Autophagy-related protein Vps34 controls the homeostasis and function of antigen cross-presenting CD8α+ dendritic cells

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The class III PI3K Vacuolar protein sorting 34 (Vps34) plays a role in both canonical and noncanonical autophagy, key processes that control the presentation of antigens by dendritic cells (DCs) to naive T lymphocytes. We generated DC-specific Vps34-deficient mice to assess the contribution of Vps34 to DC functions. We found that DCs from these animals have a partially activated phenotype, spontaneously produce cytokines, and exhibit enhanced activity of the classic MHC class I and class II antigen-presentation pathways. Surprisingly, these animals displayed a defect in the homeostatic maintenance of splenic CD8α+ DCs and in the capacity of these cells to cross-present cell corpse-associated antigens to MHC class I-restricted T cells, a property that was associated with defective expression of the T-cell Ig mucin (TIM)-4 receptor. Importantly, mice deficient in the Vps34-associated protein Rubicon, which is critical for a noncanonical form of autophagy called “Lightchain 3 (LC3)-associated phagocytosis” (LAP), lacked such defects. Finally, consistent with their defect in the cross-presentation of apoptotic cells, DC-specific Vps34-deficient animals developed increased metastases in response to challenge with B16 melanoma cells. Collectively, our studies have revealed a critical role of Vps34 in the regulation of CD8α+ DC homeostasis and in the capacity of these cells to process and present antigens associated with apoptotic cells to MHC class I-restricted T cells. Our findings also have important implications for the development of small molecule inhibitors of Vps34 for therapeutic purposes.

Dendritic cells (DCs) play a central role in the activation of naive T cells and direct the induction of adaptive immune responses against invading microorganisms. These cells capture foreign and self antigens and present them to MHC class I- and class II-restricted CD8+ and CD4+ T cells, respectively. MHC class II-associated peptides are typically generated by proteolysis of endocytosed proteins (1), whereas MHC class I-associated peptides are predominantly generated by proteolysis of cytosolic proteins (2). However, in specialized antigen-presenting cells (APCs) such as a DC subset expressing CD8α and CD103, extracellular antigens can be presented in the context of MHC class I molecules via a cross-presentation pathway whose mechanism is incompletely understood (3).

In recent years, the process of autophagy has been implicated in controlling antigen processing (4). Autophagy is a conserved catabolic process that maintains cellular energy homeostasis in response to a wide spectrum of cellular stresses (5, 6). Autophagy ensures continuous degradation of long-lived proteins, damaged cellular organelles, and protein aggregates to facilitate recycling of nutrients and hence promote cellular metabolism. The formation of autophagosomes requires an interplay between autophagy-related (Atg) gene products, which have been well characterized in yeast and are conserved in mammals (7). Defective autophagy in mammalian cells results in the accumulation of damaged cellular organelles and protein aggregates, leading to stress with pathological consequences (8). Autophagy has common features with endocytosis, with which it shares effector molecules (9). However, how these processes and their shared machinery regulate antigen presentation remains incompletely understood.

Vacular protein sorting 34 (Vps34) is a class III PI3K that plays a role in endocytosis, intracellular vesicular trafficking, and autophagosome formation during autophagy (10). Vps34-deficient cells display defective autophagic flux leading to the accumulation of aggregated cellular proteins and organelles, and Vps34 ablation in mice causes significant pathology (11–14). Although Vps34 forms multiple protein complexes that mediate its diverse cellular functions in canonical autophagy, noncanonical autophagy, and endocytosis (15), it is unclear which of these processes is responsible for the phenotypes observed in Vps34-deficient cells and mice.

In the present study, we generated mice with a DC-specific deletion of Vps34 to determine its effects on DC functions such as antigen presentation and priming of adaptive immune responses. Our findings revealed a critical role for Vps34 in the homeostasis and function of DCs that is dependent on the noncanonical autophagy pathway.

Results

DC-Specific Vps34-Deficient Mice Exhibit a Selective Reduction in CD8α+ DCs. We generated Vps34α/α;CD11c-Cre mice, which exhibited selective Vps34 ablation in DCs (Fig. S1). Although these mice displayed no signs of disease and were grossly indistinguishable

Significance

Dendritic cells (DCs) of the immune system are critical for displaying foreign antigens to T lymphocytes, a process called “antigen presentation.” This process may involve Vacuolar protein sorting 34 (Vps34), a protein implicated in diverse cellular processes, including endocytosis, an extracellular product uptake system, and autophagy, an intracellular degradation system. Here we have generated and analyzed mice in which the Vps34 gene is specifically knocked out in DCs. These animals displayed defects in the survival and function of a subset of DCs specialized in presenting antigens from dead cells to T cells. Thus our findings have revealed a critical contribution of Vps34 in DC functions that may have important implications for targeting this pathway for therapeutic purposes.

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from control littersmates, their lymphoid organs were visibly enlarged compared with control animals (Fig. 1I), and lymphoid cellularity was increased (Fig. 1B).

