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# The Small GTPase Rac2 Controls Phagosomal Alkalinization and Antigen Crosspresentation Selectively in CD8+ Dendritic Cells

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## SUMMARY

**A unique subpopulation of spleen dendritic cells (DCs) that express the CD8 surface marker efficiently present phagocytosed antigens to CD8+ T lymphocytes in a process called "crosspresentation," which initiates cytotoxic immune responses. We now show that the small GTPase Rac2 plays a critical role in antigen crosspresentation selectively in this DC subpopulation. In CD8+ DCs, Rac2 determines the subcellular assembly of the NADPH oxidase complex (NOX2) to phagosomes, whereas in CD8− DCs, Rac1 mediates the assembly of NOX2 at the plasma membrane. In the absence of Rac2, the production of reactive oxygen species (ROS) in DC-phagosomes was abolished, the phagosomal pH dropped, and the efficiency of antigen crosspresentation was reduced. We conclude that the activity of Rac1 and 2 control crosspresentation in DC subpopulations through the regulation of phagosomal oxidation and pH.**

## INTRODUCTION

Antigen processing and presentation determine the specificity of immune recognition during adaptative immune responses. Dendritic cells (DCs) are the most potent initiators of T cell-mediated immune responses, at least in part because of their unique capacity for antigen processing and presentation. Unlike other antigen-presenting cells, after antigen phagocytosis, DCs present antigenic peptides on both MHC class I and class II molecules and efficiently activate both CD4+ and CD8+ T cells. DCs are particularly efficient, as compared to other antigen-presenting cells, for the presentation of phagocytosed antigens to CD8+ T cells, a process called "crosspresentation" (Bevan, 1976). The molecular mechanisms that make DCs such a unique antigen crosspresenting cell are still unclear. Several recent studies, however, showed that DCs have adapted their endocytic and phagocytic pathways to the crosspresentation function. These adaptations seem to concur to limit the proteolytic activity in endosomes and phagosomes of DCs (Jancic et al., 2007; Savina et al., 2006). In several experimental settings in vitro and in vivo, low proteolytic activity in the internalization pathway correlates with high antigen crosspresentation, and vice versa.

The mechanisms that limit endo-phagocytic proteolysis in DCs include low expression levels and low recruitment of proteolytic enzymes to phagososmes (Lennon-Dumenil et al., 2002), limited acidification by the V-ATPase (Trombetta et al., 2003), and active alkalinization of the endosomal and phagosomal lumen by the NADPH oxidase NOX2 (Savina et al., 2006). Thus, after internalization in DCs, antigens are exposed to a nearly neutral pH, oxidative, lowly proteolytic milieu. This is in marked contrast to what happens in macrophages or neutrophils, the two types of phagocytes in which phagocytosis has been analyzed in some detail. In macrophages, the phagosomal pH drops to values around 5.5 in the first 30 min after engulfment (Lukacs et al., 1990; Yates et al., 2007). The proteolytic activity is extremely high and most proteins are rapidly degraded into amino acids. In neutrophils, a transient and alkalinization of the phagosomal pH results in a subsequent cascade of ionic fluxes that activates proteolytic enzymes before the pH acidifies strongly (Jiang et al., 1997; Reeves et al., 2002). Here too, proteins are rapidly degraded into amino acids. The results published thus far suggest that DCs limit their endo-phagosomal proteolytic capacity to "preserve" peptides from complete degradation. These "protected" peptides could then be loaded on MHC molecules and serve as a basis for immune recognition.

One of the main limitations with the studies analyzing phagocytic functions in DCs is that they have mainly been performed with in vitro generated DCs (from the bone marrow, in general). Very little, in contrast, is known about the DCs that reside in lymphoid organs. In both spleen and lymph nodes, murine DCs can be divided into several subpopulations via different surface markers (Vremec et al., 1992). One of these subpopulations expresses CD8αα homodimers and is referred to as "CD8+" DCs. This subpopulation was shown in many different experimental systems to crosspresent antigens more efficiently than

did other DCs subsets (collectively referred to as "CD8$^-$") (Iyoda et al., 2002; Pooley et al., 2001; Schnorrer et al., 2006; Schulz and Reis e Sousa, 2002).

We have analyzed the phagocytic pathway in these two main DC subpopulations of the spleen. In CD8$^-$, but not in CD8$^+$, DCs, phagosomes acidified in the first hours of phagocytosis. High phagosomal pH was maintained in CD8$^+$ DC phagosomes by the assembly of the NADPH oxidase NOX2 cytosolic subunits (including p47phox) to the membrane subunits (gp91phox and gp22phox). Phagosomal assembly of the complex in CD8$^+$ DCs caused the production of ROS inside phagosomes and prevented acidification. In CD8$^-$ DCs, in spite of very high amount of total ROS production, no phagosomal assembly of NOX2 or production of ROS was observed. These differences in the phagosomal function in CD8$^+$ and CD8$^-$ DCs were, at least in part, because of Rac2. Indeed, in Rac2-defective DCs, like in gp91phox-defective DCs, the production of ROS, the phagosomal pH, and the crosspresentation capacities were all similar in CD8$^+$ and CD8$^-$ spleen DCs.

## RESULTS

### NOX2 Alkalinizes Phagosomes and Endosomes in CD8$^+$ but Not in CD8$^-$ Spleen DCs

By using bone marrow-derived DCs, we have previously shown that crosspresentation requires high pH in phagosomes (Jancic et al., 2007; Savina et al., 2006). In the spleen, crosspresentation is a specific attribute of a particular DC subpopulation, the CD8$^+$ DCs (Pooley et al., 2001; Schnorrer et al., 2006). In order to determine the pH in the endo-phagocytic pathway in spleen DC populations, enriched CD11c$^+$ splenocytes were allowed to phagocytose latex beads bearing a mixture of pH-sensitive and -insensitive dyes. The phagosomal pH was measured by FACS as described previously (Savina et al., 2006). As shown in Figure 1A (left), phagosomes in CD8$^+$ DCs maintain a pH above 7 during the first hours after phagocytosis. In contrast, the pH in CD8$^-$ phagosomes was more acidic (by more than one pH unit) than in the CD8$^+$ DC phagosomes.

Because we have already demonstrated that bone marrow-derived DCs generated in vitro bear a phagosomal NOX2-dependent alkalinization system for the regulation of the pH (Savina et al., 2006), we hypothesize that NOX2 could also be responsible for the high pH in splenic CD8$^+$ phagosomes. We therefore tested the pH in phagosomes from CD8$^+$ and CD8$^-$ DCs from gp91phox-defective mice (*Cybb*$^{-/-}$ mice). As shown in Figure 1A (right), the phagosomal pH in NOX2-defective CD8$^+$ DCs dropped to values similar to the ones found in CD8$^-$ DCs. The phagosomal pH in CD8$^-$ DCs, in contrast, was not appreciably affected in the absence of NOX2. Similar results were obtained in phagosomes from WT DCs after treatment with DPI, a potent inhibitor flavin-containing enzymes, such as NOX2 (Figure 1C). Therefore, NOX2 activity is required to maintain the high pH observed in CD8$^+$ DCs phagosomes, but its absence does not affect phagosomal pH in CD8$^-$ DCs.

We also measured the endosomal pH in both cell populations with fluorescent dextrans and FACS-based approach on enriched CD11c$^+$ splenocytes. After 10 min of endocytosis in CD8$^+$ DCs, the endosomal pH was around 7 (Figure 1B, left). The pH in endosomes then acidified slowly, to reach values around 6.5 after 2 hr

