DCs serve a critical role in microbial and tumor immunity by presenting exogenous antigens on MHC I to elicit CTL responses, a process that is referred to as cross-presentation. Although the importance of cross-presentation for efficient priming of CTL responses has been recognized for >30 yr (1), the signal transduction pathways that regulate cross-presentation in DCs remain to be elucidated.

DCs have developed several specialized mechanisms of antigen processing that promote cross-presentation. Whereas soluble antigens are internalized by constitutive macropinocytosis, uptake of particulate antigens, such as dying cells and microbes, requires receptor-mediated phagocytosis. Numerous receptors expressed on DCs are implicated in phagocytic uptake of particulates that include complement receptors, FcRs, and scavenger receptors (2). In this context, the results of our previous studies implicated Vav family guanine nucleotide exchange factors (GEFs) in the uptake of particulates (3). After antigen uptake, cross-presentation involves the processing of antigen and loading onto MHC I, which can proceed via several distinct pathways (4–7). For example, soluble antigens taken up by macropinocytosis are thought to enter the endosomal pathway, and they can be processed and loaded onto MHC I in a TAP- and proteasome-independent manner, whereas particulate antigens taken up by phagocytosis enter the phagolysosomal pathway (4, 8). Recent reports also suggest that fusion of phagosomes with the ER may be involved in the loading of antigenic peptides onto MHC I in a TAP-dependent manner (9–13). In addition, phagosome maturation and antigen processing may also be regulated by TLR-mediated pathways (14, 15).

A recent study implicated a role for NOX2 and reactive oxygen species (ROS) production in antigen processing in the early phagosomal compartment during cross-presentation by DCs (16).
However, the mechanism of NOX2 activation in DCs has not been elucidated, and which receptors and signaling intermediates regulate ROS production in DCs remains unclear. In this regard, recent reports indicate that ROS production in neutrophils is largely dependent on immunoreceptor tyrosine-based activation motif (ITAM)–mediated signaling pathways triggered by DAP12- and FcRγ-associated receptors (17, 18). Thus, similar to ITAM-mediated antigen receptor signaling in T and B lymphocytes, ITAM signaling in myeloid cells involves phosphorylation of conserved ITAM tyrosine residues by Src family kinases providing docking sites for the tandem SH2 domains of Syk family kinases (for review see references [19, 20]). These observations are notable, as they raise the possibility that ITAM-dependent mechanisms may also be involved in regulation of ROS production and antigen presentation in DCs. In this regard, recently published works indicate the importance of Vav in ROS production and oxidative burst in macrophages and neutrophils (21–23); however, it is not known if Vav and/or DAP12 and FcRγ are involved in the regulation of ROS production, or antigen processing and presentation, in DCs.

We present evidence that Vav GEFs link DAP12 and FcRγ ITAM–containing adaptors with antigen processing and cross-presentation in DCs via a mechanism that is dependent, in part, on Nox2-dependent ROS generation.

RESULTS
Defective cross-presentation of particulate antigens by Vav<sup>NULL</sup> dendritic cells
One of the critical functions of DCs is to prime CTL responses by cross-presentation of exogenous antigens, such as pathogen-infected or dying cells. Given the results of our previous studies, which implicated Vav family GEFs in the uptake of particulates (3), we sought to determine if Vav proteins were involved in antigen uptake and/or processing by DCs. To test the requirement for Vav proteins (Vav1, Vav2, and Vav3) in cross-presentation of MHC I–associated antigens to CD8 T cells by DCs, we used mice lacking the entire Vav family (Vav<sup>NULL</sup>) (24). Bone marrow–derived DCs from wild-type and Vav<sup>NULL</sup> mice were cultured with either OVA–peptide (spanning the OT-1 T cell epitope SIINFEKL) or OVA protein. DCs were then cocultured with purified OT1 TCR-transgenic CD8<sup>+</sup> T cells, and OT-1 T cell proliferation was monitored by CFSE dye-dilution assay (Fig. 1 A). As a control, purified OT1 T cells were incubated with OVA–peptide in the absence of any exogenously added DCs, and no T cell proliferation was observed under these conditions (unpublished data).