Conventional DCs in lymphoid organs are a heterogeneous population of CD11b<sup>+</sup>CD11c<sup>+</sup> cells that can be further subdivided into populations expressing CD8α and CD103 (16). Although we found no differences in the frequency of total DCs (Fig. 1C), the absolute numbers of DCs were significantly increased in the lymphoid organs of Vps34<sup>f/f;CD11c-Cre</sup> mice (Fig. 1C). The prevalence of the DC population expressing CD103 and CD8α in Vps34<sup>f/f;CD11c-Cre</sup> mice was sharply reduced (Fig. 1D and E), although absolute numbers were comparable in both groups of mice (Fig. 1E). CD11b<sup>+</sup>CD103<sup>+</sup> cells are a major population of DCs in the lungs (17) that are developmentally related to conventional CD8α<sup>+</sup> DCs in the spleen (16). We observed a trend for reduced frequency of such DCs in the lungs of Vps34<sup>f/f;CD11c-Cre</sup> mice (Fig. 1F and G). The frequency of other populations of myeloid cells, including CD11b<sup>+</sup>CD11c<sup>−</sup> and CD11b<sup>+</sup>CD11c<sup>−</sup> cells, was similar in the two groups of mice (Fig. S2). Immunophenotyping of lymphocytes revealed that the frequencies of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, B cells, and natural killer (NK) cells were similar in the two groups of mice, but absolute numbers of these cells were profoundly increased in Vps34<sup>f/f;CD11c-Cre</sup> mice (Fig. S3). Thus, these data suggest that the increased numbers of DCs in the lymphoid organs of Vps34<sup>f/f;CD11c-Cre</sup> mice are associated with concomitant increases in T, B, and NK cells, thereby increasing the overall cellularity and size of the lymphoid organs.

To determine if the selective reduction of CD8α<sup>+</sup> DCs in the spleen is related to defects in their development or homeostasis, we analyzed young mice that contained comparable numbers of cells, suggesting normal DC development (Fig. 1H). To determine if the loss of CD8α<sup>+</sup> DCs in older mice was cell intrinsic,

![Image of DC subpopulations in Vps34<sup>f/f;CD11c-Cre</sup> mice](https://www.pnas.org/cgi/doi/10.1073/pnas.1706504114)

Fig. 1. DC subpopulations in Vps34<sup>f/f;CD11c-Cre</sup> mice. (A) Spleens and a pair of inguinal lymph nodes from representative 12-wk-old Vps34<sup>f/f</sup> and Vps34<sup>f/f</sup>; CD11c-Cre mice are shown. (Scale bar, 1 cm.) (B) Single-cell suspensions of spleens and a pair of inguinal lymph nodes were prepared and counted. Results from three independent experiments (five to seven animals per group) are shown. *P < 0.01. (C) Percent and absolute numbers of DCs in the spleen and lymph nodes of Vps34<sup>f/f</sup> or Vps34<sup>f/f;CD11c-Cre</sup> mice. Results from three independent experiments (five to seven animals per group) are shown. *P < 0.01. (D) Spleocytes from 12-wk-old Vps34<sup>f/f</sup> or Vps34<sup>f/f;CD11c-Cre</sup> mice were stained with anti-CD11c, -CD103, -CD8α, and -CD11b antibodies. The CD11c<sup>+</sup>CD11b<sup>+</sup> cells were evaluated for CD103 expression, and levels of CD8α on CD103<sup>−</sup> and CD103<sup>+</sup> cells were measured. *P < 0.01. (E) Summary of the percentage and absolute numbers of CD103<sup>+</sup>-expressing DCs in the spleen. Pooled results from two independent experiments (five to seven mice in each group) are shown. *P < 0.01. (F and G) Single-cell suspensions of lungs from the indicated mice were stained with anti-CD11c, -CD103, -CD8α, and -CD11b antibodies. A representative experiment (F) and a summary of the data pooled from two independent experiments (six mice in each group) (G) are shown. (H) Frequency of CD8α<sup>+</sup> DCs in 4-wk-old mice. (I) Bone marrow chimeras were generated in lethally irradiated B6 mice (CD45.2) by transfer of 10<sup>7</sup> cells bone marrow cells from wild-type B6.SJL mice (CD45.1) and Vps34<sup>f/f;CD11c-Cre</sup> (CD45.2) or Vps34<sup>f/f</sup> (CD45.2) mice mixed at a 1:1 ratio. At the end of 12 wk spleen cells from the chimera mice were tested for the frequency of CD8α<sup>+</sup> cells among CD45.2<sup>+</sup> DCs. A summary of two independent experiments (five mice per group) is shown. *P < 0.05. (J) Bone marrow-derived Flt3L-driven DCs were generated in the presence of 150 ng/mL of Flt3L for 9–21 d in vitro culture. Cells analogous to splenic CD8α<sup>+</sup> DCs were identified as CD45RA<sup>−</sup> “CD24<sup>−</sup>Sirpα<sup>+</sup>” cells. Representative plots from three individual experiments with two mice per group are shown.
we generated mixed bone marrow chimeras using wild-type and Vps34<sup>f/f</sup>;CD11c-Cre or Vps34<sup>f/f</sup> bone marrow cells. We found a selective reduction in Vps34-deficient CD8<sup>α</sup> DCs (Fig. 1I), indicating that Vps34 is required for the normal homeostatic maintenance of CD8<sup>α</sup> DCs. To validate these results further, we differentiated Flt3L-driven bone marrow-derived DCs (BMDCs) in vitro and analyzed CD45RA<sup>−</sup>CD24<sup>+</sup>Sirp-α<sup>−</sup> DCs, which are considered analogous to CD8<sup>α</sup> DCs in the spleen (18). We found that this population of DCs from Vps34<sup>f/f</sup>;CD11c-Cre mice was present at normal levels early (day 9) after culture but was reduced at a later time point (day 21) compared with the control cultures (Fig. 1J). Despite differences in the timing of DC development in vitro vs. in vivo, these findings are consistent with defects in DC homeostasis rather than development.