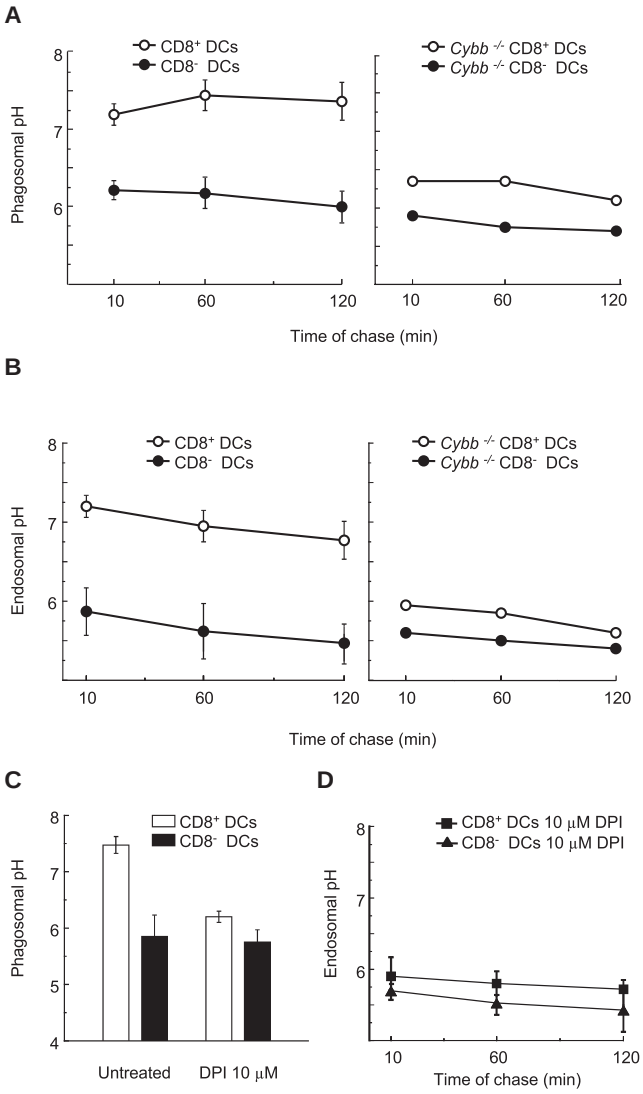



**Figure 1. Only CD8$^+$ DCs Alkalinize Their Phagosomes and Endosomes in a NOX2-Dependent Manner**

(A and B) Enriched WT (left) or *Cybb*$^{-/-}$ (right) CD11c$^+$ splenocytes were allowed to phagocytose latex beads bearing a mixture of pH-sensitive and -insensitive dyes (A) or to internalize a mix of fluorescent pH-sensitive and -insensitive 40 KD dextrans (B). After a pulse and different times of chase, the cells were stained with anti-CD8 and analyzed by FACS. pH values were determined for CD8$^+$ and CD8$^-$ splenic DCs populations by FACS as described in Experimental Procedures.

(C) Enriched CD11c$^+$ splenocytes were allowed to phagocytose latex beads bearing a mixture of pH-sensitive and -insensitive dyes in the presence or not of 10 μM DPI for 15 min and then extensively washed in cold PBS-BSA. Cells were resuspended in complete medium containing or not 10 μM DPI and chased for 45 min. After CD8 staining, the pH was determined by FACS as described in Experimental Procedures.

(D) Enriched CD11c$^+$ splenocytes were allowed to intrenalize a mixture of pH-sensitive and -insensitive 40KD dextrans in the presence or not of 10 μM DPI for 15 min. After washing, cells were chased for different times. After CD8 staining, the pH was determined by FACS as described in Experimental Procedures.

Data show mean ± SEM from triplicates values and are representative of three or more independent experiments.

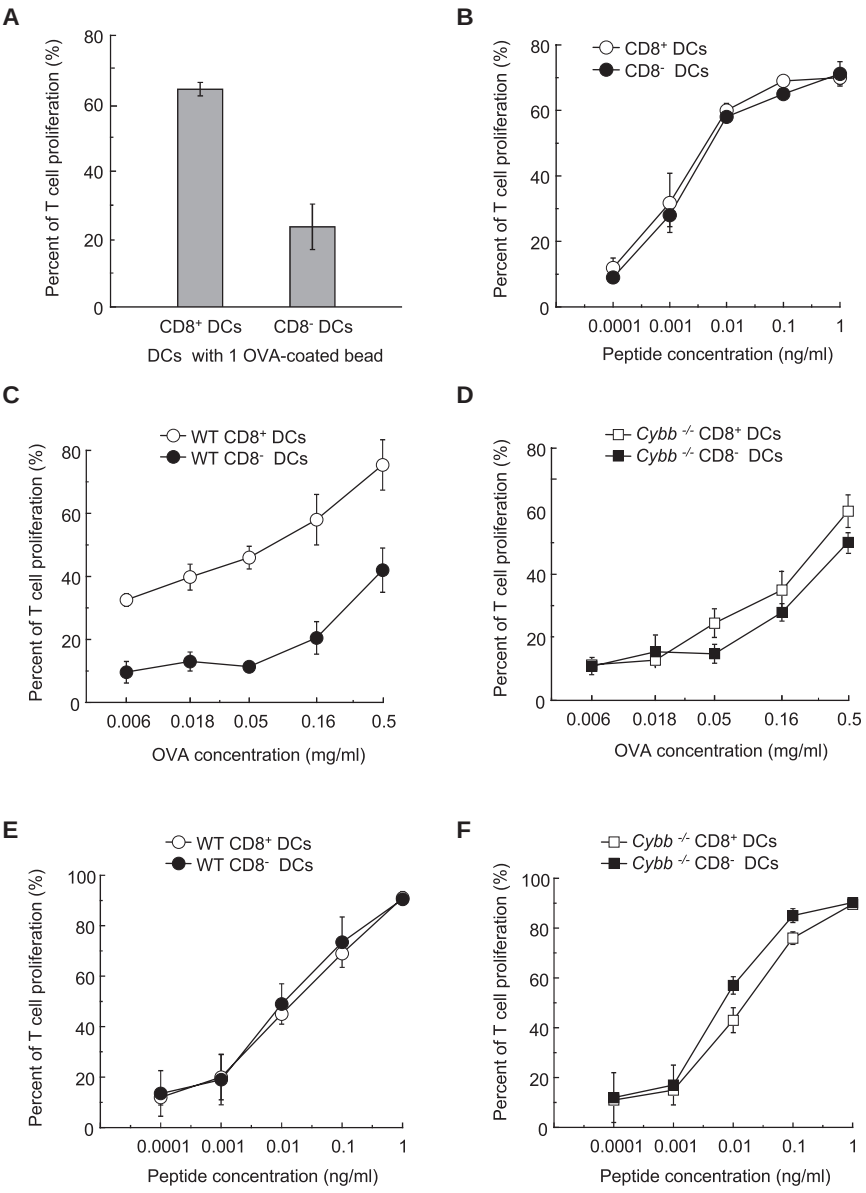



**Figure 2. Crosspresentation Ability of CD8$^+$ DCs Is NOX2 Dependent**

(A) CD8$^+$ and CD8$^-$ DCs having phagocytosed one single OVA-coated latex bead were sorted by FACS. Equal numbers of the two DC subpopulations were then incubated with CFSE-labeled OVA-specific transgenic T cells (OT-I) and crosspresentation was assessed through T cell proliferation (as evaluated by CFSE staining).

(B) A control of OVA peptide (SIINFEKL, the minimal peptide already processed) presentation was performed with the two DC populations after sorting.

(C and D) CD8$^+$ and CD8$^-$ DCs from WT mice (C) and *Cybb*$^{-/-}$ mice (D) were sorted and incubated with different concentrations of OVA for 45 min. After washing, cells were incubated with CFSE-labeled OVA-specific transgenic T cells (OT-I) and crosspresentation was assessed through T cell proliferation (as evaluated by CFSE staining).

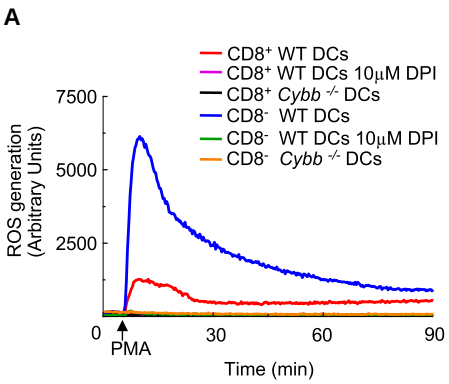
(E and F) Sorted CD8$^+$ and CD8$^-$ DCs from WT mice (E) and *Cybb*$^{-/-}$ mice (F) were incubated with different concentration peptide control for 45 min. After washing, cells were incubated with CFSE-labeled OVA-specific transgenic T cells (OT-I) and presentation of minimal peptide was assessed through T cell proliferation.

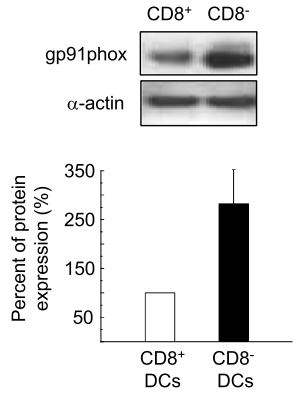
of internalization. In CD8$^-$ DCs, the pH after 10 min of endocytosis was already lower than 6, and acidified to reach 5.5 after 2 hr of endocytosis. In the DCs from NOX2-defecient mice (Figure 1B, right) or in WT DCs in the presence of DPI (Figure 1D), the endosomal pH in CD8$^+$ DCs was decreased to values similar to the ones found in CD8$^-$ DCs. Neither DPI, nor the absence of NOX2, affected the endosomal pH in CD8$^-$ DCs. We conclude that NOX2 alkalinizes phagosomes and endosomes selectively in CD8$^+$, but not in CD8$^-$, spleen DCs. In the absence of NOX2, both DC subpopulations display similar pH in their endocytic and phagocytic pathways.