Similar to WT DCs, Vav<sup>NULL</sup> DCs cultured with OVA–peptide efficiently induced OT-1 T cell proliferation over a broad range of peptide concentrations (Fig. 1 B). Consistent with this observation, Vav<sup>NULL</sup> DCs showed normal morphology and cell surface marker expression, including that of costimulatory molecules before and after maturation with LPS (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20071283/DC1, and not depicted). Given that Vav<sup>NULL</sup> DCs were capable of inducing T cell proliferation in response to exogenously added antigenic peptides, we next examined whether or not Vav is required for processing and presentation of whole OVA protein. Notably, both WT and Vav<sup>NULL</sup> DCs presented soluble OVA protein to OT-1 T cells with the same efficiency (Fig. 1). As a control, OT-1 T cells were stimulated with OVA protein in the presence of fixed DCs, and no T cell proliferation was observed at concentrations inducing maximal proliferation with live DCs (unpublished data). Collectively, these experiments demonstrate that Vav proteins are not required for the presentation of peptides or intracellular processing of soluble protein antigens by the class I MHC pathway in DCs.

To determine if Vav is required for processing and presentation of particulate antigens to CTLs by DCs, we loaded WT and Vav<sup>NULL</sup> DCs with latex beads coupled to OVA

Figure 1. Vav is required for cross-presentation of particulate, but not soluble, antigen. (A) WT and Vav<sup>NULL</sup> BMDCs were cultured with CFSE-labeled OT-1 T cells and the indicated antigens for 72 h. T cell proliferation was determined by CFSE dye dilution and FACS analysis. (B) T cell proliferation was determined as in A and plotted as antigen dose versus the percentage of T cells that had undergone at least one division.
protein and examined their ability to elicit proliferation of OT-1 T cells. In striking contrast to WT DCs, which induced vigorous T cell proliferation, Vav\textsuperscript{NULL} DCs loaded with bead-coupled OVA completely lacked the ability to induce proliferation of OT-1 T cells (Fig. 1). Importantly, Vav\textsuperscript{NULL} and WT DCs cultured with OT-1 T cells and OVA beads (or OVA peptide) showed similar levels of expression of costimulatory molecules, including B7-1 and -2, indicating that their maturation state was similar (Fig. S2).

To examine presentation of a more physiologically relevant form of particulate antigen, we cultured DCs with heat-killed \textit{Listeria monocytogenes} expressing OVA (LM-OVA) and monitored OT-1-T cell proliferation. In contrast to WT DCs, Vav\textsuperscript{NULL} DCs lacked the ability to induce OT-1 T cell responses to bacteria-associated antigens such as LM-OVA (Fig. 1). Therefore, given that Vav\textsuperscript{NULL} DCs could efficiently present antigenic peptides and soluble protein to naive T cells (Fig. 1 and not depicted), our results indicate a selective requirement in uptake and/or processing of particulate antigens by DCs.

Selective defects in antigen uptake by Vav\textsuperscript{NULL} DCs

Dendritic cells internalize antigens by macrophagocytosis and phagocytosis. Previous studies showed that DCs undergo constitutive macropinocytosis to sample the environment and efficiently internalize soluble protein antigens (2, 25, 26). To determine if Vav GEFs are required in this process, we tested the efficiency of macropinocytosis in Vav\textsuperscript{NULL} DCs. These experiments showed that both WT and Vav\textsuperscript{NULL} DCs were equally efficient at internalizing 70-kD dextran-FITC at various time points, indicating that constitutive macropinocytosis in DCs does not require Vav proteins (Fig. 2 A). Moreover, we observed no differences in endosomal loading with Dextran-FITC by single-cell imaging (Fig. 2 B).