**Spontaneous DC Activation in Vps34<sup>f/f</sup>;CD11c-Cre Mice.** We next assessed the DC activation status during steady-state conditions and found that Vps34-deficient DCs expressed modestly increased levels of CD40 and MHC class I and class II molecules but not CD80 or CD86, suggesting a partially activated state (Fig. 2A). Additionally, we found that Vps34-deficient DCs spontaneously secrete copious amounts of both pro- and anti-inflammatory cytokines such as TNFα, IL-6, and IL-10 (Fig. 2B), as is consistent with a prior study performed in vitro with a small-molecule inhibitor of Vps34, SAR405 (19). Upon activation with Toll-like receptor (TLR) ligands, no substantial enhancement of cytokine secretion by Vps34-deficient DCs was observed compared with control DCs. We obtained similar results for activated BMDCs, but these cells did not spontaneously produce cytokines (Fig. 2C and D), perhaps because of the lack of continuous exposure to TLR ligands, as may be the case for splenic DCs in vivo. Collectively, these results indicate that DCs from Vps34<sup>f/f</sup>;CD11c-Cre mice exhibit a partially activated phenotype with spontaneous production of both pro- and anti-inflammatory cytokines.

![Fig. 2. Spontaneous DC activation in Vps34<sup>f/f</sup>;CD11c-Cre mice.](image-url)
Enhancement of the Classic MHC Class I and Class II Antigen-Presentation Pathways. Because autophagy has been implicated in antigen presentation (4), we analyzed this function of Vps34-deficient DCs. These cells were as effective as wild-type DCs in presenting ovalbumin (OVA)-derived peptide to H-2Kb–restricted OT-I T cells (Fig. 3A and B). However, Vps34-deficient DCs exhibited enhanced capacity to present cytoplasmic OVA antigens to OT-I cells (Fig. 3B). We repeated this experiment with sorted CD8α+ and CD8α− DC populations and found that both subsets of Vps34-deficient DCs exhibited enhanced antigen presentation (Fig. 3C and D). We obtained comparable results with BMDCs (Fig. 3E). Similarly, Vps34-deficient DCs showed enhanced capacity to present immunodominant lymphocytic choriomeningitis virus (LCMV) epitopes to cytotoxic T lymphocyte (CTL) lines (Fig. 3F). To test whether the partially activated phenotype of Vps34-deficient DCs plays a role, we activated purified splenic DCs with reagents that up-regulate MHC class I expression. We found that IFN-γ enhanced up-regulation of MHC class I (but not MHC class II) expression (Fig. S4) on Vps34-deficient DCs compared with control cells (Fig. 3G and H), suggesting that the partially activated phenotype of Vps34-deficient DCs renders them even more sensitive to IFN-γ-mediated up-regulation of MHC class I and its associated antigen-processing machinery.

Next, we evaluated the MHC class II antigen-processing pathway. We found that Vps34-deficient and wild-type DCs had similar capacity to present an OVA-derived peptide to OT-II
T cells (Fig. 4A), but Vps34-deficient DCs showed an enhanced capacity to present soluble OVA protein (Fig. 4B). This finding was true for both the CD8α+ and CD8α- DC subsets (Fig. 4 C and D). Similar results were obtained for BMDCs (Fig. 4 E and F). To determine whether the observed differences in MHC class II-restricted antigen presentation were to the result of the role of Vps34 in endocytosis, we tested the capacity of Vps34-deficient and wild-type BMDCs to endocytose particulate matter (fluorescent microbeads), soluble proteins (chimeric Es-GFP protein), and bacteria (Citrobacter rodentium) in the presence or absence of the phagocytosis inhibitor cytochalasin D. We found that Vps34-deficient DCs exhibited modestly reduced uptake (Fig. S5), suggesting that these cells do not possess a major inherent defect in endocytosis or phagocytosis.