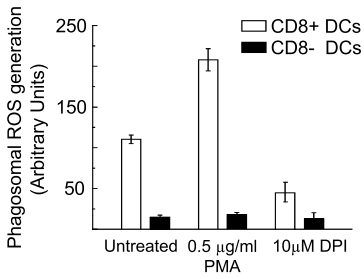
## CD8$^+$ DC Crosspresentation Capacity Is Lost in the Absence of NOX2 Activity

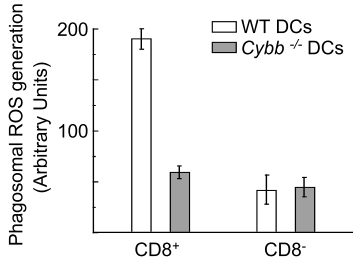
The high crosspresentation ability of CD8$^+$ DCs is due both to the phagocytic receptors they express (Iyoda et al., 2002; Schulz and Reis e Sousa, 2002) and to post-internalization differences in antigen processing (Dudziak et al., 2007; Schnorrer et al., 2006). CD8$^+$ and CD8$^-$ DCs that have phagocytosed one single ovalbumin-coated bead were FACS sorted (Figure S1 available online) and crosspresentation was assayed with OVA-specific OT-I T cells. As shown in Figure 2A, CD8$^+$ DCs crosspresented OVA more efficiently than did CD8$^-$ DCs, although both subpopulations presented the synthetic peptide with similar efficiencies (Figure 2B).

Because pH regulation and protein degradation are intimately related and considering that CD8$^+$ and CD8$^-$ DCs display different pH in their internalization pathways, we next analyzed proteolytic degradation in the two DC subpopulations. Degradation of OVA was followed in vitro on purified CD8$^+$ and CD8$^-$ DCs after different times of internalization via immunoblot analysis. As shown in Figures S2A and S2B, CD8$^-$ DCs degraded internalized OVA more efficiently than did CD8$^+$ DCs, suggesting that CD8$^+$ DCs display reduced activity of endolysosomal proteases, as compared to CD8$^-$ DCs. In order to measure the phagosomal proteolytic activity directly, phagosomes were isolated after 1 hr of phagocytosis from purified CD8$^+$ or CD8$^-$ DCs, and the activity of lysosomal enzymes at two different fixed pHs (7.4 and 6.5, the actual pH found in CD8$^+$ and CD8$^-$ phagosomes, respectively) was quantified with synthetic fluorescent substrates. As shown in Figure S2C via a mixture of substrates specific for cathepsins B-L, L, H, G, and asparagine endopeptidase (AEP), the proteolytic activity at pH 6.5 was higher than at pH 7.4 in both phagosomes from CD8$^+$ and CD8$^-$ DCs. As expected, the



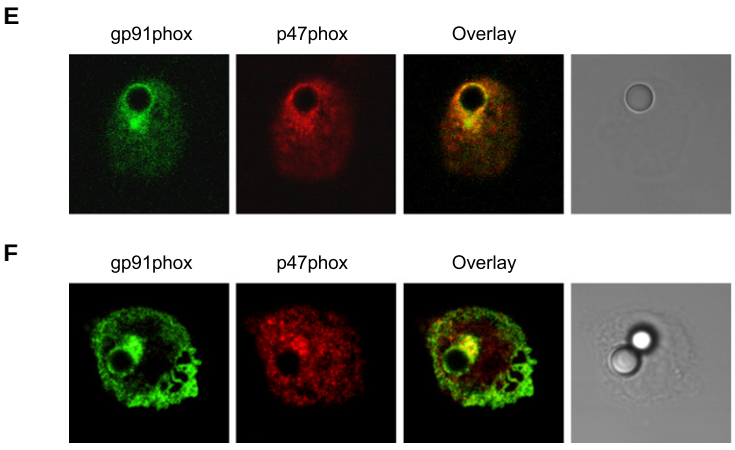




**Figure 3. NOX2 Activity Is Differentially Regulated in Spleen DC Subtypes**

(A) Total ROS production in purified DCs subpopulations from WT (pretreated or not with 10 μM DPI) or *Cybb*$^{-/-}$ mice upon stimulation with 0.5 μg/ml PMA was measured over time via luminol-amplified chemiluminiscence assay.

(B) gp91phox was detected in whole-cell lysates from purified CD8$^+$ and CD8$^-$ DCs by immunoblot. α-actin was used as a loading control. Densitometry quantification normalized as percentage of CD8$^+$ DC level of five different experiments is shown in the lower panel.

(C and D) Enriched CD11c$^+$ spleen DCs from WT mice (C) or gp91phox-deficient mice (D) were allowed to phagocytose latex beads coupled to dihydrorhodamine 123 (DHR) for a 15 min pulse and 45 min chase in the presence or absence of PMA . Phagosomal oxidation in CD8$^+$ and CD8$^-$ subtypes was detected by FACS as described in Experimental Procedures. A set of WT cells was pretreated with DPI as negative control. Data show mean ± SEM from triplicate values.

(E and F) gp91phox and p47phox were detected by immunofluorescence after 1 hr of phagocytosis in purified CD8$^+$ (E) and CD8$^-$ DCs (F) via confocal microscopy.

Data are representative of three or more independent experiments.

proteolytic activity in phagosomes is therefore strongly dependent on the pH. At the physiological pH values in phagosomes from CD8$^+$ (7.4) and CD8$^-$ (6.5) DCs (see the asterisk in Figure S2C), phagosomes from CD8$^-$ DCs showed stronger proteolytic activity than did phagosomes from CD8$^+$ DCs. The activity of cathepsin S (CatS), in contrast, was higher in phagosomes from CD8$^+$ than from CD8$^-$ DCs, especially at pH 7.4, the optimal pH for this enzyme (Claus et al., 1998) (Figure S2D). Consistently, the expression of CatS was also higher in CD8$^+$ than in CD8$^-$ DCs (Figures S2E and S2F). We conclude that the proteolytic activity in phagosomes from CD8$^+$ and CD8$^-$ DCs is regulated by the pH and that at their physiological pH, the overall proteolytic activity is superior in phagosomes from CD8$^-$ DCs, whereas the activity of CatS is greater in phagosomes from CD8$^+$ DCs.

In order to address whether the unique capacity of CD8$^+$ DCs for crosspresentation is due to their pH, we subsequently tested OVA crosspresentation in the different DC subsets from NOX2-deficient spleens. As expected, in WT mice, CD8$^+$ DCs crosspresented OVA much more efficiently than did CD8$^-$ DCs (Figure 2C). NOX2-deficient CD8$^+$ DCs, however, lost their capacity for OVA crosspresentation (Figure 2D), although the presentation of the minimal peptide was not affected (Figures 2E and 2F). The low levels of crosspresentation observed in CD8$^-$ DCs were also not affected by the absence of NOX2 activity (Figures 2C and 2D). Thus, efficient crosspresentation in CD8$^+$ DCs requires active NOX2. In the absence of NOX2, both DC subtypes crosspresent OVA with similar low efficiencies.

## Subcellular Control of NOX2 Assembly

In contrast to CD8$^+$ DCs, the absence of NOX2 activity in CD8$^-$ DCs affected neither the phagosomal pH nor crosspresentation. The simplest explanation for this lack of effect is that NOX2 is not expressed or not active in CD8$^-$ DCs. In order to address this possibility, we measured the total cellular ROS production by CD8$^+$ and CD8$^-$ DCs. We used the conventional luminol-based assay and PMA as stimulus, which induces strong ROS production. Surprisingly, total ROS production is much stronger in CD8$^-$ than in CD8$^+$ DCs (Figure 3A). Both DC subsets from *Cybb*$^{-/-}$ or DPI-treated WT DCs fail to show any activity, confirming that

ROS production is due to NOX2 (Figure 3A). Consistently, $CD8^-$ DCs express higher amounts of gp91phox, as detected by immunoblot (Figure 3B).

To measure the phagosomal production of ROS, we used dihydrorhodamine (DHR, a nonfluorescent dye that becomes fluorescent upon oxidation) bound to latex beads and FACS analysis, as described previously (Savina et al., 2006). ROS production in phagosomes followed a very different pattern. After 1 hr of phagocytosis, phagosomal ROS was undetectable in $CD8^-$ DCs, whereas effective ROS activity was found in phagosomes from $CD8^+$ DCs (Figure 3C). Treatment with PMA (which activates all cellular NOX2) increased ROS production in $CD8^+$ DC phagosomes, but it had no effect on phagosomal ROS in $CD8^-$ DCs, showing the inherent inability of this DCs subtype to produce ROS in the lumen of their phagosomes. As expected, ROS production in $CD8^+$ DC phagosomes was inhibited by DPI (Figure 3C). In $CD8^+$ DCs from *Cybb*$^{-/-}$ mice, phagosomal ROS generation was undetected, confirming that the oxidation of DHR in those phagosomes is due to the activity of NOX2. We conclude that in spite of the high amounts of NOX2 expression and total ROS production, $CD8^-$ DCs are not competent for ROS generation in phagosomes. $CD8^+$ DCs, in contrast, display relatively low total NOX2 activity, but strong phagosomal ROS production, as compared to $CD8^-$ DCs.

