C under 5% or 7% CO_2, for cultures performed at pH 7.3 or 6.5, respectively. Then, DCs were collected, washed four times in PBS containing 1% FCS and four times in PBS alone with one tube change, lysed with 0.05% Triton X-100 in 10 mM Tris buffer, pH 7.3, for 30 min, and the enzyme activity of the lysate was measured using *o*-phenylenediamine and H_2O_2 as substrates with reference to a standard curve, at 492 nm. The amount of HRP taken up by DCs was determined as the difference between HRP activities in disrupted and nondisrupted cells. HRP activity in nondisrupted DCs were always <15% compared with disrupted cells.

Endocytosis of FITC-OVA and FITC-dextran

Cells were suspended in complete medium adjusted to pH 7.3 or 6.5. FITC-OVA and FITC-dextran were added at the final concentration of 0.1, 1, 10 or 100 μg/ml, and cells were incubated for different times 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 (cells incubated with FITC-OVA or FITC-dextran at 0°C) was always subtracted. In some experiments we used the dye trypan blue to quench extracellular fluorescence, as described (31, 32). Endocytosis assays were performed as previously indicated, but acquisition of samples was performed 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). In all cases, we observed that fluorescence intensity was diminished by >85% when the acquisition of the samples was performed in the presence of trypan blue. In experiments directed to evaluate whether macropinocytosis should account for the increased uptake of Ags under acidosis, we used amiloride, cytochalasin B, NCDC, and wortmannin. In all cases, these drugs were added to cell cultures 15 min before the addition of the markers. The results obtained are expressed as MFI values. In a separate set of experiments the uptake of FITC-OVA was measured in cell lysates (0.25% SDS) adjusted at pH 9.6 with 0.5 M of carbonate buffer. Fluorescein fluorescence was measured with a spectrofluorometer (SPF-500C; SLM/AMINCO, Urbana, IL) at an excitation wavelength of 495 nm and an emission wavelength of 514 nm.

Measurement of intracellular pH

It was performed using carboxy-SNARF-1-AM as previously described (33). DCs (5×10^6/ml in PBS) were loaded with 10 μM carboxy-SNARF-1-AM during 30 min at 37°C, washed in PBS, and resuspended in the same buffer at 5×10^6 in 100 μl. Then, 10 μl of the cell suspension were added to 2 ml of the culture medium at pH 7.4, and allowed to equilibrate at 37°C during 20 min in a steady water bath. The time course of changes in internal pH was evaluated before and after the addition of a precalculated volume of isotonic HCl solution to adjust the extracellular pH to 6.5. Assays were performed by flow cytometry, with excitation at 488 nm and emission analysis at FL2 and FL3. Ten thousand events were collected. The intracellular pH was estimated from the ratio of emission intensities at the two wavelengths, standardizing by comparison with the fluorescence intensity ratios of cells for which intracellular pH values were fixed by incubation with nigericin (10 μM) in high potassium buffers, as previously described (34).

Ag pulsing of DCs and immunization protocols

For Ag pulsing, DCs (7.5×10^5 cells/ml) were cultured in complete medium containing 100 μg/ml OVA for 3 h at 37°C at pH 7.3 or 6.5. After washing, cells were suspended at 1.5×10^6 cells/ml. Nonimmunized C57BL/6 mice received an i.v. injection of 3×10^5 OVA-pulsed syngeneic DCs. Control mice were injected with unpulsed DCs.

Cytotoxicity assays

Splenocytes were harvested from mice 8–10 days after immunization with OVA-pulsed or unpulsed DCs. After the removal of RBC by hypotonic lysis, they were restimulated by coculture with the K^b-binding peptide $OVA_{257–264}$ (SIINFEKL, 10 μmol/L) and cells were harvested 5 day later. The standard ^{51}Cr release assay was performed as described (35) using DCs as target cells. They were pulsed with the peptide SIINFEKL (10 μmol/L) for 2 h at 37°C at pH 7.3 and labeled with ^{51}Cr sodium chromate (100 μCi; NEN, Boston, MA) for 1 h at 37°C. For the assay, 10^4 target cells were added to 96-well U-bottom plates along with different numbers of effector cells (at the ratios given in the figure) in a total volume of 200 μl. After 4 h, 100 μl of supernatants from triplicate cultures was collected and measured in a gamma counter (Clinigamma 1272; LKB Wallac, Turku,

Finland). The percentage of specific lysis was calculated as: 100 × [(experimental release − spontaneous release)/(maximum release − spontaneous release)]. Spontaneous release was determined for target cells in medium alone and maximum release was determined by incubating target cells in 1% Triton X-100. Spontaneous release was typically <12%.

Measurement of Ab production

Mice were bled 14 days after in vivo administration of DCs, and the amount of serum-specific Abs directed to OVA was determined by ELISA. Briefly, 96-well flat-bottom plates were coated with OVA (10 μg/ml in PBS) and incubated overnight at 4°C. After washing three times with TBST and blocked with TBST plus BSA, 100 μl per well of serum dilutions (1/30 and 1/300) from immunized mice were added in triplicates. The plates were incubated 90 min at room temperature, washed again with TBST and 100 μl of biotinylated rat anti-mouse Igκ L chain mAb (1/500 in PBS; BD PharMingen) was added to each well. After 1 h incubation at room temperature, plates were washed, streptavidin-peroxidase was applied and reactions were finally developed with the chromogenic substrate *o*-phenylenediamine (Sigma-Aldrich). Negative controls included wells with all reagents except serum dilutions or OVA. Results are expressed as mean absorbance values at 492 nm.

Ag presentation assay

Presentation of $OVA_{257–264}$ epitope on K^b was detected using the T cell hybridoma B3Z, which carries a β-galactosidase construct driven by NF-AT elements from the IL-2 promoter (36). For Ag presentation assays, DCs were exposed to different concentrations of OVA during 3 h at 37°C at pH 7.3 or 6.5. Then, cells were washed, suspended in complete medium at pH 7.3 and cultured in the presence of the T cell hybridoma B3Z. After 18 h of culture, cells were washed with PBS, and a colorimetric assay using *o*-nitrophenyl-P-D-galactoside (ONPG; Sigma-Aldrich) as a substrate was used to detect LacZ activity in B3Z lysates.

MHC class II-restricted response to OVA was assessed using the T cell hybridoma BO97.10, specific for $OVA_{323–339}$ peptide on $I\text{-}A^b$ on DCs (36). DCs were exposed to OVA (100 μg/ml) for 3 h at 37°C at pH 7.3 or 6.5. After washing, cells were suspended in complete medium at pH 7.3, and BO97.10 cells were added for 24 h. Then, the production of IL-2 by BO97.10 was measured by ELISA (R&D Systems, Minneapolis, MN).

Statistical analysis

Student's paired *t* test was used to determine the significance of differences between means, and $p < 0.05$ was taken as indicating statistical significance.

Results

Extracellular acidosis increases endocytosis by DCs

DCs use two main different mechanisms for Ag capture: macropinocytosis, which occurs in a constitutive fashion in DCs and allows continuous internalization of Ags present in the fluid phase, and receptor-mediated endocytosis. Immature DCs express C-type lectin receptors, such as mannose receptors and DEC-205, receptors for the Fc portion of IgG (FcγRI, II, and III) and IgE (FcεRI and FcεRII), and also express scavenger receptors. These receptors contribute to Ag capture by DCs via classical clathrin-mediated endocytosis (25, 30, 37).