**Defective Cell Corpse-Associated Antigen Cross-Presentation.** In addition to presenting endogenous antigens on MHC class I molecules, CD8α+ DCs can present exogenous antigens to MHC class I-restricted T cells in a process called “cross-presentation” (20). We sorted CD8α+ and CD8α- DCs to measure their capacity to cross-present the model antigen OVA delivered in four different forms: soluble, targeted to DCs with anti-DEC205 antibodies, cross-present the model antigen OVA delivered in four different forms: soluble, targeted to DCs with anti-DEC205 antibodies, and contained by apoptotic cells. We found that Vps34-deficient CD8α+ DCs were equally as efficient as wild-type DCs in cross-presenting free OVA (Fig. 5A), OVA delivered to DCs via DEC205 (Fig. 5B), and OVA produced by L. monocytogenes (Fig. 5C). In sharp contrast, Vps34-deficient DCs displayed a marked defect in cross-presenting OVA antigens associated with apoptotic cells (Fig. 5D). Next, we measured cross-presentation of OVA-associated cell corpses in vivo after mice were injected with OVA-loaded, sublethally irradiated TAPγ− splenocytes and demonstrated a profound defect in Vps34−/−CD11c-Cre mice (Fig. 5E). Collectively, these results showed that Vps34-deficient DCs possess a competent cross-presentation pathway, but their capacity to cross-present cell corpse-associated antigens is impaired.

**Defective Uptake of Cell Corpses Correlates with Reduced T-Cell Ig Mucin-4 Expression.** We next explored the mechanism of defective cross-presentation of apoptotic cells by Vps34-deficient DCs. First, we determined the capacity of DCs to take up apoptotic cells in vivo and in vitro. For in vivo experiments, we injected sublethally irradiated splenocytes into groups of Vps34−/−;CD11c-Cre and Vps34−/− mice and found that Vps34-deficient CD8α+ DCs were defective in taking up apoptotic cells (Fig. 6 A and B). A similar apoptotic cell uptake experiment was carried out in vitro and showed that Vps34-deficient CD8α+ DCs were less efficient than control DCs in phagocytosing apoptotic cells (Fig. 6 C and D). Thus, we concluded that Vps34-deficient CD8α+ DCs are selectively defective in efferocytosis.

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Effects of Vps34 deficiency on the MHC class II antigen-presentation pathway in DCs. (A) Sorted splenic DCs were pulsed with MHC class II-restricted OVA peptide for 1 h in complete medium and were washed. OT-II T cells were added, and culture supernatants were collected after 24 h to measure IL-2 by CBA. Representative plots from two experiments with four mice per group are shown. Error bars indicate the means ± SD of triplicate wells. *P < 0.05. (C and D) Splenic CD8α+ (C) and CD8α- (D) DCs were cultured with varying amounts of soluble OVA and OT-II T cells for 24 h, and IL-2 in the culture supernatant was measured. Representative graphs from three experiments with five mice per group are shown. Error bars indicate the means ± SD of triplicate wells. *P < 0.05. (E) BMDCs (106) were cultured with OVA (100 μg/mL) in the presence of OT-II cells (105). Culture supernatants were collected at 24 h, and IL-2 and IFNγ were measured by ELISA. Representative plots from two experiments with five mice per group are shown. Error bars indicate the means ± SD of triplicate wells. *P < 0.05. (F) BMDCs (2 × 107) were incubated with 50 μg/mL of GFP-Ex chimeric protein for 3 h, and cells were stained with YAe antibodies specific for Ex-derived E952-68 peptide bound with I-Aα molecules. Representative plots from two experiments with four mice per group are shown. *P < 0.05.

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Cre expression, we analyzed Vps34-deficient CD8+ cells. For this purpose, we generated mixed bone marrow chimeras and found that CD8α+ DCs expressed higher levels of TIM-4 receptor on the cell surface than did CD8α- DCs from either group of mice. Interestingly, TIM-4 expression was consistently reduced on Vps34-/-CD8α+ DCs compared with control DCs (Fig. 6F and G). To rule out potential artifacts mediated by CBA, we analyzed Vps34+/f/f, Vps34+/-, and Vps34-/- control animals used throughout these studies (Fig. S6), indicating that the observed defects were mediated by Vps34 ablation.

We next performed experiments to determine whether reduced TIM-4 expression on DCs from Vps34-/-CD11c-Cre+ mice might be caused by the cytokines that are constitutively produced by these cells. For this purpose, we generated mixed bone marrow chimeras using Vps34-/-CD11c-Cre- and Vps34+/- derived donor cells and lethally irradiated wild-type recipient animals. The results showed that Vps34-deficient CD8α+ DCs were reduced in prevalence compared with wild-type CD8α+ DCs (Fig. 1J). Further, Vps34-deficient CD8α- DCs exhibited reduced TIM-4 expression and a reduced capacity to take up dead cells compared with wild-type CD8α+ DCs (Fig. S7), indicating that the cytokines produced constitutively by Vps34-deficient CD8α+ DCs fail to impair TIM-4 expression or the uptake of apoptotic cells by wild-type CD8α+ DCs in vivo. This conclusion was supported by cocultures of DCs derived from Vps34+/+CD11c-Cre and Vps34+/- mice (1:1 ratio) for 24 h, which did not affect TIM-4 expression on either mutant or wild-type DCs (Fig. S8A). In a similar experiment, coculture of DCs from Vps34+/+CD11c-Cre mice and whole splenocytes derived from Vps34+/- mice failed to influence TIM-4 expression on Vps34 mutant DCs (Fig. S8B and C). We also considered that IL-10, one of the immunosuppressive cytokines spontaneously produced by Vps34-deficient DCs (Fig. 2), may potentially impact TIM-4 expression, but we found that IL-10 treatment had no effect on TIM-4 expression by wild-type DCs (Fig. S8D).

