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An ITAM-signaling pathway controls cross-presentation of particulate but not soluble antigens in dendritic cells

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Dendritic cells (DC) possess a unique capacity for presenting exogenous antigen on major histocompatibility class I, a process that is referred to as cross-presentation, which serves a critical role in microbial and tumor immunity. During cross-presentation, antigens derived from pathogen-infected or tumor cells are internalized and processed by DCs for presentation to cytotoxic T lymphocytes (CTLs). We demonstrate that a signaling pathway initiated by the immunoreceptor tyrosine-based activation motif (ITAM)–containing adaptors DAP12 and FcRγ utilizes the Vav family of Rho guanine nucleotide exchange factors (GEFs) for processing and cross-presentation of particulate, but not soluble, antigens by DCs. Notably, this novel pathway is crucial for processing and presentation of particulate antigens, such as those associated with Listeria monocytogenes bacteria, yet it is not required for antigen uptake. Mechanistically, we provide evidence that in DCs, Vav GEFs are essential to link ITAM-dependent receptors with the activation of the NOX2 complex and production of reactive oxygen species (ROS), which regulate phagosomal pH and processing of particulate antigens for cross-presentation. Importantly, we show that genetic disruption of the DAP12/FcRγ–Vav pathway leads to antigen presentation defects that are more profound than in DCs lacking NOX2, suggesting that ITAM signaling also controls cross-presentation in a ROS-independent manner.

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Abbreviations used: GEF, guanine nucleotide exchange factor; ITAM, immunoreceptor tyrosine-based activation motif; LM, Listeria monocytogenes; MFI, mean fluorescence intensity; ROS, reactive oxygen species.

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 Vavnull 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 (Vavnull) (24). Bone marrow–derived DCs from wild-type and Vavnull mice were cultured with either OVA- or peptide (spanning the OT-1 T cell epitope SIINFEKL) or OVA protein. DCs were then cocultured with purified OT1 TCR-transgenic CD8+ 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, Vavnull 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, Vavnull 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 Vavnull 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 Vavnull 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 Vavnull DCs with latex beads coupled to OVA.
Dendritic cells internalize antigens by macropinocytosis 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 Vavnull DCs. These experiments showed that both WT and Vavnull 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 Vavnull DCs to phagocyte latex beads or heat-killed Listeria monocytogenes expressing OVA (LM-OVA) and monitored OT-1 T cell proliferation. In contrast to WT DCs, Vavnull DCs lacked the ability to induce OT-1 T cell responses to bacteria-associated antigens such as LM-OVA (Fig. 1). Therefore, given that Vavnull 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.
Given the strict requirement for Vav in the generation of ROS by DCs, we hypothesized that Vav\textsuperscript{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\textsuperscript{NULL} DCs were loaded with latex beads covalently coupled to pH-sensitive dye. We found that Vav\textsuperscript{NULL} DCs exhibited a decrease in phagosomal pH compared to WT DCs, as evidenced by the shift in fluorescence intensity. This result suggests that Vav proteins regulate phagosome pH through their role in ROS production.

In conclusion, our data support the notion that Vav proteins play a critical role in the regulation of phagosome maturation and cross-presentation of particulate antigens. The ROS-dependent mechanisms mediated by Vav proteins are essential for the efficient presentation of particulate antigens to T cells, whereas ROS-independent mechanisms may also contribute to this process. These findings have important implications for the development of immunotherapies that target the phagosome maturation and cross-presentation pathways.
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$^{\text{NULL}}$ DCs showed significantly lower ratios of FITC to Alexa Fluor 647 fluorescence over time, indicating a more acidified environment in Vav$^{\text{NULL}}$ 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$^{\text{NULL}}$ 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$^{\text{NULL}}$ 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$^{\text{NULL}}$ DCs. Given the profound defects in cross-presentation observed in Vav$^{\text{NULL}}$ 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$\gamma^{-/-}$ dendritic cells

Previous studies indicated that, in neutrophils, ROS production in response to various adhesion-dependent stimuli requires ITAM-containing DAP12 and FcR$\gamma$ 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$\gamma$, 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$\gamma$ ITAMs (17, 19).

To confirm that, under these conditions, tyrosine phosphorylation of Vav was, indeed, dependent on signals emanating from DAP12 and FcR$\gamma$, we used DCs from mice deficient in DAP12 and FcR$\gamma$. Strikingly, we found in these experiments that tyrosine phosphorylation of Vav was drastically diminished in DAP12 and FcR$\gamma$ DCs, which is consistent with the uncoupling of Vav from adhesion-induced signaling pathways in the absence of DAP12 and FcR$\gamma$ (Fig. 6 A). Moreover, both Vav$^{\text{NULL}}$ and DAP12 and FcR$\gamma$ 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$\gamma$ adaptors in DCs.

Given the recently published work indicating a critical role for DAP12 and FcR$\gamma$ adaptors in NOX2 activation and ROS production in neutrophils (17), we tested the ability of DAP12 and FcR$\gamma$ DCs to generate ROS in response to multiple adhesion-dependent stimuli, including latex beads, LM, and LPS (Fig. 6 C). Strikingly, DAP12 and FcR$\gamma$ 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$\gamma$ DCs mirrored the loss of ROS we observed in Vav$^{\text{NULL}}$ 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$\gamma$ ITAM-based signaling pathway

![Figure 5](https://example.com/figure5.png)