We studied the impact of extracellular acidosis on the endocytic capacity of DCs using three markers: FITC-OVA, FITC-dextran, and HRP. Although FITC-OVA is taken up exclusively by fluid phase endocytosis, HRP and FITC-dextran appear to be taken up via both fluid phase and mannose receptor-dependent endocytosis (30, 37–39). Flow cytometric analysis shows that extracellular acidosis (pH range from 7.0 to 6.0) increased the uptake of FITC-OVA by DCs, being the strongest effect observed at pH 6.5 (Fig. 1*A*). DCs cultured at 37°C accumulate FITC-OVA in a time-dependent fashion either at pH 7.3 or pH 6.5 (Fig. 1*B*). Extracellular acidosis did not induce any enhancement in FITC-OVA uptake at 5 or 10 min of incubation, but markedly increased endocytosis after 30 min of culture.

To analyze whether the increased fluorescence of DCs cultured with FITC-OVA under acidosis could involve a nonspecific attachment of FITC-OVA to the cellular surface, we then performed additional assays in which endocytosis was conducted as described in Fig. 1*A*, but the acquisition of samples was performed in the presence of trypan blue (200 μg/ml), a dye capable to quench extracellular fluorescence (31, 32). In agreement with data in Fig. 1*A*, we observed that the MFI of DCs cultured with FITC-OVA at pH 6.5 was higher than that of DCs cultured at pH 7.3 (265 ± 42 vs 86 ± 33, mean ± SEM, $n = 4$, $p < 0.05$), supporting that increased fluorescence of DCs cultured with FITC-OVA under acidosis is due to stimulation of endocytosis.

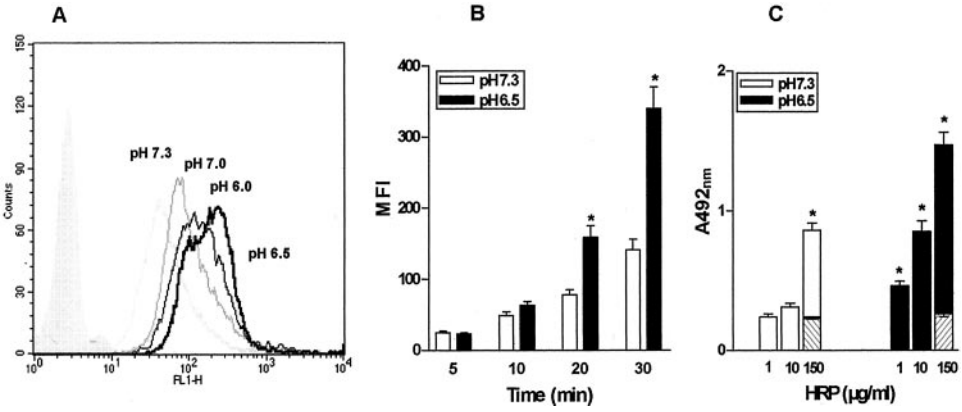

FIGURE 1. Extracellular acidosis increases endocytosis by DCs. *A*, DCs (1.5×10^6/ml) were incubated with FITC-OVA (100 μg/ml) for 30 min at 37°C in culture medium adjusted to different pH values and the amount of ligand accumulated was measured by flow cytometry. The filled histogram represents the uptake of FITC-OVA after incubation for 30 min at 0°C, which was similar at the different values of pH assessed. A representative experiment is shown. *B*, DCs were incubated with FITC-OVA (100 μg/ml) for different times at 37°C at pH 7.3 or 6.5 and analyzed by flow cytometry. Results are expressed as MFI values and represent the arithmetic mean ± SEM of five experiments. Asterisk represents statistical significance ($p < 0.05$) for pH 6.5 vs 7.3. *C*, DCs were incubated with different concentrations of HRP for 30 min at 37°C at pH 7.3 or 6.5, and the amount of HRP accumulated was measured as described in *Materials and Methods*. The uptake of HRP (150 μg/ml) by DCs, after incubation for 30 min at 0°C, was <10% of the uptake at 37°C. Stripped bars represent the uptake of HRP by DCs cultured for 48 h with LPS (1 μg/ml) (mature DCs) and then incubated with 150 μg/ml HRP for 30 min at 37°C at pH 7.3 or 6.5. Results are expressed as OD at 492 nm and represent the arithmetic mean ± SEM of six experiments. Asterisk represents statistical significance ($p < 0.05$) of pH 6.5 vs 7.3, and untreated vs LPS-treated DCs.

Lutz et al. (38) have shown that FITC-OVA taken by immature DCs was retained for long periods in a mildly acidic compartment. It was possible, however, that depending on extracellular pH values, FITC-OVA might be retained in DC vesicles of different acidity. If so, and considering that FITC fluorescence is quenched at acidic pH values (39), the endocytic responses showed in Fig. 1, *A* and *B*, might be over- or underestimated. Consequently, to avoid the problem of fluorescence quenching in intact cells, we evaluated the cellular uptake of FITC-OVA by spectrofluorometer analysis in DC lysates. DCs were cultured with FITC-OVA (100 μg/ml, 30 min at 37°C) at pH 7.3 and 6.5, and endocytosis was assessed as described in *Materials and Methods*. In agreement with the results obtained by flow cytometry in intact cells (Fig. 1, *A* and *B*), fluorometric assessment of endocytosis showed that the uptake of FITC-OVA was markedly increased at pH 6.5 compared with pH 7.3; percentage of increase is 254 ± 38 (mean ± SEM, $n = 5$).

We also analyzed whether the increased accumulation of FITC-OVA under acidosis could be related to a lower rate of marker degradation. To this aim, DCs were cultured with OVA-FITC (100 μg/ml) for 30 min at 37°C at pH 7.3 and 6.5, washed and suspended in OVA-FITC free medium at pH 7.3 and 6.5, respectively, and the loss of cell-associated fluorescence was evaluated after 30 min of culture at 37°C. In agreement with Lutz et al. (38), showing long-lasting retention of Ags such as FITC-OVA and FITC-dextran by immature DCs, we observed that the loss of cell-associated fluorescence was <20% for both experimental conditions, supporting that the increased accumulation of FITC-OVA by DCs cultured under acidosis could not be attributed to a lower rate of marker degradation.

Fig. 1*C* shows that not only the uptake of FITC-OVA but also the uptake of HRP, evaluated by measuring the enzyme activity in DC lysates, was higher at pH 6.5 compared with pH 7.3. As expected, we also observed that maturation of DCs by LPS resulted in the inhibition of endocytosis, which was evident at both pH values (Fig. 1*C*, dashed bars).

Additional experiments were conducted using different concentrations of FITC-OVA and FITC-dextran (Fig. 2). Interestingly, a comparative analysis of the amounts of the markers that were endocyted at neutral and acidic conditions showed that in agreement with the results obtained with HRP (Fig. 1*C*), to reach similar levels of endocytosis, DCs cultured at pH 7.3 required concentrations of Ag in the extracellular medium almost 10-fold higher compared with DCs cultured at pH 6.5.

As previously mentioned, macropinocytosis and receptor-mediated endocytosis constitute the two main mechanisms for Ag capture by DCs (30, 37, 40, 41). Our observations showing that acidosis increases the uptake of OVA, dextran, and HRP in a similar fashion suggest that stimulation of Ag uptake by acidosis occurs via macropinocytosis. Macropinocytosis involves the uptake of large vesicles mediated by membrane ruffling driven by the actin cytoskeleton. Previous reports have shown that macropinocytosis is dependent not only on microfilament integrity, but also on the activity of the Na^+/H^+ antiport, phospholipase C (PLC), and phosphatidylinositol 3-kinase (PI3K) (30, 42–44). To test whether the increase in the endocytic capacity of DCs under acidosis involved the stimulation of macropinocytosis, experiments were performed using cytochalasin B, an actin microfilament disrupting agent, amiloride, an inhibitor of the Na^+/H^+ antiport, NCDC, a PLC inhibitor, and wortmannin, a PI3K inhibitor (30, 38–41). As expected, the uptake of FITC-OVA and FITC-dextran by DCs at pH 7.3 was inhibited by all these compounds (Fig. 3), supporting that it is mediated, at least in part, by macropinocytosis, whereas the same drugs exerted only minor effects on the uptake of IgG immune complexes, which occurs by endocytosis through FcγR (data not shown). More interestingly, all of these drugs markedly decreased endocytosis of FITC-OVA and FITC-dextran at pH 6.5 (Fig. 3), suggesting that extracellular acidosis increases DC endocytosis via stimulation of macropinocytosis.