In an attempt to provide direct evidence for reduced TIM-4 expression as a cause of defective efferocytosis by Vps34-deficient DCs, we transfected these cells with a TIM-4-containing expression vector, but, as was consistent with similar attempts to transfect primary DCs by other investigators (23), we were unsuccessful. As an alternative approach, we used the DC2.4 cell line (24) and primary BMDCs, which lack TIM-4 expression (Fig. 6H and I). Transfection with the TIM-4 expression vector resulted in efficient TIM-4 surface expression, which in turn enhanced the uptake of apoptotic cells (Fig. 6H and I), demonstrating that TIM-4 can function as a phagocytic receptor for apoptotic cells on DCs. Although these findings point to blunted TIM-4 expression as a likely defect in Vps34-deficient DCs evoking defective efferocytosis, they do not exclude the possibility that other phagocytic receptors are involved.

DC Defects Are Independent of Light Chain 3-Associated Phagocytosis. The Beclin1–Vps34 complex is used by the canonical autophagy process as well as by light chain 3 (LC3)-associated phagocytosis (LAP) (25). The protein Rubicon is critically required for LAP but not for canonical autophagy (26). Therefore, we analyzed DCs from Rubicon-deficient mice, which exhibited normal prevalence of the CD8α+ subset (Fig. 7A and B), lack of constitutive cytokine production (Fig. 7C), normal TIM-4 surface expression (Fig. 7D), and a normal capacity to take up dead cells (Fig. 7E). These results indicate that the alterations in CD8α+ DCs observed in Vps34-mutant mice are most likely not caused by defective non-canonical autophagy. Instead, electron microscopy studies revealed autophagosomal double-membrane structures, indicating defective canonical autophagy, in wild-type but not Vps34-deficient DCs (Fig. S9A). To provide further support for defects in canonical autophagy in Vps34-deficient DCs, we measured mitochondrial and endoplasmic reticulum (ER) mass, which was enhanced in Vps34-deficient compared with wild-type DCs (Fig. S9B). Although these findings are consistent with the premise that the observed phenotypes in Vps34-deficient DCs are mediated by defects in canonical autophagy, we cannot exclude a role for other cellular processes in which Vps34 is involved.

Defective Induction of Responses to Apoptotic Cell-Associated Antigens. To investigate the functional consequences of Vps34 ablation in DCs on the induction of an immune response, we...
Fig. 6. Vps34-deficient DCs have defects in the uptake of cell corpses and TIM-4 expression. (A) For in vivo uptake of apoptotic cells by DCs, $3 \times 10^7$ CFSE-labeled apoptotic B6.SJL (CD45.1$^+$) splenocytes were injected into groups of mice. After 4 h spleen cells were prepared, and CFSE$^+$CD45.1$^+$CD45.2$^+$ DCs subsets were determined. A representative plot is shown. (B) A summary of the experiment in A is shown. Data are pooled from two individual experiments (five mice in each group). *$P < 0.05$. (C) CFSE-labeled apoptotic cells (CD45.1$^+$) were incubated with total DCs (CD45.2$^+$) derived from the indicated mice for 3 h (1:10 ratio). At the end of the culture, CFSE$^+$CD45.1$^-$CD45.2$^+$ DC subsets were determined as shown. (D) A summary of the experiment in C is shown. Data are pooled from two individual experiments (five mice in each group). *$P < 0.01$. (F) mRNA expression of the indicated phagocytic receptors in splenic DCs. Data shown are representative of three independent experiments. *$P < 0.01$. (G) The mean fluorescence intensity (MFI) of TIM-4 expression shown in F is summarized. Data are pooled for six mice from two individual experiments. *$P < 0.01$. (H and I) DC2.4 cells (H) or BMDCs (I) were transfected with a TIM-4-containing vector, CFSE$^+$ apoptotic cells were added 24 h later, and 3 h later cells were stained with an anti–TIM-4 antibody and were analyzed by flow cytometry. *$P < 0.05$. (I) A summary of the experiment in H. Four independent transfection experiments were performed.
measured CTL responses to cell corpse-associated antigens in vivo. Sublethally irradiated, OVA-loaded TAP−/− splenocytes were injected into mice, and 7 d later we measured OVA257–264-specific CTL responses. Consistent with the relative reduction of CD8α+ DCs in spleens of Vps34f/f;CD11c-Cre mice and defects in the cross-presentation of apoptotic cells (Fig. 5E), the results showed efficient induction of OVA-specific CTLs in Vps34f/f mice but a blunted response in Vps34f/f;CD11c-Cre mice (Fig. 8).