To examine internalization of particulate antigens, we next analyzed the ability of Vav\textsuperscript{NULL} DCs to phagocytose latex beads or heat-killed bacteria labeled with fluorescent dyes at different time points using confocal microscopy and FACS-based assays (Fig. 3, A and B). These experiments revealed that the kinetics of bead uptake by Vav\textsuperscript{NULL} DCs were delayed (Fig. 3 C). During a 15-min incubation with beads, \sim 30\% fewer Vav\textsuperscript{NULL} DCs internalized beads as compared with WT. However, by 60 min, uptake of beads was indistinguishable between Vav\textsuperscript{NULL} and WT DCs (Fig. 3 C). In contrast, the ability of WT and Vav\textsuperscript{NULL} DCs to internalize bacteria such as heat-killed LM was identical (Fig. 3 D). Thus, although a delay in bead internalization could conceivably contribute to defects in presentation of bead-linked OVA antigen by Vav\textsuperscript{NULL} DCs, normal intake but defective presentation of LM-OVA indicates that Vav is required for intracellular processing of physiological particulate antigens.

Critical function of Vav in the regulation of NOX2 and ROS production in DCs

A recent study implicated a role for NOX2 and ROS production in antigen processing in the early phagosomal compartment during cross-presentation by DCs (16). However, it remains unclear which signaling pathways regulate NOX2 activation in DCs during antigen processing and presentation. Previously published work indicated the importance of Vav in ROS production and oxidative burst in macrophages and neutrophils (21–23), suggesting that Vav may also be required for the regulation of NOX2 and ROS production by DCs.

To address this issue, we directly compared the generation of ROS in WT, NOX2-deficient, and Vav\textsuperscript{NULL} DCs incubated with latex beads. In contrast to WT, NOX2-deficient DCs failed to produce ROS, indicating that NOX2 is the major source of ROS generated by DCs in these assays (Fig. 4). Strikingly, Vav\textsuperscript{NULL} DCs exhibited defects in ROS production that were comparable in magnitude to NOX2-deficient DCs (Fig. 4 A). These results suggest that Vav is required for NOX2 induction and ROS production by DCs undergoing phagocytosis of latex beads. In addition, we tested the requirement of Vav in ROS production induced by various adhesion-dependent stimuli (17, 18). In contrast to WT DCs, which showed robust ROS production, Vav\textsuperscript{NULL} DCs failed to produce any detectable ROS upon adhesion to fibronectin or stimulation with LPS, peptidoglycan, or zymosan (Fig. 4, B–E). Importantly, Vav\textsuperscript{NULL}, but not NOX2-deficient, DCs responded to PMA with robust ROS production (Fig. 4 E, inset, and not depicted). Thus, collectively, these experiments indicate that although Vav\textsuperscript{NULL} DCs express all essential components of the NOX2 complex and can generate ROS in DCs.
response to phorbol esters, Vav proteins are required for NOX2 activation and ROS production in response to adhesion-dependent stimuli. Furthermore, our data also suggest that Vav-dependent ROS production is critical for cross-presentation of particulate antigens.

Given that Vav^NULL and NOX2-deficient DCs both failed to generate detectable levels of ROS in response to adhesion-mediated stimuli, we sought to examine the efficiency of cross-presentation in NOX2-deficient DCs using the same OT-1 T cell-based assay described in Fig. 1. These experiments showed that, consistent with previous studies (16), the ability of NOX2-deficient DCs to cross-present bead-linked OVA was diminished, as indicated by reduced OT-1 T cell proliferation, whereas NOX2 was not required for the induction of OT-1 T cell responses by DCs pulsed with soluble antigens such as OVA-peptide or protein (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20071283/DC1, and not depicted). As expected, defective presentation of particulate antigen in NOX2-deficient DCs was not caused by inefficient uptake of OVA-beads, as we observed no defects in bead-phagocytosis in these cells (Fig. S4), which is in agreement with a previous study (16). We note, however, that cross-presentation defects of NOX2^-/- DCs were relatively mild, as compared with Vav^NULL DCs, which showed a complete block in cross-presentation of particulates. We interpret these results as indicating that Vav proteins regulate cross-presentation via both ROS-dependent and -independent mechanisms.