**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 MHCI 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.

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 drying 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
remains 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\textsuperscript{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 phagosomal 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 MHCI-restricted T cells, as Vav\textsuperscript{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 FcR\gamma regulate Vav, and are themselves required for cross-presentation. Although the identities of the cell surface receptors associated with DAP12 and FcR\gamma 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 FcR\gamma exhibit widespread defects in adhesion-dependent ROS production (17). Similar to these findings in neutrophils, we report that DCs from DAP12 and FcR\gamma mice exhibit defects in ROS production induced by adhesion or phagocytosis. Thus, although DAP12 and FcR\gamma 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/FcR\gamma-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 FcR\gamma-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 FcR\gamma, although there are likely to be more ITAM-associated receptors yet to be identified in DCs. It is difficult to know which of
these receptors may be involved in cross-presentation of various particulate antigens. In addition, the ligands for DAP12-associated receptors like the TREMs are not known. It is possible that these ligands initiate DAP12 signals that regulate antigen presentation; however, such a model is yet to be formally proven. Although we observed that DAP12 is not required for cross-presentation of OVA beads to OT-1 T cells (Fig. S6, available at http://www.jem.org/cgi/content/full/jem.20071283/DC1), additional ITAM-containing adaptors, such as FeRγ, may compensate in the absence of DAP12. Although further investigation is required to identify the precise receptors involved in regulating cross-presentation, the data presented herein clearly indicate that signaling through a Vav-dependent ITAM pathway is critical for the regulation of cross-presentation.

MATERIALS AND METHODS

Mice. Mice genetically deficient in Vav1, Vav2, and Vav3 (Vav<sup>−/−</sup>) have been previously described (24). NOX2-deficient mice were purchased from The Jackson Laboratory. OT-1 and -2 mice were a gift from H. Virgin (Washington University, St. Louis, MO). Mice genetically deficient in DAP12 and FcRγ were a gift from M. Colonna (Washington University, St. Louis, MO). All animal work was performed in accordance with the guidelines of, and was approved by, the Animal Studies Committee of Washington University School of Medicine.

Antibodies and flow cytometry. The following antibody conjugates were used (all from BD Biosciences): APC anti-CD8 (53–6.7), FITC anti-CD11b, PE anti-CD11c (HL3), FITC anti-B7.1 (16-10A1), FITC anti-B7.2 (GL1), FITC anti-CD40 (3/23), and FITC anti-I-κBα. All samples were analyzed on a FACS Calibur flow cytometer (Becton Dickinson) with FlowJo software.

Reagents. Synthetic OT-1 peptide (SIINFEKL) and OT-2 peptide (ISQAHAAHAEINEAGR) were gifts from P. Allen (Washington University, St. Louis, MO). Purified OVA protein was obtained from Thermo Fisher Scientific. 10 mg/ml OVA was passively adsorbed to 5-μm sulfated latex beads (Invitrogen) at 37°C for at least 2 h before a thorough washing in PBS. The final concentration of beads was 7.5 × 10<sup>5</sup>/μl. Alternatively, 10 mg/ml OVA was covalently coupled with FITC and Alexa Fluor 647 succinimidyl ester (Invitrogen) at a final concentration of 2.5–5 × 10<sup>5</sup> 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 luminescence 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 luminometer (MGM Instruments, Inc.). Immediately after the baseline reading, cells were stimulated with LPS, PMA, zymosan, or peptidoglycan (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.

Dendritic cell cultures. Bone marrow was harvested from the femurs and tibias of mice and cultured in complete DME containing 10% low endotoxin PBS (HyClone) and 2% mouse GM-CSF conditioned media derived from TOPO cells. Cultures were maintained for 7–10 d and analyzed for CD11c expression by FACS before use in experiments. Alternatively, fresh DCs were purified from splenocytes by positive selection using CD11c MACS beads (Miltenyi Biotec).

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<sup>5</sup> cells/ml) were then cultured with dendritic cells (2 × 10<sup>5</sup>/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<sup>5</sup>/ml) were cultured with 70 K DITC-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 three times 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<sup>5</sup> cells/250 μl) were cultured with 2 μl FITC/Alexa Fluor 647 beads (stock solution 2.63% vol/vol). Cells were washed three times in PBS and analyzed by FACS. Alternatively, dendritic cells (4 × 10<sup>5</sup> 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<sup>5</sup>/beads/ml). Cells were washed three times 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 LM was performed similarly. Dendritic cells (6 × 10<sup>5</sup> 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 three times 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 re-suspended in HBSS supplemented with 12.3 μg/ml MgSO<sub>4</sub> and 7.2 μg/ml Ca<sub>2+</sub>/Cl<sub>−</sub> at a concentration of 2.5–5 × 10<sup>5</sup> 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 luminescence 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 luminometer (MGM Instruments, Inc.). Immediately after the baseline reading, cells were stimulated with LPS, PMA, zymosan, or peptidoglycan (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<sup>5</sup>/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<sup>+</sup> cells that had been labeled 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<sup>5</sup>/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<sup>5</sup> cells/ml in DME) were stimulated for the indicated time points on 6-well plates coated with 150 μg/ml of sheep IgG.
Online supplemental material. Fig. S1 demonstrates similar expression of activation markers in WT and Vav<sup>-/-</sup> BMDCs before and after maturation with LPS. Fig. S2 indicates that WT and Vav<sup>-/-</sup> BMDCs express similar levels of costimulatory markers in antigen presentation assays with OT-1 T cells. Fig. S3 demonstrates modest cross-presentation defects in NOX2<sup>-/-</sup> BMDCs. Fig. S4 shows equal phagocytosis of latex beads in WT and Vav<sup>-/-</sup> BMDCs. Fig. S5 shows defective MHCII presentation in Vav<sup>-/-</sup> BMDCs. Fig. S6 indicates that WT and DAP12<sup>-/-</sup> 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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