Experiments were then performed to analyze the influence of cytosolic pH on the stimulation of endocytosis by extracellular acidification. In agreement with our previous findings in neutrophils (17), we observed an abrupt fall of 0.59 ± 0.12 U (mean ± SEM, $n = 5$) in cytosolic pH, 30 s after acidification of extracellular medium from 7.3 to 6.5, in DCs cultured in bicarbonate-supplemented medium (the medium used throughout our study), but not in DCs cultured in bicarbonate-free medium, which undergo only a slight decrease of 0.13 ± 0.05 U (mean ± SEM, $n = 5$) in cytosolic pH. Taking these results into account, we next compared the uptake of FITC-OVA by DCs suspended in culture medium with or without bicarbonate. At pH 7.3, there were no differences between both conditions: MFI = 126 ± 28 vs 142 ± 24, respectively (mean ± SEM, $n = 6$). At pH 6.5, we found a significant increase in the uptake of FITC-OVA in both medium, but interestingly, it was markedly higher for DCs suspended in bicarbonate-free medium compared with bicarbonate-supplemented medium: MFI = 556 ± 29 vs 266 ± 37, respectively (mean ± SEM, $n = 6$, $p < 0.05$). These results support the notion that cytosolic acidification does not favor the stimulation of endocytosis triggered by extracellular acidification.

Extracellular acidosis up-regulates the expression of cell surface proteins involved in Ag presentation

Having shown that acidosis increases Ag uptake by DCs, we next analyzed whether it could also be able to modulate the expression

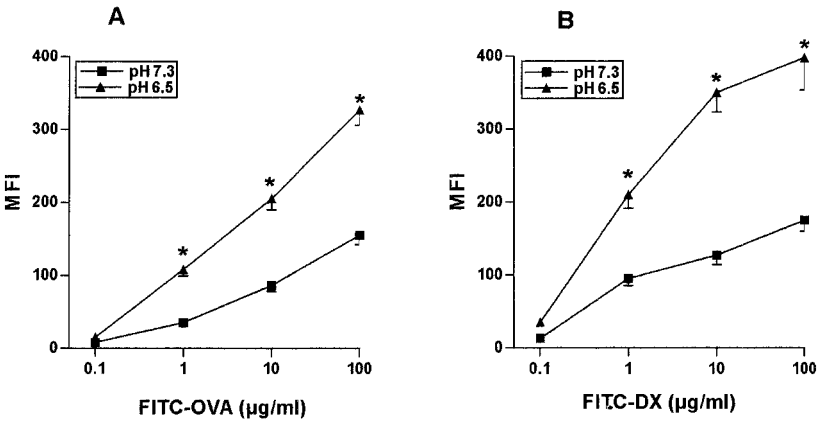

FIGURE 2. Enhancement of endocytosis by acidic pH analyzed at different concentrations of FITC-OVA and FITC-dextran. DCs (1.5×10^6/ml) were incubated for 30 min at 37°C with different concentrations of FITC-OVA (*A*) or FITC-dextran (*B*) at pH 7.3 or 6.5, and the amount of ligand accumulated was measured by flow cytometry. The uptake of either FITC-OVA or FITC-dextran (100 μg/ml) by DCs after incubation for 30 min at 0°C was <20% of the uptake at 37°C. Results are expressed as MFI values and represent the arithmetic mean ± SEM of seven experiments. Asterisk represents statistical significance ($p < 0.05$) pH 6.5 vs 7.3, for each of the concentrations of FITC-OVA and FITC-dextran used.

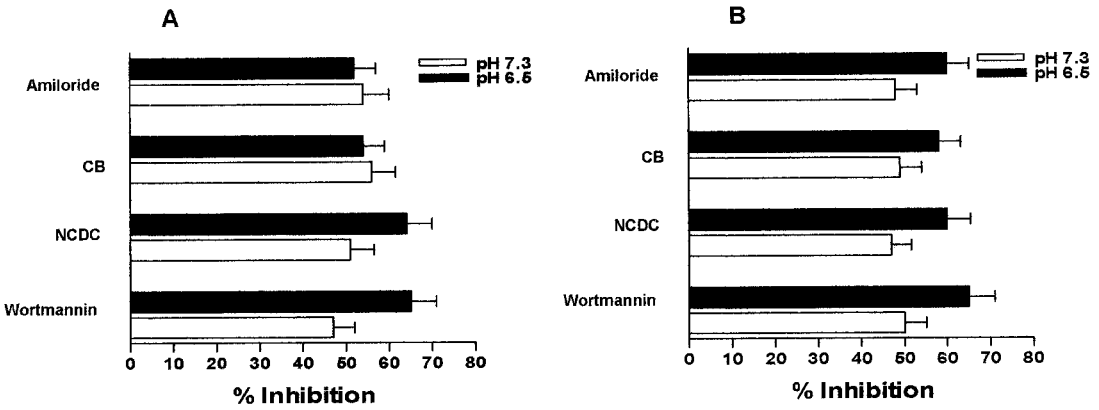

FIGURE 3. Inhibitors of macropinocytosis partially prevent stimulation of endocytosis by acidic pH. DCs (1.5×10^6/ml) were incubated for 15 min at 37°C with cytochalasin B (5 μg/ml), amiloride (100 μM), NCDC (10 μM), or wortmannin (0.5 μM). Then, cells were cultured in the presence of (100 μg/ml) of FITC-OVA (*A*) or FITC-dextran (*B*) at pH 7.3 or 6.5 for 30 min at 37°C, and the amount of ligand accumulated was measured by flow cytometry. Results are expressed as percentage of inhibition compared with untreated DCs at pH 7.3 or 6.5 ($p < 0.05$ in all cases), and represent the arithmetic mean ± SEM of six experiments.

of CD11c, MHC class I and class II, CD40, CD86, and FcγRII/III. Experiments were conducted by incubating DCs at pH 7.3 and 6.5, for 30 min and 4 h at 37°C. No changes in the expression of cell surface proteins were observed after 30 min of culture at pH 6.5 (data not shown). By contrast, exposure of DCs to pH 6.5 for 4 h resulted in a significant increase in the expression of CD11c, MHC class II, CD40, and CD86, whereas the expression of MHC class I and FcγR was not modified (Fig. 4). Similar enhancing effects on the expression of CD11c, MHC class II, CD40, and CD86 were observed after exposure of DCs to pH 6.5 for 24 h (data not shown).

Maturation of DCs is associated with several coordinated events, which include up-regulation of costimulatory molecules and MHC class II expression and the loss of endocytic ability (25, 27). Considering the results previously described, which indicate that acidosis up-regulates the expression of CD40, CD86, and MHC class II, we analyzed the endocytic ability of DCs cultured for long periods under acidosis, to determine whether acidosis might be able to trigger the transition from immature to mature DCs. To this aim, DCs were cultured at pH 7.3 or 6.5 for 24 h. Then, cells were washed, suspended at pH 7.3, and incubated with FITC-OVA (100 μg/ml) for 30 min at 37°C, and endocytosis was evaluated by flow cytometry. We found that previous exposure to acidosis did not result in the loss of endocytic ability of DC: MFI = 138 ± 25 vs 149 ± 37 (pH 7.3 and 6.5, respectively, mean ± SEM, $n = 4$). Similar observations were made for DCs preincubated at pH 6.5 for 4 and 48 h (data not shown).