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Given the strict requirement for Vav in the generation of ROS by DCs, we hypothesized that Vav^NULL DCs, similar to NOX2-deficient DCs, could exhibit a reduction in phagosomal pH caused by the loss of neutralizing activity of ROS. To measure phagosomal pH, WT and Vav^NULL DCs were loaded with latex beads covalently coupled to pH-sensitive
FITC and pH-insensitive Alexa Fluor 647. After phagocytosis of beads, cells were washed, cultured for an additional 1–4 h, and analyzed by FACS for FITC and Alexa Fluor 647 fluorescence. In these experiments, the relative fluorescence intensity of FITC versus Alexa Fluor 647 remained constant in WT DCs, indicating a neutral pH environment of phagocytosed beads; however, phagosomes of Vav ^{null} DCs showed significantly lower ratios of FITC to Alexa Fluor 647 fluorescence over time, indicating a more acidified environment in Vav ^{null} phagosomes (Fig. 5 A). Given that the acidic environment of phagosomes would be predicted to lead to increased activation of pH-sensitive proteases, such as cathepsins, we decided to test if Vav ^{null} DCs show more rapid degradation of particulate antigen than WT DCs. To this end, we loaded DCs with latex beads covalently coupled to OVA protein, as described above. After washing, DCs were cultured for an additional hour before recovering beads by lysis and quantifying OVA remaining on the beads by staining with polyclonal OVA-specific antibodies and FACS analysis. These experiments showed that beads recovered from Vav ^{null} DCs showed decreased fluorescence intensity, as compared with beads from WT DCs, indicating diminished OVA content (Fig. 5 B). We interpret these experiments as indicating that Vav is involved in the regulation of phagosomal pH and antigen degradation. We note, however, that a substantial amount of OVA remained intact in phagosomes derived from Vav ^{null} DCs. Given the profound defects in cross-presentation observed in Vav ^{null} DCs, as compared with NOX2-deficient DCs, it is possible that Vav also controls cross-presentation by mechanisms distinct from ROS production.

Defective cross-presentation of particulate antigens by DAP12/FcR ^{y} dendritic cells

Previous studies indicated that, in neutrophils, ROS production in response to various adhesion-dependent stimuli requires ITAM-containing DAP12 and FcR ^{y} adaptors (17, 19). Given that Vav proteins have been implicated in control of ITAM-mediated signaling pathways in several hematopoietic lineages, including T, B, and NK cells (27), we hypothesized that Vav would link ROS production in DCs to ITAM-dependent signaling by DAP12 and FcR ^{y}, which is triggered by integrin receptors in neutrophils (17, 19). Consistent with such a scenario, we found that Vav was inducibly tyrosine phosphorylated in DCs upon stimulation by adhesion to the integrin ligand fibrinogen (Fig. 6 A), a process that is critically dependent on the function of DAP12 and FcR ^{y} ITAMs (17, 19). To confirm that, under these conditions, tyrosine phosphorylation of Vav was, indeed, dependent on signals emanating from DAP12 and FcR ^{y}, we used DCs from mice deficient in DAP12 and FcR ^{y}. Strikingly, we found in these experiments that tyrosine phosphorylation of Vav was drastically diminished in DAP12 and FcR ^{y} DCs, which is consistent with the uncoupling of Vav from adhesion-induced signaling pathways in the absence of DAP12 and FcR ^{y} (Fig. 6 A). Moreover, both Vav ^{null} and DAP12 and FcR ^{y} DCs showed diminished ERK activation in response to integrin-mediated adhesion (Fig. 6 B and not depicted). Thus, it appears that Vav is involved in transduction of signals that emanate from DAP12 and FcR ^{y} adaptors in DCs.