To analyze the mechanism of this difference in phagosomal ROS production, we analyze the recruitment of the cytosolic subunit p47phox to phagosomal membranes in the two DC subpopulations. Confocal immunofluorescence images revealed a clear labeling of phagosomal membranes for gp91phox and p47phox in $CD8^+$ DCs, indicating effective assembly of active NOX2 on phagosomes (Figure 3E). In $CD8^-$ DCs, gp91phox was present both at the plasma membrane and at the membrane of phagosomes (Figure 3F). Nevertheless, p47phox was not detected around phagosomes in $CD8^-$ DCs, indicating that $CD8^-$ DCs fail to assemble NOX2 at the membrane of phagosomes (Figure 3F). These results indicate that the lack of ROS production in phagosomes in $CD8^-$ DCs is due to their inability to assemble the cytosolic subunits of the NOX2 complexes on their phagosomal membranes.

## Rac1 and Rac2 Expression and Subcellular Distribution in $CD8^+$ and $CD8^-$ DCs

Why, then, do $CD8^-$ DCs fail to assemble NOX2 complexes on phagosomes? We showed previously that phagosomal NOX2 activity is dependent on Rab27a. However, in the absence of Rab27a, the total NOX2 activity was also decreased (Jancic et al., 2007). Even though the expression of Rab27a was slightly higher in $CD8^+$ DCs (Figures S3A and S3B), because of the high total NOX2 activity $CD8^-$ DCs (Figure 3A), a role for Rab27a in the differences observed between the two DC subpopulations is unlikely. We therefore turned to other potential regulators of NOX2 subcellular assembly. Rac proteins are essential for reconstituted oxidase activity in vitro and in vivo, as revealed initially by chronic granulomatous disease (CGD, where NOX2 activity is absent) in patients bearing mutations in Rac GTPases (Bokoch, 2005; Bokoch and Diebold, 2002; Williams et al., 2000). Previous reports suggested that the two main Rac isoforms, Rac1 and Rac2, may also influence the intracellular sites (phagosomes versus plasma membrane) of NOX2 assembly in neutrophils and macrophages (Michaelson et al., 2001; Werner, 2004; Yamauchi et al., 2004).

To determine a possible role for Rac proteins in the differential NOX2 assembly in $CD8^+$ and $CD8^-$ DCs, we first analyzed Rac expression. As shown in Figures 4A and 4B, $CD8^+$ DCs display higher total Rac expression, as detected with an antibody that recognizes both isoforms. Strikingly, a Rac1-specific antibody revealed similar expression in the two cell types (Figures 4A and 4B, right), suggesting that $CD8^+$ DCs express increased amounts of Rac2. Because all the Rac2 antibodies tested failed to reveal Rac2 in DCs extracts by immunoblot, we used intracellular FACS labeling. As shown in the fluorescence intensity histograms in Figure 4C, Rac2 labeling is higher in $CD8^+$ than in $CD8^-$ DCs. DCs from Rac2-deficient mice displayed very low amounts of fluorescence, controlling for the specificity of the labeling (Figures 4C and 4D). Intracellular FACS labeling with the antibodies specific for total Rac and for Rac1 confirmed that the total amounts of Rac are higher in $CD8^+$ DCs, whereas the expression of Rac1 is similar in the two cell types (Figure S4). These results show that the Rac2/Rac1 ratio is higher in $CD8^+$ than in $CD8^-$ DCs.

The subcellular distribution of the two Rac isoforms was also distinct in DCs after 1 hr of phagocytosis. Rac1 was detected on disperse intracellular structures, at the plasma membrane, and around phagosomes (Figure 4E) in both $CD8^+$ and $CD8^-$ DCs (the presence of Rac1 at the plasma membrane was more evident in $CD8^-$ DCs). The distribution of Rac2 in the two DC subpopulations, in contrast, was very different. In $CD8^+$ DCs, Rac2 strongly accumulated around phagosomes, whereas it shows a disperse distribution, and no phagosomal accumulation, in $CD8^-$ DCs (Figure 4F). We conclude that the two Rac isoforms are differentially expressed and distributed in $CD8^+$ and $CD8^-$ DCs. Rac2 concentrates selectively on $CD8^+$ DC-phagosomes, suggesting that it may somehow be involved in phagosomal function in this DC subpopulation.

## The Assembly and Activation of NOX2 in $CD8^+$ DC-Phagosomes Is Rac2 Dependent

In order to analyze the role of Rac2 in the assembly and activation of NOX2, $CD8^+$ and $CD8^-$ DCs were purified from Rac2-deficient mice. Rac2-deficient DCs were phenotypically identical to WT DCs (as judged by the surface expression of CD11c, CD80-86, and CD40, not shown). The total production of ROS was partially decreased in $CD8^-$ DCs, and slightly diminished in $CD8^+$ DCs, as compared to WT DCs (Figure 5A). In contrast, the phagosomal activity of NOX2, which was only detectable in WT $CD8^+$ DCs, was decreased to background expression in the absence of Rac2 (Figure 5B). Therefore, Rac2 is dispensable for total ROS production, but absolutely required for phagosomal ROS generation, suggesting that Rac2 may selectively control the phagosomal assembly of NOX2 in $CD8^+$ DCs.

We addressed this possibility by analyzing the subcellular distribution of gp91phox and p47phox by using confocal microscopy. Accumulation of gp91phox was observed around phagosomes in WT $CD8^+$ DCs (Figures 3E and 5C), with virtually no labeling at the plasma membrane. Consistent with the sustained production of ROS in $CD8^+$ DC phagosomes (Figure 3C), p47phox was effectively recruited to the phagosomal gp91phox (Figure 5C). In contrast, in Rac2-deficient $CD8^+$ DCs, gp91phox

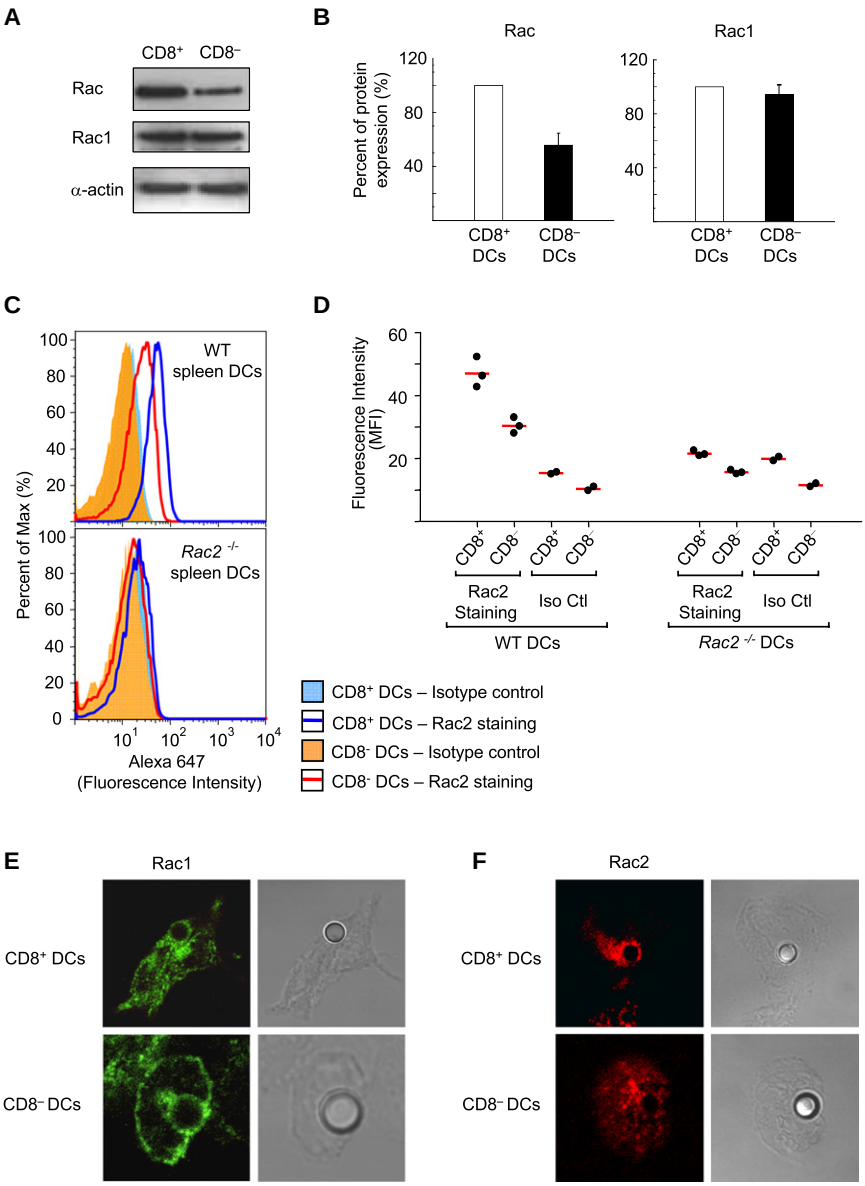



**Figure 4. Expression and Subcellular Localization of Rac Isoforms Vary between CD8$^+$ and CD8$^-$ DCs**

(A) Total Rac (top) and Rac1 (middle) were detected in whole-cell lysates from purified CD8$^+$ and CD8$^-$ DCs by immunoblot. α-actin was used as a loading control (bottom).