We also examined whether acidosis might affect DC viability. Studies performed in DCs after 4, 24, and 48 h of culture at pH 7.3 and 6.5 showed that acidosis did not reduce DC viability, which was always higher than 85%, as determined by trypan blue exclusion. We also determined the absolute cell numbers recovered after 4, 24, and 48 h of culture at pH 6.5 and 7.3 and found that cell recovery was higher than 80% at all time points, either at pH 7.3 or 6.5. Together, these data suggest that acidosis (pH 6.5) did not induce deleterious effects on DCs.

Extracellular acidosis improves MHC class-I restricted Ag presentation by DCs

We analyzed whether acidosis could improve the acquisition of Ags by DCs for MHC class II-restricted presentation. To this aim, we used the T cell hybridoma BO97.10, specific for $OVA_{323–339}$ epitope on $I\text{-}A^b$ (36). DCs (1×10^6/ml) were exposed to OVA (100 μg/ml) during 3 h at 37°C at pH 7.3 or 6.5. After washing, they were cultured with BO97.10 cells (1×10^6/ml) at pH 7.3 for 24 h, and the production of IL-2 by BO97.10 cells was measured in the supernatants by ELISA. There were no differences between the levels of IL-2 produced by BO97.10 cells stimulated with DCs

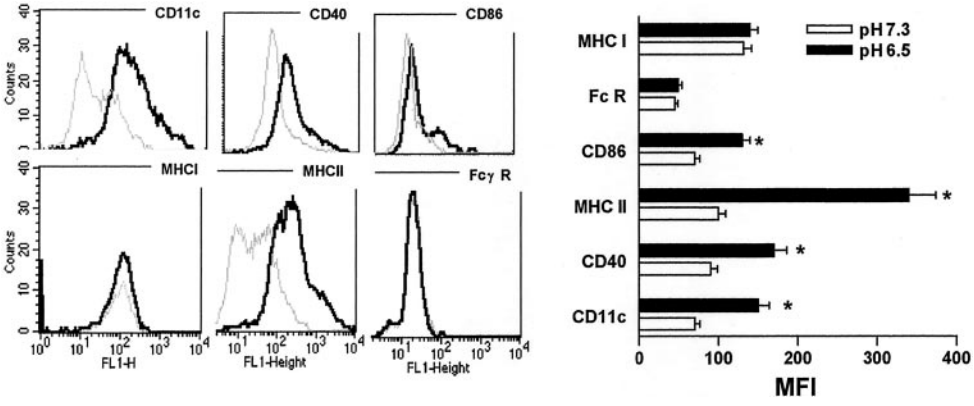

FIGURE 4. Flow cytometry profile of DCs cultured at pH 7.3 or 6.5. DCs (1.5×10^6/ml) were incubated for 4 h at 37°C at pH 7.3 or 6.5. Then, cell surface Ag expression was evaluated by single staining. The histograms (*left*) from a representative experiment are shown. Values for pH 7.3 (thin lines) and pH 6.5 (thick lines) are shown. Results (*right*) are expressed as MFI values and represent the arithmetic mean ± SEM of eight experiments. MFI of control isotype ranged between 5 and 20 in all cases. Asterisk represents statistical significance ($p < 0.05$) for pH 6.5 vs 7.3.

pulsed with OVA under neutral and acidic conditions: IL-2 in cell supernatants is 231 ± 37 pg/ml vs 267 ± 55 pg/ml (pH 7.3 vs 6.5, respectively, mean ± SEM, $n = 5$).

It has become clear that exogenous Ags may be presented not only via MHC class II but also via MHC class I molecules (25, 27, 35, 43). We next examined whether acidosis improved the acquisition of Ags by DCs for MHC class I-restricted presentation. To analyze this point, we studied presentation of OVA to a $CD8^+$ T cell hybridoma called B3Z, which carries a β-galactosidase construct driven by NF-AT elements from the IL-2 promoter that enable the analysis of T cell activation by measuring β-galactosidase activity in cell lysates (36, 45). DCs were exposed to different concentrations of OVA during 3 h at 37°C at pH 7.3 or 6.5, and presentation of the $OVA_{257–264}$-epitope/H-$2K^b$ to B3Z cells was assessed. Fig. 5 shows that acidosis improves MHC class I presentation. Indeed, to reach similar levels of Ag presentation, DCs cells cultured at pH 7.3 required concentrations of Ag in the extracellular medium almost 10- to 50-fold higher compared with those cultured at pH 6.5. As expected, no presentation was observed when DCs were fixed with glutaraldehyde before the addition of OVA, either at pH 7.3 or 6.5 (data not shown). We finally analyzed whether acidosis could modulate direct presentation of the $OVA_{257–264}$ peptide. To this aim, DCs were cultured with the peptide (10 ng/ml) for 3 h at 37°C at pH 7.3 and 6.5, and MHC class I presentation was assessed as described in Fig. 5. No differences were observed in the presentation of the peptide between DCs pulsed under neutral and acidic conditions: T cell response measured as OD at 415 nm = 0.73 ± 0.19 vs 0.65 ± 0.28 (pH 7.3 and 6.5, respectively, mean ± SEM, $n = 3$).

Priming with OVA under acidosis improves DC ability to induce specific CTL responses

We next tested whether incubation of DCs and Ag under acidosis would improve DC capacity to induce CTLs capable of lysing OVA-expressing target cells. DCs were pulsed during 3 h with OVA (100 μg/ml) at pH 7.3 or 6.5, and after washing, 3×10^5 OVA-pulsed DCs were injected i.v. into naive C57BL/6 mice. Splenocytes harvested from mice 8–10 day after immunization were restimulated by coculture with the K^b-binding peptide SIINFEKL (10 μmol/L). Cells were harvested 5 day later to test their cytotoxic activity using SIINFEKL-loaded DCs as targets, as described (35). Fig. 6 shows that splenocytes from mice immunized with DCs pulsed with OVA under acidosis elicited a strong CTL response. By contrast, a low CTL response was observed using splenocytes from mice immunized with DCs pulsed with OVA under neutral pH. As expected, no cytotoxic response was observed using unpulsed DCs as targets, or when splenocytes were depleted of $CD8^+$ T lymphocytes by using paramagnetic beads and rat anti-mouse $CD8^+$ Abs (data not shown).

Priming with OVA under acidosis improves DC ability to induce specific Ab responses in vivo

It has been shown the effectiveness of DC preparations to induce Ab responses in vivo, not only in Ag-primed, but also in naive mice, perhaps reflecting their ability to capture and retain unprocessed Ag, then transfer it to naive B cells to initiate a specific Ab response (46, 47). We next tested whether incubation of DCs and Ag under acidosis would improve DC capacity to induce B cell responses in vivo. DCs were pulsed during 3 h with OVA (100 μg/ml) at pH 7.3 or 6.5, and after washing, 3×10^5 OVA-pulsed DCs were injected i.v. into naive C57BL/6 mice. Animals were bled 14 days later, and the amount of specific Abs directed to OVA was measured by ELISA, using serum dilutions of 1/30 and 1/300. Fig. 7 shows that DCs pulsed with OVA at pH 6.5 induced higher Ab responses compared with those pulsed at pH 7.3.