Given the recently published work indicating a critical role for DAP12 and FcR ^{y} adaptors in NOX2 activation and ROS production in neutrophils (17), we tested the ability of DAP12 and FcR ^{y} DCs to generate ROS in response to multiple adhesion-dependent stimuli, including latex beads, LM, and LPS (Fig. 6 C). Strikingly, DAP12 and FcR ^{y} DCs completely lacked the ability to generate ROS under these conditions (Fig. 6 C). We note that the total loss of detectable ROS production in DAP12 and FcR ^{y} DCs mirrored the loss of ROS we observed in Vav ^{null} and NOX2-deficient DCs (Fig. 4 and not depicted). Thus, taking into consideration that Vav and NOX2 are critical for cross-presentation of particulate antigens in DCs, these results suggested an intriguing possibility that a DAP12/FcR ^{y} ITAM-based signaling pathway

Figure 5. Vav controls phagosomal pH and antigen degradation. (A) Phagosomal pH was measured in BMDCs using latex beads coupled with pH-sensitive FITC and pH-insensitive Alexa Fluor 647. DCs were loaded with beads for 30 min, washed, and cultured for the indicated time points before analysis by FACS. For analysis, cells that had internalized equal numbers of beads (based on Alexa Fluor 647 MFI) were gated and analyzed for FITC MFI. Data represent the relative fold change in FITC MFI over time. (B) Antigen degradation in phagosomes was monitored using latex beads covalently coupled to OVA. DCs were loaded with beads for 30 min, washed, and cultured for the indicated time points. Beads were then recovered by lysing the cells and stained with anti-OVA antibodies before FACS analysis.
Uptake of soluble antigens by macropinocytosis has been examined in DCs expressing dominant-negative forms of the Rho family GTPases Rac and Cdc42, both of which were implicated in macropinocytosis (25, 26); however, Rac1/2-deficient DCs have not been examined for macropinocytic activity (28). Notably, Vav has been implicated in activation of both Rac and Cdc42 by GTP exchange (29), although we find that Vav is not required for macropinocytosis in DCs. Consistent with this observation, Vav is also dispensable for cross-presentation of soluble antigen.

In contrast to soluble antigen, the uptake of particulate antigens, such as dying cells and microbes, requires receptor-mediated phagocytosis. Numerous receptors expressed on DCs can mediate phagocytosis, including complement receptors (CRs), FcRs, and scavenger receptors (2). Previously published studies implicated Vav proteins in regulating phagocytosis downstream of CRs and FcRs in macrophages and neutrophils, respectively (3, 22, 30). Consistent with a role for Vav in phagocytosis, we observed a moderate delay in the kinetics of latex bead-uptake by Vav-null DCs, although it is not clear which receptor pathways may be involved in this process. In contrast, phagocytosis of bacteria was unaffected in the absence of Vav, which is consistent with our previously published work (21). Importantly, LM-OVA was efficiently phagocytosed by Vav-null DCs, yet it was not processed and presented to OT-1 T cells.

After antigen uptake, cross-presentation involves processing of antigen and loading onto MHC I by several distinct pathways (4, 6, 7). Although the precise mechanisms still may control this process. To test this hypothesis, we examined the ability of DAP12 and FcRγ DCs to cross-present antigens using OT-1 T cell-based assays. Remarkably, we found that similar to Vav-null DCs, DAP12 and FcRγ DCs could efficiently present soluble OVA peptide and OVA protein to OT-1 T cells, yet they completely failed to present OVA coupled to beads or expressed in LM (LM-OVA; Fig. 7, A and B). Collectively, our data implicate a DAP12- and FcRγ-dependent pathway involving Vav GEFs as critical for NOX2 activation and cross-presentation of particulate antigens in DCs.