(B) Densitometry quantifications normalized as percentage of CD8$^+$ DC amounts in five different DC purifications are shown.

(C) Typical FACS profiles for Rac2 intracellular staining (and isotype antibody control) for each cell type from one DC purification are shown.

(D) Quantification of Rac2 (and isotype control) intracellular staining of CD11c$^+$ purified spleen cells from three different mice. Data are representative of three independent experiments.

(E and F) Confocal images of immunofluorescent detection of Rac1 (C) and Rac2 (D) on purified CD8$^+$ and CD8$^-$ DCs after 1 hr phagocytosis of latex beads are shown.

accumulated mainly at the plasma membrane and no labeling of p47phox around phagosomes was observed, even when several confocal planes were analyzed (Figure 5D). In Rac2-deficient CD8$^-$ DCs, we observed no differences, as compared to their WT counterparts, in the subcellular distribution of gp91phox and p47phox (Figure S5). We conclude that Rac2 controls the phagosomal assembly and activation of NOX2 complex selectively in CD8$^+$ DCs. In the absence of Rac2 (when only Rac1 is present), NOX2 assembly in phagosomes is not observed.

Interestingly, in the absence of any stimuli, gp91phox was mainly present at the plasma membrane in WT CD8$^-$ DCs and on disperse cytoplasmic structures in CD8$^+$ DCs. The distribution of these two NOX2 subunits was not affected in *Rac2*$^{-/-}$ CD8$^-$ DCs (Figure 5E, top), but a strong redistribution of gp91phox from the cytoplasm to the plasma membrane was observed in CD8$^+$ DCs lacking Rac2 (Figure 5E, bottom). Therefore, at the steady state, the distribution of gp91phox in Rac2-deficient CD8$^-$ and CD8$^+$ DCs was indistinguishable. As expected, p47phox remained mainly cytosolic in all cases, because there is almost no active NOX2 under these steady-state conditions. Together, these results show that Rac2 was essential to the phagosomal assembly and ROS production in CD8$^+$ DCs, whereas Rac1 directed the assembly of the complex to plasma membrane in both CD8$^-$ DCs and Rac2-deficient CD8$^+$ DCs.

## Rac2 Controls the Phagosomal pH and Crosspresentation in DCs

Contrary to our expectations, the phagosomal pH was only slightly decreased (by 0.5 pH units) in CD8$^+$ DCs (Figure S6A) or in bone marrow-derived DCs (not shown) from Rac2-deficient mice. To investigate the possible reasons of this low degree of acidification in spite of the absence of ROS production in phagosomes, we analyzed the expression of the V-ATPase in the Rac2-deficient DCs. Strikingly, the expression of the Vo membrane subunit of the V-ATPase, an essential player in phagosome acidification, was strongly decreased in *Rac2*$^{-/-}$ DCs (Figure S6B). Downmodulation of the V-ATPase could explain the low degree of phagosomal acidification observed in Rac2-deficient DCs, suggesting that CD8$^+$ DCs somehow compensate the absence of Rac2 expression by limiting acidification through decreased expression of the V-ATPase. Consistent with this observation, no important inhibition of crosspresentation was observed in CD8$^+$ DCs (Figure S6C) and BMDC (data not shown) from *Rac2*$^{-/-}$ mice.

We reasoned that if CD8$^+$ DCs indeed compensate the absence of Rac2 by decreasing the expression of the V-ATPase, the effect of reducing Rac2 expression directly in immature DCs should have stronger effects on the phagosomal pH and on crosspresentation. We therefore genetically targeted Rac2 by

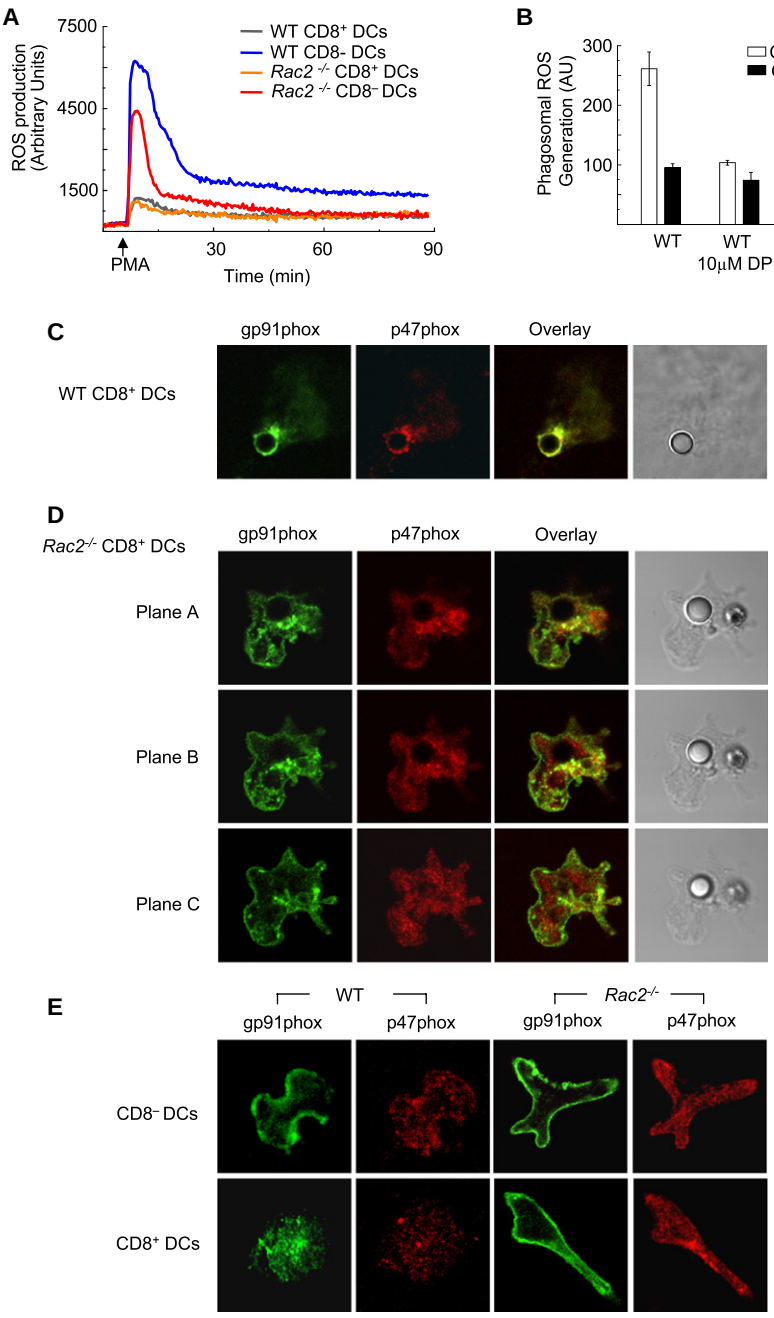



**Figure 5. Rac2 Controls the Phagosomal Activity of NOX2 in CD8$^+$ DCs**

(A) Total ROS production in purified DCs subpopulations from WT or *Rac2*$^{-/-}$ mice was evaluated during 90 min after PMA stimulation via a luminol-amplified chemiluminiscence assay.

(B) Enriched CD11c$^+$ spleen DCs from WT or Rac2-deficient mice were allowed to phagocytose DHR-coated beads for 15 min. After washing, the cells were chased for 45 min. Oxidation in phagosomes in CD8$^+$ and CD8$^-$ subtypes was detected by FACS as described in Experimental Procedures. A set of WT cells was pretreated with DPI as negative control. Data show mean ± SEM from triplicate values.

(C and D) gp91phox and p47phox were detected by immunofluorescence after 1 hr phagocytosis of latex beads on purified CD8$^+$ and CD8$^-$ DCs from WT (C) or CD8$^+$ *Rac2*$^{-/-}$ (D) mice. Three different confocal planes are depicted in (D).

(E) gp91phox and p47phox were detected by immunofluorescence in steady-state purified CD8$^+$ and CD8$^-$ DCs from WT or *Rac2*$^{-/-}$ mice.