Enhanced B16 Lung Melanoma Metastases. Recent studies on CD8α+ DCs have shown that these DCs have a critical role in cross-presenting tumor-associated antigens and in inducing tumor-specific CTLs (27). We therefore challenged Vps34f/f;CD11c-Cre and Vps34f/f mice with B16 melanoma cells and scored the animals 15–17 d later for lung tumor metastases. Vps34f/f;CD11c-Cre mice possessed significantly higher tumor burdens than Vps34f/f control mice (Fig. 9).

CD11b+CD11chi DCs in the lungs are developmentally related to splenic CD11c+CD8α+CD103+ DCs (16). This population of DCs in the lungs was recently shown to be critically required for preventing B16 lung metastases (28). We therefore analyzed distinct lung myeloid cell populations and found high TIM-4 expression on CD11b+CD11chi DCs from wild-type mice but not on those from Vps34f/f;CD11c-Cre mice (Fig. S10A). These results suggest that the TIM-4-expressing CD11b+CD11chi population of DCs in the lungs plays a role in controlling tumor metastasis.

We considered the possibility that the increased tumor burden in Vps34f/f;CD11c-Cre mice may be related to spontaneous cytokine production by DCs in these animals. We therefore evaluated CD11b+Gr1+ myeloid-derived suppressor cells, which promote tumor growth and are induced in response to inflammatory stimuli (29), but found no difference in the frequency of these cells in mutant and wild-type animals (Fig. S10B).

Discussion

The class III PI3K Vps34 plays a role in endocytosis, intracellular vesicular trafficking, and autophagy, key processes that control the presentation of self and foreign antigens by DCs to naïve T lymphocytes. Here we analyzed DC functions in mice with a DC-specific Vps34 gene ablation. DCs from these animals exhibited a decrease in the ratio of CD8α+ to CD8α− subsets that was cell intrinsic and mediated by impaired DC homeostasis. Although the frequency of CD8α+ DCs was reduced in Vps34f/f;CD11c-Cre mice, these cells retained their capacity to cross-present the model antigen OVA to MHC class I-restricted T cells when delivered in a soluble form through the DEC205 receptor and via expression in bacteria but were profoundly impaired in their capacity to cross-present OVA antigens associated with dying cells. This impaired capacity was associated with reduced expression of TIM-4, a receptor predominantly expressed by CD8α+ DCs that binds with phosphatidylserine to engulf apoptotic cells (22, 27). Therefore, the combined defect in the homeostatic maintenance of CD8α+ DCs and blunted efferocytosis in Vps34f/f;CD11c-Cre mice resulted in impaired induction of CTL responses to antigens associated with dying cells and defective antitumoral immunity. Although our findings suggest defective TIM-4 expression as a likely cause for the impaired efferocytosis by Vps34-deficient DCs, additional phagocytic receptors for apoptotic cells may be involved.
In addition to autophagy, Vps34 plays a role in endocytosis (9, 30). Our findings showed that DCs from Vps34<sup>-/-</sup>CD11c-Cre mice lack significant defects in endocytosis or phagocytosis but exhibit abnormalities in autophagosomal double-membrane structures and mitochondrial ER mass, suggesting defective autophagy as the main process responsible for the observed alterations in DC functions.

Vps34 has been implicated in both canonical and noncanonical autophagy (31). During the induction of canonical autophagy the preinitiation complex triggers recruitment of the Beclin1–Vps34 complex, followed by induction of Vps34 kinase activity, recruitment of multiple Atg proteins, and lipidation of LC3. This process allows the formation of double-membrane autophagosomal structures, which ultimately fuse with lysosomes to degrade the contents of the autophagosomes (9). In our previous studies we demonstrated that deletion of Vps34 in T cells, heart, or liver results in profound defects in canonical autophagy and in the loss of normal cellular function in vivo (11, 13). Vps34 deficiency in DCs similarly caused defects in canonical autophagy, resulting in the complete loss of visible double-membrane autophagosomal structures, the accumulation of cellular organelles, and increases in ER and mitochondrial mass. In the noncanonical autophagy pathway, TLR activation induces the recruitment of several Atg proteins such as LC3 to a single-membrane phagosome. Such LC3-enriched phagosomes degrade their contents more efficiently and thus modulate immune responses triggered by the engulfed cargo (32). This process of noncanonical autophagy, also known as “LAP,” requires the activity of the Beclin1–Vps34 complex before recruitment of LC3 to phagosomes (33, 34). A recent study has demonstrated that the Vps34 complex is critically required for the induction of LAP and for canonical autophagy in macrophages (26). Therefore the spontaneous activation and cytokine secretion by Vps34-deficient DCs may be potentially attributed to deficiencies in the anti-inflammatory activity of canonical autophagy, to LAP, or to both. Our results with mice deficient in Rubicon, which binds Vps34 and is required for LAP but not for canonical autophagy (26), suggest that defects in Vps34-deficient DCs are not caused by defective LAP. Although these findings are consistent with defective canonical autophagy being the cause of the observed phenotype of Vps34-deficient DCs, defects in additional cellular processes that involve Vps34 may contribute as well.