**DISCUSSION**

In this study, we identify an ITAM-mediated signaling pathway that is critically dependent on DAP12 and FcRγ adaptors and Vav GEFs that controls ROS production and cross-presentation of particulate antigens by DCs. Although the importance of cross-presentation for microbial and tumor immunity has been appreciated since the late 1970s (1), the exact mechanism by which DCs process exogenous antigens for cross-presentation on MHC I to CTLs remains controversial, and little is known about signal transduction pathways that regulate this process. We show that in DCs, Vav GEFs link ITAM-dependent receptors with the processing of particulate antigens for cross-presentation, although the identities of the cell surface receptors associated with DAP12 and FcRγ that regulate this process remain to be elucidated. In addition, our results highlight a differential requirement for ITAM signaling, Vav, and ROS production in cross-presentation of particulate versus soluble antigens.

Figure 6. Adhesion-dependent Vav phosphorylation and ROS production in DCs requires DAP12 and FcRγ. (A) WT and DAP12 and FcRγ (DF) BMDCs were stimulated on fibrinogen-coated plates (150 μg/ml) and lysed at the indicated time points. Vav was immunoprecipitated from the lysates, resolved by SDS-PAGE, and detected by Western blot with anti-phospho-tyrosine antibody. Blots were subsequently stripped and reprobed with anti-Vav antibody to demonstrate equal protein loading. (B) Alternatively, whole-cell lysates were blotted for phospho-ERK, stripped, and reprobed for total ERK. (C) ROS production in WT and DF BMDCs was determined with the chemiluminescent substrate lucigenin. Cells were stimulated with latex beads, LM, or LPS (10 μg/ml). RLU, relative light units.
remain controversial, it is thought that the mode of antigen uptake dictates the pathway by which antigen is processed. Specifically, soluble antigen taken up by macropinocytosis enters the endosomal pathway and can be processed and loaded onto MHC I in a TAP- and proteosome-independent manner (4, 8). Given that Vav$^{\text{NULL}}$ DCs show no defects in uptake or processing of soluble antigen, Vav appears to be dispensable for endosomal processing of antigen. In contrast, Vav appears to be strictly required for cross-presentation of particulate antigens, which are taken up by phagocytosis and enter the phagolysosomal pathway (4).

During the initial phase of antigen processing in the DC phagosome, NOX2-derived ROS were recently shown to regulate phagosomal pH (16). The production of ROS by NOX2 coincides with phagocytosis and consumes protons in the process, thus neutralizing the phagosome. Upon phagosome neutralization, pH-sensitive proteases are partially inactivated, thus limiting the extent to which protein antigens are initially degraded. Consequently, potential T cell epitopes are preserved for further processing by the proteosomes upon transport out of phagosomes (8). Our data indicate that ROS production during antigen processing is regulated by ITAM signals propagated through Vav proteins, although this ITAM pathway may also regulate a ROS-independent mechanism of antigen processing. In addition, this pathway may also be required for efficient presentation of particulate antigens to MHCII-restricted T cells, as Vav$^{\text{NULL}}$ DCs were defective in presentation of bead-linked OVA to OT-2 T cells (Fig. S5, available at http://www.jem.org/cgi/content/full/jem.20071283/DC1), and NOX2-deficient DCs were also inefficient at presenting antigen to CD4 T cells (16). Collectively, these data are consistent with the model in which ROS production is needed to prevent acidification and antigen degradation in the phagosomes.

Recent evidence suggests that fusion of phagosomes with the ER may promote loading of antigenic peptides onto MHC I in a TAP-dependent manner (9–12), whereas TLR-mediated pathways may also be involved in the regulation of phagosome maturation (14, 15). Our data do not directly address a potential role for Vav in later stages of antigen processing, such as in phagosome maturation and/or trafficking, although Vav proteins may very well participate in these processes. Nevertheless, our data clearly identify the Vav family as a critical regulator of ROS production and cross-presentation in DCs.