Data are representative of three or more independent experiments.

using lentivirus-delivered short hairpin RNAs (shRNA). As shown in Figure 6A, two different shRNAs targeting Rac2 inhibit the expression of Rac2, as compared to DCs transduced with the scramble shRNA. Rac2 depletion caused a strong inhibition of ROS production in phagosomes (Figure 6B). Likewise, the phagosomal pH acidified strongly (by around 2 pH units) after 1 hr of phagocytosis, as compared to cells infected with the scrambled shRNA control lentivirus (Figure 6C). No inhibition of the expression of the V-ATPase was detected in the DCs infected with the Rac2 shRNA lentivirus, as compared to the control infected DCs (Figure S7A). Crosspresentation was assayed with latex beads coated with different ratios of OVA/BSA or with soluble OVA. As shown in Figure 6D, crosspresentation was strongly inhibited by the Rac2 shRNAs, as compared to the control shRNA. No effect of the shRNAs on the presentation of the peptide was observed (Figure 6E). In one of the experiments, the inhibition of Rac2 expression was partially achieved with one of the shRNAs (Rac2 # 1 construct; Figure S8A). The phagosomal pH and crosspresentation were only partially affected (Figures S8B–S8D) by this shRNA, indicating that the effects of the shRNAs on pH and crosspresentation are a direct consequence of the amount of Rac2 expression. In all cases, the phagocytic capacity of the infected DCs was not modified in absence of Rac2 (Figure S7B). Crosspresentation of soluble OVA was also defective in absence of Rac2 (Figure S9A). However, antigen presentation in the MHC class II context was not importantly affected (Figure S9B). We conclude that Rac2 controls ROS production in phagosomes and endosomes, thereby preventing effective acidification of endo-phagocytic pathway and promoting antigen crosspresentation.

## DISCUSSION

The functional organization of the phagocytic pathway is a characteristic of different phagocytes. Accordingly, macrophages, neutrophils, and DCs show quite different phagosomal pathways in terms of oxidation, pH, and degradation. Neutrophils and macrophages have highly cytotoxic and degradative phagocytic

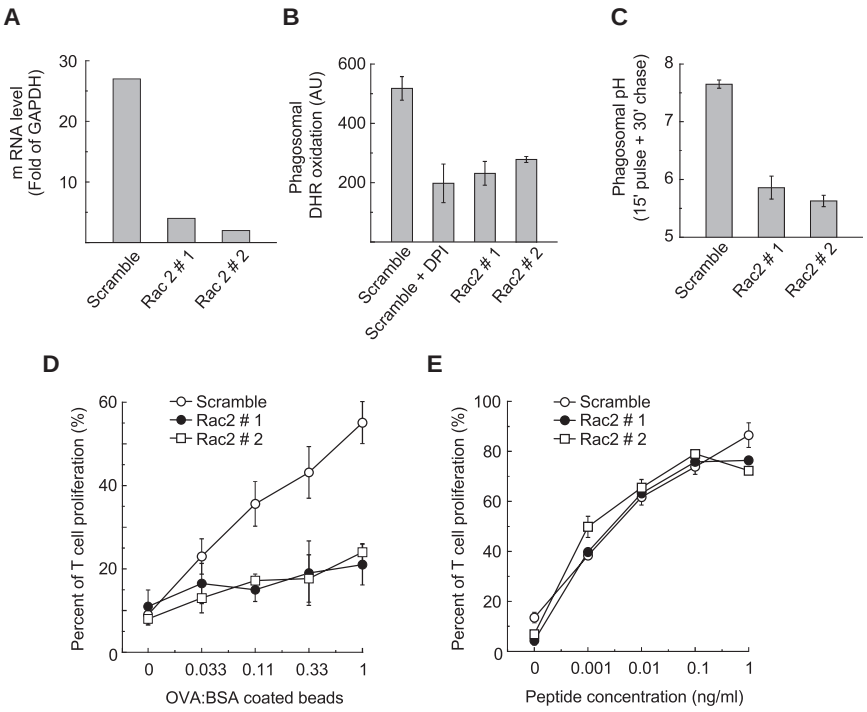



**Figure 6. Phagosomal pH and Crosspresentation Are Controlled by Rac2 in DCs**

(A) DCs were infected with three different lentivirus encoding a random sequence (Scramble) and two different shRNA targeted to Rac2 (Rac2 # 1 and Rac2 # 2). After selection, DCs were analyzed at day 7 by quantitative RT-PCR to evaluate the decreased expression of Rac2. Results are expressed as a ratio relative to GAPDH.

(B) Transduced DCs at day 7 were allowed to phagocytose DHR-coated beads for 15 min. After washing, the cells were chased for 45 min. Oxidation in phagosomes was detected by FACS as described in Experimental Procedures. A set of WT cells was pretreated with DPI as negative control. Data show mean ± SEM from triplicate values.

(C) Phagosomal pH of transduced DCs was measured by FACS after 15 min of pulse and 30 min of chase of latex beads coated with pH-sensitive and nonsensitive dyes. Data show mean ± SEM from triplicate values.

(D) Crosspresentation in transduced DCs was assayed via beads (dilution 1:200) coated with different ratios of OVA/BSA protein concentrations. T cell proliferation was evaluated by CFSE staining of OVA-specific transgenic T cells (OT-I).

(E) A control with OVA peptide was done with the same DCs. Data show mean ± SEM from triplicate values.

Data are representative of at least three independent lentivirus infections.

pathways, adapted to their role in the killing of microbes and clearance of apoptotic cells. DCs, in contrast, have a nonacidic, lowly degradative phagocytic pathway, most likely devoted at producing partial proteolytic products for immune recognition. The present study shows that different DC populations display different functional phagocytic organisations. Although both subpopulations display "mild" phagocytic pathways as compared to macrophages or neutrophils, $CD8^+$ DCs seem to "borrow" certain functional features from neutrophils, whereas the phagocytic pathway of $CD8^-$ DCs seems to be closer to macrophages' phagocytic pathways. The distinction between these two phagocytic organisations relies on Rac2: in the absence of Rac2, $CD8^+$ and $CD8^-$ DCs are very similar in terms of phagosomal acidification, ROS production, and crosspresentation.

The functional relevance of the existence of different myeloid DC subpopulations is still unclear. These subpopulations express different sets of receptors, including receptors for microorganisms (lectins, PRRs, including TLRs), cytokines and chemokine receptors, and receptors for dead cells (Edwards et al., 2003). The different subpopulations also produce different cytokines and chemokines and present antigens more or less efficiently to different types of T lymphocytes (Maldonado-Lopez et al., 2001). The heterogeneity in DC subpopulations therefore seems to correspond to a double functional purpose. First, because they express different phagocytic receptors, the two main spleen DC subpopulations take up different pathogens or, more generally, different types of particles. $CD8^+$ DCs, for example, phagocytose apoptotic cells much more efficiently than do $CD8^-$ DCs (Iyoda et al., 2002; Schulz and Reis e Sousa, 2002). *Leishmania*, as another example, infects $CD8^-$ DCs preferentially (Ritter et al., 2004). Second, because they express different TLRs or T cell costimulation molecules, and because they produce different cytokines, different DC populations may initiate different types of immune responses (Maldonado-Lopez et al., 1999). There is therefore no question that myeloid DC subpopulations may play selective roles in the initiation of adaptative immune responses.

The specialization of different subpopulations of DCs for antigen presentation was reported by several groups (Iyoda et al., 2002; Pooley et al., 2001; Schulz and Reis e Sousa, 2002), but direct evidence showing that their differential presentation abilities are a consequence of postinternalization events was published only recently. Indeed, Villadangos's group analyzed antigen presentation to $CD8^+$ and $CD4^+$ T cells by DCs from either subpopulation that had phagocytosed on a single antigen-coupled latex bead (the cells were FACS sorted). Even though all the DCs had internalized the same amount of antigen, $CD8^+$ DCs presented them more efficiently to $CD8^+$ T cells, whereas the opposite was found in $CD8^-$ DCs (Schnorrer et al., 2006). Dudziak et al. (2007) showed subsequently that in vivo targeting of ovalbumin to the two DC subpopulations with two different monoclonal antibodies also resulted in more efficient $CD8^+$ T cell activation by $CD8^+$ DCs and more efficient activation of $CD4^+$ T cells by $CD8^-$ DCs. Although this group showed that the overall expression of certain genes involved in MHC class I versus MHC class II-restricted antigen presentation were slightly overexpressed in the corresponding DC subpopulations, no evidence that these differences were responsible for the selectivity of antigen presentation were presented.

Our results show that the absence of expression of gp91phox or Rac2, both causing a loss of phagosomal ROS production in $CD8^+$ DCs, also abolish the high efficiency of antigen

crosspresentation to CD8$^+$ T cells. Importantly, both gp91phox and Rac2 deficiencies caused the endophagocytic pathway of CD8$^+$ DCs to resemble that of CD8$^-$ DCs (i.e., reduced ROS production and increased acidification). As a consequence, CD8$^+$ DCs failed to crosspresent antigens efficiently, indicating that high ROS production and low acidification in endosomes and phagosomes are required for efficient crosspresentation. These results establish that the functional organization of DCs' internalization pathway critically controls their crosspresentation capacities.