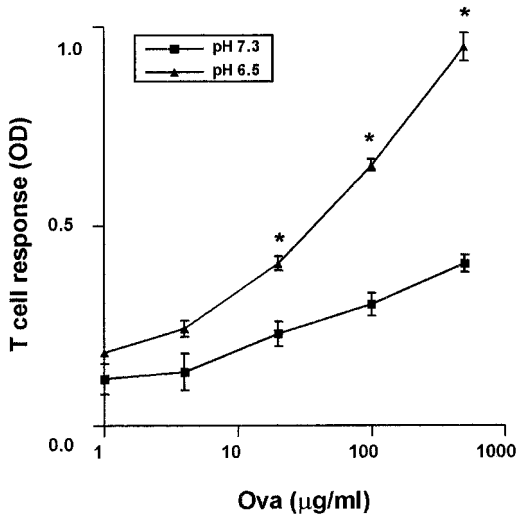

FIGURE 5. Acidosis improves the acquisition of Ags by DCs for MHC class I-restricted presentation. DCs (1×10^6/ml) were cultured with different concentrations of OVA for 3 h at 37°C at pH 7.3 or 6.5. Then, cells were washed, suspended in complete medium at pH 7.3, and cultured for 18 h at 37°C in the presence of B3Z cells (1×10^6/ml), a T cell hybridoma specific for OVA-K^b, which carries a β-galactosidase construct driven by NF-AT elements from the IL-2 promoter. T cell activation was measured using a colorimetric assay for LacZ activity with *o*-nitrophenyl-P-D-galactoside as a substrate. Background absorbance values obtained for DCs cultured in the absence of OVA were subtracted. Statistical significance is shown for pH 6.5 vs 7.3, *, $p < 0.05$.

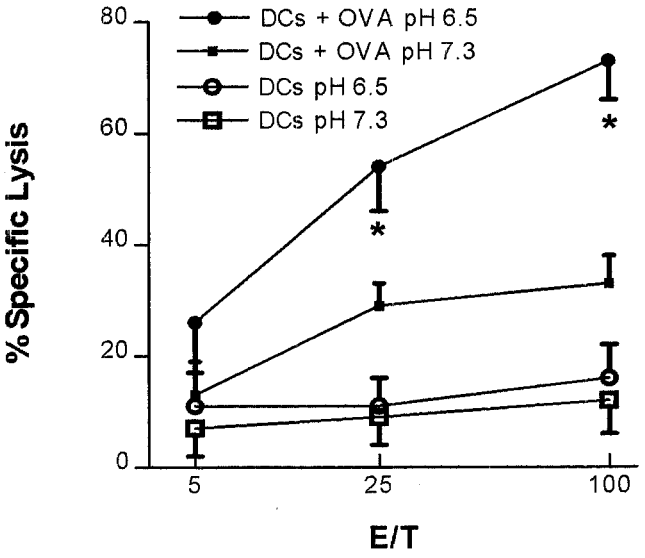

FIGURE 6. DCs pulsed with OVA under acidosis improves their ability to induce specific CTL responses. DCs (1×10^6/ml) were cultured with or without OVA (100 μg/ml) for 3 h at 37°C, at pH 7.3 or 6.5. Then, cells were washed and suspended at 1.5×10^6 cells/ml. Nonimmunized C57BL/6 mice received an i.v. injection of 3×10^5 unpulsed (controls) or OVA-pulsed syngeneic DCs. Splenocytes were harvested from mice 8–10 day after immunization and were restimulated by coculture with the K^b-binding peptide SIINFEKL (10 μmol/L) and effector cells were harvested 5 day later. Cytotoxic assays were performed using SIINFEKL-loaded DCs as targets, at different E:T ratios. Results are expressed as a percentage of specific lysis, and represent the mean ± SEM of five to eight animals in each group. Asterisk represents statistical significance ($p < 0.05$) for pH 6.5 vs 7.3.

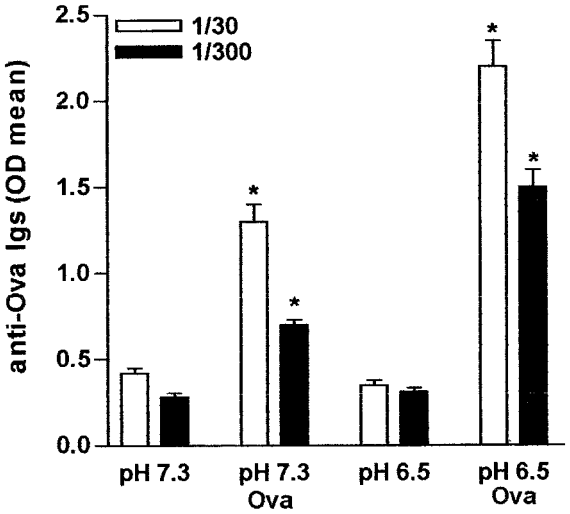

FIGURE 7. DCs pulsed with OVA under acidosis improves their ability to induce specific Ab responses in vivo. DCs (1×10^6/ml) were cultured with or without OVA (100 μg/ml) for 3 h at 37°C, at pH 7.3 or 6.5. Then, cells were washed and suspended at 1.5×10^6 cells/ml. Nonimmunized C57BL/6 mice received an i.v. injection of 3×10^5 unpulsed (controls) or OVA-pulsed syngeneic DCs. Mice were bled 14 days later, and the amount of serum specific Abs directed to OVA was determined by ELISA using serum dilutions of 1/30 and 1/300. Results are expressed as mean absorbance values at 492 nm, and represent the mean ± SEM of six to nine animals in each group. Asterisk represents statistical significance ($p < 0.05$) for pH 6.5 vs 7.3.

Discussion

Our observations reveal that exposure of DCs to pH 6.5 stimulates macropinocytosis, the acquisition of extracellular Ags for MHC class I-restricted presentation and the expression of CD11c, CD40, CD86, and MHC class II. Of note, DCs pulsed with Ag under acidosis showed an improved efficacy to prime specific $CD8^+$ CTL responses as well as to stimulate the production of specific Abs in vivo.

Given the critical role of DCs in the induction of immunity, and considering that many inflammatory responses against infectious agents are associated to the development of acidic microenvironments in peripheral tissues (1–7), it is surprising that no previous work has analyzed the impact of acidosis on DC physiology. This point appears to be relevant not only in anti-infectious immunity, but also in antitumor immunity because acidic regions with values of pH ranging from 5.8 to 7.4 have been described within the solid tumors (8–15). Interestingly, observations performed in breast carcinoma tissue showed that mature DCs are preferentially located in peritumoral areas, whereas a high number of immature DCs reside within the tumor (48).

In contrast with what is found in macrophages and epithelial cells in which macropinocytosis is transiently induced by phorbol esters or growth factor stimulation, this activity is constitutive in immature DCs (37). Sensitivity to a variety of pharmacological agents clearly distinguishes macropinocytosis from clathrin-dependent micropinocytosis, a process that account not only for receptor-mediated endocytosis, but also appears to contribute for a substantial fraction of fluid-phase micropinocytosis (30, 42). Like phagocytosis, macropinocytosis clearly depends on actin polymerization at the plasma membrane. Direct dependence of macropinocytosis on actin is in marked contrast with clathrin-dependent micropinocytosis, which is not impaired by cytochalasins (30, 49). Moreover, whereas macropinocytosis is suppressed by pharmacological inhibitors of PLC and PI3K, as well as by inhibition of the Na^+/H^+ antiport, these pathways are usually not required for clathrin-dependent micropinocytosis (30, 50, 51). Our results show that extracellular acidosis markedly increases Ag uptake by DCs, and that this effect involves, at least in part, stimulation of macropinocytosis, as judged by the action of drugs able to inhibit actin polymerization, the Na^+/H^+ antiport, PLC, and PI3K activities. Interestingly, macropinocytosis appears to be one of the major pathways that allows receptor-independent cross presentation of exogenous soluble Ags by DCs (45, 52), supporting that the enhancement of MHC class I-restricted presentation observed for DCs cultured with OVA under acidosis occurs via stimulation of macropinocytosis. However, other mechanisms should also be considered. In fact, acidosis also increased the expression of CD40 and CD86 by DCs, which might favor MHC class I-restricted presentation.