In addition to implicating Vav in cross-presentation by dendritic cells, our data indicate that signals generated by ITAM-containing adaptors DAP12 and FcRγ regulate Vav, and are themselves required for cross-presentation. Although the identities of the cell surface receptors associated with DAP12 and FcRγ that regulate cross-presentation are unknown, one candidate is the integrin family. Recent reports indicated that adhesion-mediated integrin signaling controls myeloid cell activation (17, 18). Thus, neutrophils deficient in DAP12 and FcRγ exhibit widespread defects in adhesion-dependent ROS production (17). Similar to these findings in neutrophils, we report that DCs from DAP12 and FcRγ mice exhibit defects in ROS production induced by adhesion or phagocytosis. Thus, although DAP12 and FcRγ are clearly not essential for phagocytosis of latex beads or LM, together they are critically required for the induction of ROS production by these stimuli. These data indicate that a DAP12/FcRγ-dependent pathway is activated during phagocytosis, even though it is not required for phagocytosis itself.

Based on our findings, a model can be proposed in which an integrin and ITAM-mediated pathway, in cooperation with additional DAP12 and FcRγ-associated receptors enables cross-presentation of particulates in DCs. Among the candidates are TREMs and SIRPs, which associate with DAP12 (31), and Fc receptors, which associate with FcRγ, although there are likely to be more ITAM-associated receptors yet to be identified in DCs. It is difficult to know which of...
In vitro antigen presentation. T cells were purified from OT-1 or -2 spleen and lymph nodes by two rounds of negative selection using MACS columns (Miltenyi Biotec) or one round of negative selection followed by one round of positive selection on MACS columns. Purified T cells were labeled with CFSE (Vybrant CFDA SE cell tracer kit; Invitrogen) according to the manufacturer’s recommendation. T cells (10^6 cells/well) were then cultured with dendritic cells (2 × 10^5/well) and antigen for 3 d in 96-well round bottom plates before analysis of CFSE dye dilution by FACS.

Macropinocytosis assays. Dendritic cells (5 × 10^5/ml) were cultured with 70 kD FITC-Dextran (Invitrogen) at a final concentration of 1 mg/ml in complete DME. Cells were incubated at 37°C or 4°C for the indicated time points, washed thrice in PBS, and analyzed by FACS for FITC intake. Alternatively, cells were allowed to adhere to poly-l-lysine-coated slides, fixed in 4% paraformaldehyde, and visualized by fluorescence microscopy. Cells were visualized on a fluorescence microscope (E400; Nikon). Images were acquired using a 60× objective lens with a 10× ocular lens. Image processing was performed in Photoshop CS (Adobe).

Phagocytosis assays. Dendritic cells (2 × 10^5 cells/250 μl) were cultured with 2 μl FITC/Alexa Fluor 647 beads (stock solution 2.63% vol/vol). Cells were washed thrice in PBS and analyzed by FACS. Alternatively, dendritic cells (4 × 10^5 cells/200 μl) were stained with FITC anti-CD11b for 15 min at 4°C, washed in PBS, resuspended in 200 μl DME, and mixed with the indicated volume of 5-μm sulfated latex beads (stock solution 7.5 × 10^6 beads/ml). Cells were washed thrice in PBS, distributed onto poly-l-lysine–coated slides, and fixed in 4% paraformaldehyde before imaging by confocal microscopy. Cells were visualized on a confocal microscope equipped with LSM image analysis software (Carl Zeiss, Inc.). Images were acquired using a 60× objective lens with a 10× ocular lens. Image processing was performed in Photoshop CS. Phagocytosis of LMs was performed similarly. Dendritic cells (6 × 10^5 cells/200 μl) were cultured with LM (1 μl of stock) that had been labeled with CFSE, as described in In vitro antigen presentation for T cells. Cells were incubated at 37°C for the indicated time points, washed thrice in PBS, distributed onto poly-l-lysine–coated slides, fixed in 4% paraformaldehyde, and imaged by fluorescence microscopy.