How do the two splenic DC subpopulations regulate the subcellular localization of ROS production? Rac proteins have previously been shown to participate in the assembly of the NADPH oxidase in neutrophils and macrophages (Bokoch and Zhao, 2006). In mammalian cells, three Rac isoforms have been identified: Rac1, Rac2, and Rac3. Rac1 and Rac3 are widely expressed whereas Rac2 expression is highly restricted to hematopoietic cells (Werner, 2004). In neutrophils, Rac-dependent NOX2 activation is selective for Rac2, even though these cells express both Rac1 and Rac2 at similar amounts as in mice (unlike human neutrophils, which express predominantly Rac2) (Werner, 2004). In contrast, in human and murine macrophages, Rac1 is the most abundant Rac isoform. Rac1 and Rac2, however, display extremely high amino acid identity (92%). The main difference between them is in the hypervariable C-terminal tail, which is believed to confer distinct localization of small GTPases and specificity for ROS production (Yamauchi et al., 2004). Indeed, previous reports showed that Rac1 has a preferential localization to the plasma membrane and Rac2 localizes mainly to intracellular membranes (Filippi et al., 2004; Michaelson et al., 2001; Yeung et al., 2008). In DCs, we observed that Rac1 was mainly present at the plasma membrane (with some faint labeling around phagosomes), whereas Rac2 was found exclusively on phagosomes.

Because it is well known that Rac1 is essential for the phagocytosis process and regulates the actin cytoskeleton (Greenberg, 1999; Niedergang and Chavrier, 2005), its presence around phagosomes in DCs is not surprising. The observation that Rac1 is present on CD8$^-$ DC phagosomes, which are incompetent to assemble and activate NOX2, suggests that this GTPase is not involved in phagosomal ROS production. Indeed, in Rac2-deficient DCs (which express normal amounts of Rac1), the phagosomal assembly of NOX2 was not observed, confirming that Rac1 fails to mediate the assembly NOX2 on phagosomes. In contrast, in Rac2-deficient DCs, the plasma membrane assembly of NOX2 was more evident than in the WT, suggesting that the two Rac isoforms somehow compete for the assembly at these two alternative subcellular locations. These experiments also show that CD8$^+$ DCs express all the machinery required for the plasma membrane assembly of NOX2. Nevertheless, it is also possible that the reason why CD8$^-$ DCs (which also express Rac2, although at lower amount) fail to assemble NOX2 on phagosomes is related to the expression of Rac effectors. The selective expression of Rac1-specific GEFs (such as β-Pix) (ten Klooster et al., 2006) or the lack of Rac2-specific effectors, GAPs, and/or GEFs (P-Rex1 and Vav1 are Rac GEFs that favor activation of Rac2 versus Rac1) (Dong et al., 2005; Ming et al., 2007; Welch et al., 2002) could contribute to NOX2 assembly at different sites. Indeed, we found that Vav1 expression is higher (by at least 2-fold) in CD8$^+$ than CD8$^-$ DCs (not shown). Accordingly, it has recently been shown that Vav is required for both maintaining the phagosomal pH in DCs and for OVA crosspresentation (Graham et al., 2007). It is also possible that under certain conditions, such as stimulation of particular TLRs, the activation of the two Rac isoforms is modified, inducing changes in the phagosomal fate and in crosspresentation. Such a regulation of the phagosomal function could explain why CD8$^-$ DCs can crosspresent antigens in vivo after injection of OVA immune complexes (Pooley et al., 2001). Nevertheless, our results show that after stimulation, CD8$^-$ DCs produce ROS at the plasma membrane, suggesting that extracellular ROS production may be involved in some other biological process, such as inflammation, as shown in macrophages (Forman and Torres, 2001).

Interestingly, all our observations for the regulation of the phagosomal pH are also valid for endosomes. This is quite surprising because phagocytosis is known to activate NOX2, but this was not the case for endocytosis (a constitutive process). Several different possibilities could account for these observations. First, DCs, in contrast to other phagocytes, could display low basal NOX2 assembly on endosomes. Indeed, we also showed that the NOX2 assembles on endosomes in human DCs (Mantegazza et al., 2008). Second, NOX2 activation could occur selectively on macropinosomes, which are known to form constitutively in DCs, and not in macrophages. Finally, very small LPS contamination in the dextrans (which, however, fail to cause complete DC activation) used to measure endosomal pH could eventually cause the activation of ROS.

The most direct, but not the only, explanation for the critical role of ROS production and the pH in crosspresentation, relates to the proteolytic activity in the endocytic and phagocytic pathways of DCs. Several groups showed previously that the proteolytic activity in DC-lysosomes (Trombetta et al., 2003) and -phagosomes (Lennon-Dumenil et al., 2002) is lower than in macrophages. Limited proteolysis is in part due to low levels of expression of certain proteolytic enzymes in DCs (Delamarre et al., 2005). Likewise, a recent paper shows slight differences in the levels of mRNA encoding several cathepsins in between CD8$^+$ and CD8$^-$ DCs (Dudziak et al., 2007). This is not, however, the case for all proteases, as shown by the fact that CatS is clearly overexpressed in CD8$^+$ DCs. Interestingly, and consistent with our pH measurements in DC phagosomes and endosomes, CatS has optimal activity at neutral pH, in contrast to many other lysosomal proteases. According to this, CD8$^+$ phagosomes displayed higher CatS activity than phagosomes from CD8$^-$ DCs, especially at 7.4. Overall, however, the proteolytic activity of the rest of proteases tested was lower in CD8$^+$ than in CD8$^-$ DCs, in part at least due to the pH differences. Taken together, our findings show that the differences in the phagocytic environment between CD8$^+$ and CD8$^-$ DCs are complex. Not only is the pH regulated differentially, but the relative abundance and the activity of different cathepsins are also selectively controlled. Whereas the phagosomes from CD8$^-$ DCs are somehow similar to phagosomes from macrophages (although probably less acidic and less degradative), phagosomes from CD8$^+$ DCs are very different, with low levels and low activity of most cathepsins, whereas CatS is both very abundant and very active at the high pH found in CD8$^+$ DC phagosomes. The observation that the

pH in phagosomes and endosomes in DCs specialized in crosspresentation is maintained at the optimal values for CatS, and not for other proteases, suggests that CatS is a critical player in antigen breakdown for crosspresentation (as already suggested by Shen et al. [2004]). Interestingly, the activity of this protease decreases with the reduction of the peptide length (Lutzner and Kalbacher, 2008), indicating that CatS is a self-limiting protease and may be specialized in the production of relatively long peptides. Besides the proteolytic activity, phagosomal ROS production and high pH may favor other intracellular steps involved in crosspresentation, such as peptide loading (if it can occur in phagosomes, which is still unclear) or antigen export to the cytosol.

This study provides evidence that the differentiation of myeloid DC subpopulations implicates specializations of their endophagocytic pathways. Such a degree of control of the internalization pathway suggests an evolutionary pressure on this particular function. If the main purpose of this phagocytic specialization was related to crosspresentation (which we do not know, but is suggested by our results), these results would support a critical role of crosspresentation in immunological defense against pathogens.

## EXPERIMENTAL PROCEDURES

### Mice

C57BL/6 mice (WT) and C57BL/6 recombination activating gene 1-deficient OT-1 TCR (Vα2, Vβ5) transgenic mice were obtained from Charles River Laboratories and Centre de Distribution, Typage et Archive Animal (Orleans, France). *Cybb*$^{-/-}$ mice (C57BL/6 background) were originally provided by M. Mallat (Hôpital de la Salpêtriere, Paris) and raised in the Curie Institute animal facility. *Rac2*$^{-/-}$ mice (C57BL/6 background) (Roberts et al., 1999) were provided by D. Williams (Cincinnati Children's Research Foundation, Cincinnati, OH) and V. Tybulewicz (National Institute for Medical Research, London, UK). All mice were used according to the guidelines and regulations of the French Veterinary Department.

### Crosspresentation Assay

For crosspresentation assays with soluble OVA (Worthington Biochemical Corporation), sorted DCs were incubated with different concentrations of soluble OVA or different concentrations of the minimal peptide as control of surface MHC class I amount. 45 min later, cells were washed three times with PBS plus 0.5%BSA and cocultured with CFSE-OT-I T cells for 3 days. For monitoring T cell proliferation, diminution of CFSE staining on TCR$^+$-CD8$^+$ population was measured by FACS. Alternatively, OVA-BSA-coated beads were prepared by attaching different ratios of OVA and BSA protein concentrations (OVA 10 mg ml$^{-1}$ alone; OVA 3.33 mg ml$^{-1}$/BSA 6.67 mg ml$^{-1}$; OVA 1.11 mg ml$^{-1}$/BSA 8.89 mg ml$^{-1}$; OVA 0.37 mg ml$^{-1}$/BSA 9.63 mg ml$^{-1}$; and BSA 10 mg ml$^{-1}$ alone) to 3 μm latex beads by passive adsorption in PBS at 4°C ON, and extensive washed in PBS. DCs were incubated with 3 μm OVA-BSA-coated latex beads or with the specific OVA peptide 257-264 (SIINFEKL) (Neosystem) for 45 min. After washing with PBS plus 0.5%BSA and cocultured with CFSE-OT-I T cells for 3 days, T cell proliferation was assayed by FACS.