In contrast to the classical DC activation induced by LPS, TNF-α, CD40 ligand, or IgG immune complexes (24–27), the response triggered by acidosis does not lead to down-regulation of endocytosis. In fact, after exposure to pH 6.5 for 4, 24, or 48 h DCs showed an increased expression of CD40, CD80, and MHC class II, together with a high endocytic ability. Similar contrasting effects were described in human monocyte-derived DCs activated through the triggering receptor expressed on myeloid cells-2, the first receptor associated with the adaptor molecule DAP12 identified in DCs. Ligation of triggering receptor expressed on myeloid cells-2 induced increased cell surface expression of CD40, CD86, and MHC class II but Ag-capturing mechanisms were not down-regulated (53). This unusual pattern of activation could be explained considering that maturation of DCs is usually associated with several coordinated events that are regulated by different signal transduction pathways (36, 53, 54). In this regard, it has been shown that the inhibition of the p38 stress-activated protein kinase (p38SAPK) reduces the LPS-induced up-regulation of CD80, CD83, and CD86 in human monocyte-derived DCs, but has no effect on the LPS-induced down-regulation of macropinocytosis (55). Studies are underway in our laboratory to characterize the signal transduction pathways activated by extracellular acidosis in DCs.

We also found that DCs pulsed with OVA at pH 6.5 induced in vivo Ab responses higher than those induced by DCs pulsed at pH 7.3. Because both preparations of DCs were equally able to mediate MHC class II-restricted presentation, as evaluated by measuring activation of the T cell hybridoma BO97.10, it is unlikely that they may differ in their intrinsic ability to activate virgin T $CD4^+$ cells specific for OVA. Supporting this presumption, we also observed that splenocytes from mice immunized with OVA-pulsed DCs, prepared at pH 7.3 or pH 6.5, produced similar levels of IL-2 when cultured in vitro in the presence of OVA (data not shown). In contrast, it should be considered that, besides activating naive T cells, Ag-pulsed DCs directly activate naive B lymphocytes during the initiation of the immune response. Indeed, DCs can capture and retain Ag in native form for long periods and can subsequently transfer it to naive B cells to initiate a specific Ab response (25, 46, 47). We have analyzed whether endocytosis of HRP by DCs at pH 6.5 resulted in the retention of higher amounts of native HRP for long periods, compared with pH 7.3. It was found that HRP activity in DC lysates, evaluated 24 h after endocytosis, was 2- to 4-fold higher in DCs pulsed with HRP at pH 6.5 compared with pH 7.3 (M. Vermeulen, unpublished observations), supporting that the increased ability of DCs pulsed with Ag at pH 6.5 to induce Ab responses in vivo could be due, at least in part, to their higher content of intact Ag.

A large body of evidence in a variety of cell lines suggests that acidification of the cytosol inhibits endocytosis (56–60). Using

three different methods to acidify the cytosol to pH values below 6.5, Sandvig et al. (56, 57) found a marked decrease in the internalization of transferrin and epidermal growth factor, whereas the endocytic uptake of the fluid phase marker lucifer yellow was only slightly reduced. In studies directed to analyze the influence of low extracellular pH on endocytosis in baby hamster kidney cells, Davoust et al. (58) showed that when the extracellular medium was adjusted to pH 5.7, the intracellular pH decreased within 2 min to pH 6.2 and the endocytosis of HRP in the fluid phase dropped to undetectable levels. By contrast, with an external pH of 6.3, the internal pH dropped to pH 6.8 and HRP was internalized at a normal rate for 5 min but accumulation during longer incubation times did not occur. They also showed, in a fibroblast mutant cell line lacking the Na^+/H^+ antiporter, that cytoplasmic acidification blocked the uptake of lucifer yellow and HRP, as well as the internalization and the recycling of transferrin (59). Not only constitutive endocytosis, but also the stimulation of macropinocytosis by growth factors appears to be inhibited by cytoplasmic acidification, as judged by the findings of West et al. (60), showing that the increase in the rate of pinocytosis of HRP induced by epidermal growth-factor in the epidermoid carcinoma A431 cells was dramatically inhibited by imposing an outward gradient of NH3/$NH4^+$ across the plasma membrane, the most common method used to induce cytosol acidification.

Our present data in DCs appear to be in discrepancy with the findings previously described in different cell lines because we observed that, although extracellular acidosis induces cytosol acidification, it stimulates endocytosis. These contrasting results could be explained considering that the mechanisms that regulate endocytosis might differ in different cells. Supporting this possibility it has been described that, not only the routes for macropinocytosed Ags, but also the sensitivity of macropinocytosis to microtubule and microfilament destabilizing drugs differ between professional phagocytes and other cell types (38, 59, 61–63). However, our observations made in bicarbonate-free medium support that, as observed in other cell types, acidification of cytosol exerts an inhibitory effect on DC endocytosis. In fact, when cultured at pH 6.5 in bicarbonate-free medium, DCs undergo a much lower decrease in cytosolic pH, but a more pronounced stimulation of endocytosis, compared with DCs cultured in bicarbonate-supplemented medium. Also supporting an inhibitory effect of cytosolic acidification on DC endocytosis, we observed that cytosolic acidification induced by imposing an outward gradient of NH3/$NH4^+$ across the plasma membrane, resulted in the inhibition of endocytosis by DCs either at pH 7.3 or 6.5 (M. Vermeulen, unpublished observations).

The mechanism through which extracellular acidosis stimulates endocytosis by DCs has not been addressed in our study. In this regard, however, it should be mentioned that low extracellular pH is able to activate mitogen-activated protein kinases extracellular signal-regulated kinase 2, c-Jun N-terminal kinase, and p38 in a variety of cell types (64, 65). More interestingly, changing extracellular pH from 7.4 to 6.1 has shown to trigger the activation of PLC with the subsequent production of inositol trisphosphates and Ca^{2+} mobilization, in human fibroblasts, endothelial, smooth muscle, and neuroblastoma cells, but not in human epidermoid carcinoma (A431) cells (66). Because decreasing cytosolic pH failed to trigger activation of PLC, it appears that extracellular acidosis acts protonating a functional group, possibly imidazolium (pKa of 6–7), in a cell surface receptor (66), which might act as a chemosensor for acid, as described for bacteria and taste cells (67–69). Whether exposure to acidosis also results in the stimulation of PLC in DCs is an attractive hypothesis to be tested. This question is relevant because the activation of PLC appears to play a critical role in the dramatic reorganization of the actin cytoskeleton resulting in macropinocytosis (50).

In summary, our results show that acidosis increases the acquisition of extracellular Ags by DCs for MHC class I-restricted presentation and the ability of Ag-pulsed DCs to induce both specific $CD8^+$ CTL responses and the production of specific Abs in vivo. Further studies are required to determine whether targeting tumor Ags to DCs under acidosis might represent a suitable method to improve vaccination protocols used for cancer immunotherapy.