Fig. 8. Defective CTL responses to dead cell-associated antigens in Vps34<sup>-/-</sup>CD11c-Cre mice. The indicated mice were immunized with apoptotic, OVA-loaded TAP<sup> +/-</sup> splenocytes. Seven days later, spleen cells were stimulated with or without OVA<sub>323-339</sub> peptide for 6 h, and IFN-γ-producing CD8<sup>+</sup> T cells were detected by flow cytometry. As a control, cells were stimulated with phorbol myristate acetate plus ionomycin (PMA +ION). Representative flow cytometry plots from two individual experiments (A) and a summary of the percentage of IFN-γ<sup>-</sup>CD8<sup>+</sup> cells (B) are shown; n = 5 per group. *P < 0.01.

Autophagy has been shown to play a role in processing antigens for presentation to MHC class I- and class II-restricted T cells (4). Surprisingly, we found enhanced antigen presentation via the conventional MHC class I and class II pathways by Vps34-deficient DCs. Our electron microscopic analyses of Vps34-deficient DCs revealed increased accumulation of ER membranes. Such an expanded ER compartment, rich in the components of the MHC class I processing machinery, may allow efficient processing of cytosolic proteins for presentation onto MHC class I-restricted T cells. Thus, a combination of factors, including increased sensitivity of partially activated Vps34-deficient DCs to IFN-γ stimulation, together with defects in autophagy resulting in an expansion of the MHC class I peptide-loading compartment, may contribute to the observed enhancement in MHC class I-restricted antigen presentation. In professional APCs such as DCs, MHC class II-presented antigens reach phagosomes via endocytosis or pinocytosis. These phagosomes then fuse with MHC class II-containing endosomal compartments in which the antigens are degraded and peptides are loaded onto MHC class II-restricted T cells (35). Although our results showed enhanced MHC class II-restricted antigen presentation by Vps34-deficient DCs, we found a modest defect rather than enhancement in the uptake of antigens by Vps34-deficient DCs. A recent study provided evidence that autophagosomal vesicles constantly fuse with the MHC class II peptide-loading compartment and facilitate antigen presentation (36). The increased accumulation of electron-dense lysosomal structures observed in Vps34-deficient DCs might explain the enhanced MHC class II-restricted antigen presentation.

Targeting Vps34 to inhibit autophagy has recently been considered as an anticancer therapy (37). Autophagy protects cancer cells against metabolic stress such as nutrient starvation and hypoxic conditions. Inhibiting autophagy in cancer cells therefore may reduce resistance to chemotherapy and radiation therapy. A selective Vps34 inhibitor, SAR405, in combination with the mammalian target of rapamycin (mTOR) inhibitor everolimus, was shown to inhibit the proliferation of renal tumor cell lines in vitro (38). Our results suggest that Vps34 inhibition may lead to impaired T-cell–mediated immunity in tumors that may limit the utility of SAR405 in cancer therapy.

Materials and Methods

Reagents, electron microscopy, analyses of mitochondrial and ER stress, Western blotting, lung cell preparation, DC activation experiments, antigen-presentation assays, endocytosis and phagocytosis assays, quantitative real-time PCR, TIM-4 overexpression, and induction of lung metastases are described in SI Materials and Methods.

Mice. Vps34<sup>-/-</sup> (11, 13), Rubicon<sup>-/-</sup> (26), and TAP<sup> +/-</sup> (39) mice have been described. CD11c-Cre, OT-I, and OT-II transgenic mice were obtained from The Jackson Laboratory. All mice were housed under specific pathogen-free conditions and in compliance with guidelines from the Institutional Animal Care and Use Committee at Vanderbilt University.
Isolation of Splenic DCs and Generation of BMDCs. Splenocytes were prepared from wild-type B6.SJL (CD45.1) mice by gentle teasing and were cultured in 6 cm dishes in DMEM containing 10% FCS, 100 U/mL penicillin, and 100 mg/mL streptomycin for 2 h at 37°C. DCs were purified based on the expression of CD11c and MHC class II using FACS as described previously (40), at a final purity greater than 1%.

In Vitro and In Vivo Phagocytosis of Cell Corps. In vitro and in vivo dead cell uptake assays were performed as described (44), with sublethally irradiated, carbofluorescein succinimidyl ester (CFSE)-labeled B6.SJL (CD45.1) splenocytes. In vitro assays apoptotic cells were incubated with DCs (CD45.2) for 3 h at a 1:10 ratio in U-bottomed plates. The cells were washed, and CFSE− cells among CD45.2+CD45.1− cells were examined by flow cytometry. For in vivo assays, CFSE-labeled apoptotic cells were injected into mice (3 × 10^6 cells per mouse), and after 4 h spleen cells were prepared and CFSE− DCs among CD45.2+CD45.1− cells were examined.

In Vivo CD8+ T-Cell Responses. Splenocytes from TAP−/− mice were loaded with 10 ng/mL OVA by osmotic shock, irradiated sublethally, and washed, and mice were injected with 2 × 10^6 cells per mouse. Seven days later, mice were killed, splenocytes were cultured with 1 μM OVA 257−264 peptide for 6 h in the presence of Brefeldin A, and intracellular IFN-γ levels in CD8+ T cells were detected by flow cytometry.