For some experiments, OVA-Biotin was conjugated to streptavidin fluorescent latex beads (Streptavidin fluoresbrite YG Microspheres 1.0 micron from Polyscience, Inc.) as described elsewhere (Schnorrer et al., 2006). After isolation from spleens, enriched CD11c$^+$ cells were allowed to phagocytose OVA-Biotin-coated fluorescent latex beads for 15 min. After extensive washing, the cells were chased for 45 min more. Phagocytosis was stopped by adding cold PBS-BSA. The cells were then stained with anti-CD11c and anti-CD8 and subsequently sorted in CD8$^+$ and CD8$^-$ populations for cells that have phagocytosed one latex bead. Equal numbers of the two DC subpopulations were then incubated with CFSE-labeled OVA-specific transgenic T cells (OT-I) and crosspresentation was assessed through T cell proliferation (as evaluated by CFSE staining).

### Intracellular Staining

Purified CD11c$^+$ cells from spleens were staining with an FITC anti-CD8 at 4°C. After washing, the cells were treated for intracellular staining according to manufacturer procedures with the kit Foxp3 Fixation/Permeabilization concentrate and diluent (eBioscience). Anti-Rac, anti-Rac1, anti-Rac2, and isotype antibodies were used at 1.5 μg/ml and the secondary antibodies (Alexa 647 labeled) at 1 μg/ml. The cells were subsequently analyzed by FACS.

### Immunofluorescence Assay

For the detection of Rac1, Rac2, gp91phox, and p47phox, purified DCs were pulsed or not with latex beads (Polysciences, Inc) (dilution 1:100) for 15 min at 37°C. After extensive washing with cold PBS, the cells were placed on poly-L-lysine-coated glass coverslips at 37°C in an atmosphere of 5% $CO_2$ during a short period. Complete medium was added and cells were chased for another 45 min. After washing, cells were fixed with 2% paraformaldehyde for 10 min at 4°C and quenched by adding 0.1 M glycine. Cells were permeabilized in PBS-0.05% saponin-0.2% BSA for 20 min, washed, and then incubated with the primary and secondary antibodies. After labeling and extensive washing with PBS-0.05% saponin-0.2% BSA, the coverslips were mounted with Fluoromount G and the cells were analyzed by confocal microscope (Zeiss confocal microscope [LSM Axivert 720], 63 3 1.4 NA oil immersion objective). For z-stack acquisition, several images (step 0.3 μm) to cover most of the cell volume were acquired.

### pH Measurement

pH in phagosomes was determined as described previously (Savina et al., 2006). In brief, 3 μm polybeads amino were covalently coupled with 1 mg/ml FITC (pH sensitive) and 1 mg/ml FluoProbes 647 (pH insensitive) in the presence of sodium hydrogen carbonate buffer at pH 8 for 2 hr at room temperature. After extensively washing with glycine 100 mM, the beads were suspended in PBS. Enriched CD11c$^+$ spleen DCs were pulsed in a small volume $CO_2$-independent medium without serum with the coupled beads for 15 min in a water bath at 37°C and then extensively washed (2 times) in a large volume of cold PBS-0.5% BSA. In each wash, the cells were centrifuged at 1000 rpm for 4 min to pull down only the cells avoiding the no internalized beads. After washing, the cells were resuspended in complete medium incubated at 37°C ("chased") in the incubator for the indicated times and immediately placed on ice. CD8α (1 μg/ml) staining was done and then the cells were analyzed by FACS, via a gating FSC-CD8α$^+$ selective for cells that have phagocytosed one latex bead. The ratio of the mean fluorescence intensity (MFI) emission between the two dyes was determined. Values were compared with a standard curve obtained by resuspending the cells that had phagocytosed beads for 30 min at fixed pH (ranging from pH 5.5 to 8) and containing 0.1% Triton X-100 for 8 min. The cells were immediately analyzed by FACS, to determine the emission ratio of the two fluorescent probes at each pH. Alternatively, phagosomal pH was measured in Rac2-depleted BMDC. For this purpose, after 15 min pulse and 30 min chase, infected BMDC were placed on ice and stained for CD11c. The FACS analysis for pH measurement was done gating CD11c$^+$ cells.

pH in endosomes was determined as follows. Enriched CD11c$^+$ spleen DCs were pulsed in a small volume $CO_2$-independent medium containing a mix of 40,000 MW Dextran fluorescein (5 mg/ml) and 40,000 MW Dextran Alexa 647 (5 mg/ml) for 7 min in a water bath at 37°C and then extensively washed in a large volume of cold PBS-0.5% BSA. After washing, the cells were resuspended in complete medium incubated at 37°C ("chased") in the incubator for the indicated times and immediately placed on ice. To determine the endocyting population, a control at 4°C during pulse and chase was done. CD8α (1 μg/ml) staining was done and then the cells were analyzed by FACS, via a gating FSC-CD8α$^+$ selective for cells that have internalized dextrans. The ratio of the mean fluorescence intensity (MFI) emission between the two dyes was determined. Values were compared with a standard curve obtained by resuspending the cells that had phagocytosed beads for 30 min at fixed pH (ranging from pH 5.5 to 8) and containing 0.1% Triton X-100 for 8 min. The cells were immediately analyzed by FACS to determine the emission ratio of the two fluorescent probes at each pH.

### Analysis of NOX2 Activity

#### Detection of Phagosomal ROS Production via a Fluorescent Probe

3 μm polybeads amino were covalently coupled with DHR (1 mg/ml) and FluoProbes 647 (1 mg/ml) in the presence of sodium hydrogen carbonate buffer at pH 8 for 2 hr at room temperature. After extensively washing with glycine 100 mM, beads were resuspended in PBS. Enriched $CD11c^+$ spleen DCs were pulsed in a small volume $CO_2$-independent medium containing DHR-coupled beads during 15 min and then extensively washed in a large volume of cold PBS-0.5%BSA. In each wash, the cells were centrifuged at 1000 rpm for 5 min. After washing, the cells were resuspended in complete medium incubated at 37°C ("chased") in the incubator for the indicated times and immediately placed on ice. CD8α (1 μg/ml) staining was done and then the cells were analyzed by FACS, via a gating FSC/$CD8^+$ selective for cells that have phagocytosed one latex bead. Because mean fluorescence intensity (MFI) of FluoProbes 647 is constant over time, only the variation of DHR MFI was determined. For DPI assay, cells were incubated in the presence of 5 μM DPI for 30 min before and during the 15 min pulse.

Alternatively, phagosomal oxidation was measured in Rac2-depleted BMDC. For this purpose, after 15 min pulse and 30 min chase with DHR/Fluoprobes 647-coated beads, infected BMDC were placed on ice and stained for CD11c. The FACS analysis was done gating $CD11c^+$ cells.

#### Measurement of Total NOX2 Activity by Chemiluminiscence

$5 \times 10^5$ purified $CD8^+$ DCs and $CD8^-$ DCs were resuspended in 200 μl of $CO_2$-independent medium containing 10 μM luminol (the probe which releases energy as visible light upon oxidation) and 5 units of horseradish peroxidase. Cell suspensions were preheated to 37°C in the thermostated chamber of the luminometer (Berthold Centro LB 960) and allowed to stabilize. Changes in chemiluminiscence were measured over the indicated times. An injection of 0.5 μg/ml PMA after 5 min was used as stimulus.

### Lentiviral shRNA Knock Down of Rac2

#### Generation and Titer of Lentivirus

Plasmids encoding lentiviruses expressing shRNAs were obtained from the library of The RNAi Consortium (TRC) (Moffat et al., 2006). Plamids were purified with the QiaPrep miniprep kit (QIAGEN, Valenica, CA). Plasmids were then transfected into HEK293T cells with a three-plasmid system to produce lentivirus with a very high titer of $\sim 10^7$ CFU/ml (Moffat et al., 2006; Naldini et al., 1996). We initially tested five different hairpins directed against Rac2 and compared their efficiency by RT-PCR against a control hairpin (a scramble sequence against GFP). We found Rac2#2 (target sequence GCCAAGT GGTTCCCTGAGGTA) and Rac2#1 (target sequence GATTCAGTCAAGTAC TTGGAA) to be the most efficient.

#### Dendritic Cell Infection

Mouse bone marrow cells were plated on a 96-well plate (round bottom) at a concentration of $10^5$ cells per well with 200 μl of GM-CSF medium. After 48 hr, the medium was carefully removed without disturbing the cells at the bottom. We added 10 μl of virus and the pellet was resuspended 3 to 5 times. 40 μl of polyB in GM-CSF medium were added (1:1000 final concentration) and we centrifuged the plate at 2200 rpm for 90 min at 37°C. After the centrifugation, all medium was removed and 200 μl/well of fresh GM-CSF medium were added. The plates were incubated for 48 hr and then the cells were selected with 5 μg/ml of puromycin. Cells were collected for analysis 72 hr after selection.

## SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures and nine figures and can be found with this article online at http://www.immunity.com/supplemental/S1074-7613(09)00137-X.

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