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References

1. Dubos, R. J. 1955. The micro-environment of inflammation or Metchnikoff revisited. *Lancet 2:1*.
2. Edlow, D. W., and W. H. Sheldon. 1971. The pH of inflammatory exudates. *Proc. Soc. Exp. Biol. Med. 137:1328*.
3. Ward, T. T., and R. T. Steibigel. 1978. Acidosis of synovial fluid correlates with synovial fluid leukocytosis. *Am. J. Med. 64:933*.
4. Bryant, R. E., A. L. Rashad, J. A. Mazza, and D. Hammond. 1980. β-lactamase activity in human pus. *J. Infect. Dis. 142:594*.
5. Abbot, N. C., V. A. Spence, J. Swanson-Beck, F. M. Carnochan, J. H. Gibbs, and J. G. Lowe. 1990. Assessment of the respiratory metabolism in the skin from transcutaneous measurements of pO_2 and pCO_2: potential for non-invasive monitoring of response to tuberculin skin testing. *Tubercle 71:15*.
6. Simmen, H. P., and J. Blaser. 1993. Analysis of pH and PO_2 in abscesses, peritoneal fluid, and drainage fluid in the presence or absence of bacterial infection during and after abdominal surgery. *Am. J. Surg. 166:24*.
7. Hunt, J. F., K. Fang, R. Malik, A. Snyder, N. Malhotra, T. A. E. Platts-Mills, and B. Gaston. 2000. Endogenous airway acidification: implications for asthma pathophysiology. *Am. J. Respir. Crit. Care Med. 161:694*.
8. Ashby, B. S. 1966. pH studies in human malignant tumors. *Lancet 2:312*.
9. Vaupel, P., F. Kallinowsky, and P. Okunieff. 1989. Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review. *Cancer Res. 49:6449*.
10. Griffiths, J. R. 1991. Are cancer cells acidic? *Br. J. Cancer 64:425*.
11. Helmlinger G., F. Yuan, M. Dellian, and R. K. Jain. 1997. Interstitial pH and pO_2 gradients in solid tumors in vivo: high resolution measurements reveal a lack of correlation. *Nat. Med. 3:177*.
12. Newell, K. J., and I. F. Tannock. 1989. Reduction of intracellular pH as a possible mechanism for killing cells in acidic regions of solid tumors: effects of carbonylcyanide-3-chlorophenylhydrazone. *Cancer Res. 49:4477*.
13. Crowther, M., N. J. Brown, E. T. Bishop, and C. E. Lewis. 2001. Microenvironmental influence on macrophage regulation of angiogenesis in wounds and malignant tumors. *J. Leukocyte Biol. 70:478*.
14. Tannok, I. F., and D. Rotin. 1989. Acidic pH in tumors and its potential for therapeutic exploitation. *Cancer Res. 49:4373*.
15. Gerweck, L. E., and K. Seetharaman. 1996. Cellular pH gradient in tumor versus normal tissue: potential exploitation for the treatment of cancer. *Cancer Res. 56:1194*.
16. Lardner, A. 2001. The effect of extracellular pH on immune function. *J. Leukocyte Biol. 69:522*.
17. Trevani, A. S., G. Andonegui, M. Giordano, D. H. López, R. Gamberale, F. Minucci, and J. R. Geffner. 1999. Extracellular acidification induces human neutrophil activation. *J. Immunol. 162:4849*.
18. Bidani, A., C. Z. Wang, S. J. Saggi, and T. A. Heming. 1998. Evidence for pH sensitivity of tumor necrosis factor-α release by alveolar macrophages. *Lung 176:111*.
19. Baud, L., A. Bellocq, C. Philippe, and B. Fouqueray. 1997. Low extracellular pH has a role in the induction of NO synthase type 2 in macrophages. *Bull. Acad. Natl. Med. 181:247*.
20. Ratner, S. 1992. Motility of IL-2-stimulated lymphocytes in neutral and acidified extracellular matrix. *Cell. Immunol. 139:399*.
21. Severin, T., B. Muller, G. Giese, B. Uhl, B. Wolf, S. Hauschildt, and W. Kreutz. 1994. pH-Dependent LAK cell cytotoxicity. *Tumour Biol. 15:304*.
22. Loeffler, D. A., P. L. Juneau, and G. H. Heppner. 1991. Natural killer-cell activity under conditions reflective of tumor micro-environment. *Int. J. Cancer 48:895*.
23. Loeffler, D. A., P. L. Juneau, and S. Masserant. 1992. Influence of tumour physico-chemical conditions on interleukin-2-stimulated lymphocyte proliferation. *Br. J. Cancer 66:619*.
24. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature 392:245*.
25. Banchereau, J., F. Briere, C. Caux, J. Davoust, S. Lebecque, L. Yong-Jun, B. Pulendran, and K. Palucka. 2000. Immunobiology of dendritic cells. *Annu. Rev. Immunol. 18:767*.
26. Lanzavecchia, A., and F. Sallusto. 2001. Regulation of T cell immunity by dendritic cells. *Cell 106:263*.
27. Guermonprez, P., J. Valladeau, L. Zitvogel, C. Thery, and S. Amigorena. 2002. Antigen presentation and T cell stimulation by dendritic cells. *Annu. Rev. Immunol. 20:621*.