Oxidative burst assays. Dendritic cells were washed in PBS and resuspended in HBSS supplemented with 12.3 μg/ml MgSO_4 and 7.2 μg/ml CaCl_2 at a concentration of 2.5–5 × 10^6 cells/ml. Lucigenin (Invitrogen) was added to the cells to achieve a final concentration of 150 μM, and cells were distributed into 1-ml aliquots in 5-ml polystyrene lumimeter tubes coated with or without 1 μg/ml of fibronectin (Sigma-Aldrich). Baseline luminescence was measured in each sample for 10 s in an OptocompII lumimeter (MGM Instruments, Inc.). Immediately after the baseline reading, cells were stimulated with LPS, PMA, zymosan, or poly(I:C) (all from Sigma-Aldrich). Subsequently, luminescence was measured in each sample at the indicated time points. Luminescence is expressed as relative light units detected over 10 s.

Phagosomal pH measurement. Dendritic cells (4 × 10^6/200 μl DME) were loaded with FITC/Alexa Fluor 647 beads (1 μl) for 30 min at 37°C, washed, resuspended in 500 μl DME, and cultured for the indicated time points. Cells were then stained with PE anti-CD11c and analyzed by FACS. For analysis, FITC fluorescence was measured on a linear scale in CD11c+ cells that had internalized equal numbers of beads, as determined by Alexa Fluor 647 fluorescence intensity. Relative FITC intensity was calculated based on changes in FITC mean fluorescence intensity (MFI) over the indicated time points.

Antigen degradation. Dendritic cells (4 × 10^6/200 μl DME) were loaded with OVA-coupled beads (1 μl) for 30 min at 37°C, washed, resuspended in 500 μl DME, and cultured for the indicated time points. Cells were then stained with biotinylated anti-OVA rabbit serum (Abcam), followed by APC-streptavidin (Invitrogen). Cells were washed and subsequently lysed in 1% TX-100 in PBS containing complete protease inhibitor tablets (Boehringer), followed by filtering through nylon mesh. The recovered beads were then stained with biotinylated anti-OVA rabbit serum, followed by PE-streptavidin (BD Biosciences). Beads were then analyzed by FACS. For analysis, PE intensity was evaluated on only the beads that had been internalized (APC negative).

Biochemistry. Dendritic cells (12 × 10^6 cells/ml in DME) were stimulated for the indicated time points on 6-well plates coated with 150 μg/ml of sheep IgG.
fibrogen (Sigma-Aldrich) and primed with 50 ng/ml of mouse TNF-α (Peprotech). Cells were lysed for 5 min at 4°C on ice with lysis buffer (1% TX-100, 0.15 M NaCl, and 25 mM Hepes, pH 7.4). Whole-cell lysates were resolved by PAGE, transferred to PVDF membranes (Millipore), and Western blotted with mouse anti-phospho-ERK (Cell Signaling Technology). Blots were subsequently stripped and reprobed with rabbit anti-ERK2 antisera (Santa Cruz Biotechnology). After immunoprecipitation for 16 h at 4°C, beads were washed twice in lysis buffer, eluted with sample buffer, and resolved by SDS-PAGE. Proteins were then transferred from the gel to PVDF membranes and blotted with anti-phosphotyrosine (clone 4G10; Millipore). Membranes were subsequently stripped and reprobed with mouse anti-Vav (CHEMICON International, Inc.). Primary antibodies were detected with HRP-coupled anti-rabbit IgG (GE Healthcare) or HRP-coupled anti-mouse IgG (Invitrogen) and ECL chemoluminescent substrate (GE Healthcare).

Online supplemental material. Fig. S1 demonstrates similar expression of activation markers in WT and Vav$^{-/-}$ BMDCs before and after maturation with LPS. Fig. S2 indicates that WT and Vav$^{-/-}$ BMDCs express similar levels of costimulation markers in antigen presentation assays with OT-1 T cells. Fig. S3 demonstrates modest cross-presentation defects in NOX2$^{-/-}$ BMDCs. Fig. S4 shows equal phagocytosis of latex beads in WT and NOX2$^{-/-}$ BMDCs. Fig. S5 shows defective MHCII presentation in Vav NULL BMDCs. Fig. S6 indicates that WT and DAP12$^{-/-}$ BMDCs perform cross-presentation with similar efficiencies. The online version of this article is available at http://www.jem.org/cgi/content/full/jem.20071283/DC1.

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