28. Lutz, M. B., C. U. Assmann, G. Girolomoni, and P. Ricciardi-Castagnoli. 1996. Different cytokines regulate antigen uptake and presentation of a precursor dendritic cell line. *Eur. J. Immunol. 26:586*.
29. Inaba, K., M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara, S. Muramatsu, and R. M. Steinman. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med. 176:1693*.
30. Sallusto, F., M. Cella, C. Danieli, and A. Lanzavecchia. 1995. Dendritic cells use macropynocitosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: down regulation by cytokines and bacterial products. *J. Exp. Med. 182:389*.
31. Sahlin, S., J. Hed, and I. Rundquist. 1983. Differentiation between attached and ingested immune complexes by a fluorescence quenching cytofluorometric assay. *J. Immunol. Methods 60:115*.
32. Van Strijp, J. A., K. P. Van Kessel, M. E. Van der Tol, and J. Verhoef. 1989. Complement-mediated phagocytosis of herpes simplex virus by granulocytes: binding or ingestion. *J. Clin. Invest. 84:107*.
33. Gottlieb, R. A., H. A. Giesing, J. Y. Zhu, R. L. Engler, and B. M. Babior. 1995. Cell acidification in apoptosis: granulocyte colony-stimulating factor delays programmed cell death in neutrophils by up-regulating the vacuolar H^+-ATPase. *Proc. Natl. Acad. Sci. USA 92:5965*.
34. Chow, S., D. Hedley, and I. Tannock. 1996. Flow cytometric calibration of intracellular pH measurements in viable cells using mixtures of weak acids and bases. *Cytometry 24:360*.
35. Brossart, P., and M. J. Bevan. 1997. Presentation of exogenous protein antigens on major histocompatibility complex class I molecules by dendritic cells: pathway of presentation and regulation by cytokines. *Blood 90:1594*.
36. Regnault, A., D. Lankar, V. Lacabanne, A. Rodriguez, C. Théry, M. Rescigno, T. Saito, S. Verbeek, C. Bonnerot, P. Ricciardi-Castagnoli, and S. Amigorena. 1999. Fcγ Receptor-mediated induction of dendritic cell maturation and major histocompatibility complex class-I-restricted antigen presentation after immune complex internalization. *J. Exp. Med. 189:371*.
37. Théry, C., and S. Amigorena. 2001. The cell biology of antigen presentation in dendritic cells. *Curr. Opin. Immunol. 13:45*.
38. Lutz, M. B., P. Rovere, M. J. Kleijmeer, M. Rescigno, C. U. Assmann, V. M. J. Oorschot, H. J. Geuze, J. Trucy, D. Demandolx, J. Davoust, and P. Ricciardi-Castagnoli. 1997. Intracellular routes and selective retention of antigens in mildly acidic cathepsin D/lysosome-associated membrane protein-1/MHC class II-positive vesicles in immature dendritic cells. *J. Immunol. 159:3707*.
39. Becker, D., U. Kuhn, U. Lempertz, A. Enk, J. Saloga, and J. Knop. 1997. Flow-cytometric screening for the modulation of receptor-mediated endocytosis in human dendritic cells: implications for the development of an in vitro technique for predictive testing of contact sensitizers. *J. Immunol. Methods 203:171*.
40. Steinman, R. M., and J. Swanson. 1995. The endocytic activity of dendritic cells. *J. Exp. Med. 182:283*.
41. Lanzavecchia, A. 1996. Mechanisms of antigen uptake for presentation. *Curr. Opin. Immunol. 8:348*.
42. Araki, N., M. T. Johnson, and S. J. Swanson. 1996. A role for phosphoinositide 3-kinase in the completion of macropinocytosis and phagocytosis by macrophages. *J. Cell Biol. 135:1249*.
43. Norbury, C. C., L. J Hewlett, A. R. Prescott, N. Shastri, and C. Watts. 1995. Class I MHC presentation of exogenous soluble antigen via macropinocytosis in bone marrow macrophages. *Immunity 3:783*.
44. Terebiznik, M. R., O. V. Vieira, S. L. Marcus, A. Slade, C. M. Yip, W. S. Trimble, T. Meyer, B. B. Finlay, and S. Grinstein. 2002. Elimination of host cell PtdIns(4,5)P(2) by bacterial SigD promotes membrane fission during invasion by Salmonella. *Nat. Cell. Biol. 4:766*.
45. Shastri, N., and F. Gonzalez. 1993. Endogenous generation and presentation of the ovalbumin peptide/K^b complex to T cells. *J. Immunol. 150:2724*.
46. Sornasse, T., V. Flamand, G. De Becker, H. Bazin, F. Thielemans, K. Thielemans, J. Urbain, O. Leo, and M. Moser. 1992. Antigen-pulsed dendritic cells can efficiently induce an antibody response in vivo. *J. Exp. Med. 175:15*.
47. Wykes, M., A. Pombo, C. Jenkis, and G. MacPherson. 1998. Dendritic cells interact directly with naive B lymphocytes to transfer antigen and initiate class switching in a primary T-dependent response. *J. Immunol. 161:1313*.
48. Bell, D., P. Chomarat, D. Broyles, G. Netto, G. M. Harb, S. Lebecque, J. Valladeau, J. Davoust, K. A. Palucka, and J. Banchereau. 1999. In breast carcinoma tissue, immature dendritic cells reside within the tumor, whereas mature dendritic cells are located in peritumoral areas. *J. Exp. Med. 190:1417*.
49. Lamaze, C., L. M. Fujimoto, H. L. Yin, and S. L. Schmid. 1997. The actin cytoskeleton is required for receptor-mediated endocytosis in mammalian cells. *J. Biol. Chem. 15:20332*.
50. Amyere, M., B. Payrastre, U. Krause, P. Van Der Smissen, A. Veithen, and P. J. Courtoy. 2000. Constitutive macropinocytosis in oncogene-transformed fibroblasts depends on sequential permanent activation of phosphoinositide 3-kinase and phospholipase C. *Mol. Biol. Cell 11:3453*.
51. Hewlett, L. J., A. R. Prescott, and C. Watts. 1994. The coated pit and macropinocytic pathways serve distinct endosome populations. *J. Cell Biol. 124:689*.
52. Norbury, C. C., B. J. Chambers, A. E. Prescott, H. G. Ljunggren, and C. Watts. 1997. Constitutive macropinocytosis allows TAP-dependent major histocompatibility complex class I presentation of exogenous soluble antigen by bone marrow-derived dendritic cells. *Eur. J. Immunol. 27:280*.
53. Bouchon, A., C. Hernandez-Munain, M. Cella, and M. Colonna. 2001. A DAP12-mediated pathway regulates expression of CC chemokine receptor 7 and maturation of human dendritic cells. *J. Exp. Med. 194:1111*.
54. Puig-Kroger, A., M. Relloso, O. Fernandez-Capetillo, A. Subyaga, A. Silva, C. Bernabeu, and A. L. Corbi. 2001. Extracellular signal-regulated protein kinase signaling pathway negatively regulates the phenotypic and functional maturation of monocyte-derived human dendritic cells. *Blood 98:2175*.
55. Ardeshna, K. M., A. R. Pizzey, S. Devereux, and A. Khwaja. 2000. The PI3 kinase, p38 SAP kinase, NFκB signal transduction pathways are involved in the survival and maturation of lipopolysaccharide-stimulated human monocyte-derived dendritic cells. *Blood 96:1039*.
56. Sandvig, K., S. Olsnes, O. W. Peterson, and B. Van Deurs. 1987. Acidification of the cytosol inhibits endocytosis from coated pits. *J. Cell Biol. 105:679*.
57. Sandvig, K., S. Olsnes, O. W. Peterson, and B. Van Deurs. 1988. Inhibition of endocytosis from coated pits by acidification of the cytosol. *J. Cell. Biochem. 36:73*.
58. Davoust, J., J. Gruenberg, and K. Howell. 1987. Two threshold values of low pH block endocytosis at different stages. *EMBO J. 6:3601*.
59. Cosson, P., I. de Curtis, J. Pouysségur, G. Griffiths, and J. Davoust. 1989. Low cytoplasmic pH inhibits endocytosis and transport from the trans-Golgi network to the cell surface. *J. Cell Biol. 108:377*.
60. West, M. A., M. S. Bretscher, and C. Watts. 1989. Distinct endocytic pathways in epidermal growth factor-stimulated human carcinoma A431 cells. *J. Cell Biol. 109:2731*.
61. Haigler, H. T., J. A. McKanna, and S. Cohen. 1979. Rapid stimulation of pinocytosis in human carcinoma cells A431 by epidermal growth factor. *J. Cell Biol. 83:82*.
62. Racoosin, E. L., and J. A. Swanson. 1989. Macrophage colony-stimulating factor (rM-CSF) stimulates pinocytosis in bone marrow-derived macrophages. *J. Exp. Med. 170:1635*.
63. Racoosin, E. L., and J. A. Swanson. 1993. Macropinosome maturation and fusion with tubular lysosomes in macrophages. *J. Cell Biol. 121:1011*.
64. Xue, L., and J. M. Lucocq. 1997. Low extracellular pH induces activation of MAP kinases ERK 2, JNK, and p38 in A431 and Swiss 3T3 cells. *Biochem. Biophys. Res. Commun. 241:236*.
65. Xu, L., D. Fukumura, and R. K Jain. 2002. Acidic extracellular pH induces vascular endothelial growth factor (VEGF) in human glioblastoma cells via ERK1/2 MAPK signaling pathway: mechanism of low pH-induced VEGF. *J. Biol. Chem. 277:11368*.
66. Smith, J. B., S. D. Dwyer, and L. Smith. 1989. Lowering extracellular pH evokes inositol polyphosphate formation and calcium mobilization. *J. Biol. Chem. 264:8723*.
67. Tso, W. W., and J. Adler. 1974. Negative chemotaxis in *Escherichia coli*. *J. Bacteriol. 118:560*.
68. Adler, J., and W. W. Tso. 1974. "Decision"-making in bacteria: chemotactic response of *Escherichia coli* to conflicting stimuli. *Science 184:1292*.
69. Autrum, H. 1971. In *Handbook of Sensory Physiology 4, Part 2, Taste*. Biedler, L. M. ed. Springer-Verlag, New York, p. 200.