Generation of Bone Marrow Chimeras. B6 (CD45.2) mice were lethally irradiated (1,000 cGy) and 6 h later were injected with 10^6 bone marrow cells derived from wild-type B6.SJL (CD45.1) and Vps34−/−CD11cCre or Vps34+−/− (CD45.2) mice mixed at a 1:1 ratio. Mice were used for experiments at 12 wk after injection of bone marrow cells.

Statistical Analyses. Statistical significance was determined by an unpaired two-tailed Student t test or one-way ANOVA using GraphPad Prism software; P < 0.05 was considered significant.

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Supporting Information

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SI Materials and Methods

**Reagents.** Anti–CD11b-FITC, –CD11c-APC, –CD8α-PE, –IFN-γ-FITC, –CD4-PerCP, –K′-FITC, –D7-FITC, –IAa-FITC, –B20-2-PE, –Gr-1-PE, –CD80-PE, –TIM-4-PE, and –CD4-PE antibodies and isotype controls were obtained from BD Biosciences. Anti-CD11b-APC, –CD11c-PerCP or –Cy5.5, –Sipr-α, –DEC-205-biotin, –CD8α-PE, –CD103-FITC, –F4/80-PE, and –CD86-FITC antibodies and isotype controls were obtained from eBioscience. PMA and ionomycin were obtained from ICN Pharmaceuticals, Inc. OVA, LPS, and lipopolysaccharide acid (LTA) were obtained from Sigma. Flagellin (FLG), poly IC (IC), imiquimod (IMQ), and CpG were obtained from Invivogen. Recombinant GM-CSF and Flt3-L were obtained from PeproTech, and IFN-γ was obtained from BD biosciences.

**Electron Microscopy.** Electron micrographs of BMDCs were generated as previously described for T cells (11).

**Mitochondrial and ER Mass.** Mitochondrial mass and ER mass were measured by staining with MitoTracker red and ER-Tracker green dyes, respectively, according to the manufacturer’s protocol (Molecular Probes). The intensity of the staining was determined by flow cytometry as described (11).

**Western Blotting.** DCs, macrophages, and B cells from the spleens of Vps34f/f or Vps34+ mice were purified by magnetic sorting and were washed three times with PBS before cellular proteins were prepared for Western blotting with anti-Vps34 or anti-β-actin antibodies (Sigma-Aldrich) as described previously (11).

**Preparation of Lung Cells.** Single-cell suspensions of total lung were prepared by collagenase digestion. In brief, paired lungs were minced with a blade and were treated with 1 mg/mL of collagenase D (Sigma) and 0.01 mg/mL of DNase I (Sigma) in plain RPMI 1640 medium for 1 h with occasional stirring. The collagenase digestion was stopped by diluting the cells with complete RPMI medium containing 10% FBS (R-10), and single-cell suspensions were obtained by repeated pipetting. The cells were washed two times with complete medium and were analyzed by flow cytometry.

**In Vitro DC Activation.** Splenic DCs were purified by FACS and cultured in complete medium either alone or in the presence of LPS (1 μg/mL), LTA (10 μg/mL), FLG (1 μg/mL), IMQ (1 μg/mL), IC (1 μg/mL), or CpG (oligodeoxynucleotides, 0.5 μg/mL) for 24 h. Culture supernatants were collected to measure IL-6, TNFα, and IL-10 by CBA (BD Biosciences). BMDCs were activated as described above, and culture supernatants were collected at 48 h for measurement of IL-6 and TNFα by CBA.

**In Vitro Antigen-Presentation Assays.** MHC class I-restricted presentation of OVA antigens was tested by loading BMDCs with OVA by osmotic shock as described (45). These OVA-loaded cells (1–2 × 10^6) were cultured overnight with 10^5 B3Z hybridoma cells (obtained from N. Shastri, University of California, Berkeley, CA) or with SINF6K1-specific OT-I T cells for 24 h, and IL-2 in the supernatant was measured by ELISA. For cross-presentation of OVA to OT-I T cells, splenic CD8α+ and CD8α− DCs were incubated with free OVA (50–250 μg/mL) and OT-I T cells. For cross-presentation of OVA expressed by L. monocytogenes, splenic CD8α+ and CD8α− DCs were incubated with the bacteria (10^5–10^6 cfu) for 2 h, followed by coculture with OT-I T cells. For cross-presentation of OVA by DCs delivered via the DEC205 receptor, CD8α+ and CD8α− DCs were first incubated with anti-DEC-205-biotin antibody at 4 °C, followed by incubation with OVA-antigen delivery reagent (Milenyi Biotec) according to the manufacturer’s instructions. The DCs were further cultured for 1 h at 37 °C and were cocultured with OT-I T cells. To determine the presentation of LCMV-specific peptides to MHC class I-restricted T cells, short-term CD8+ T-cell lines specific for the LCMV peptides NP205-312 and NP33-41 were generated as described previously (46). Splenic DCs were infected with LCMV virus for 3 h as described (42) and were washed twice with complete medium. Varying numbers of infected DCs were cultured with 10^5 LCMV-specific T cells, and culture supernatants were collected at 36 h for measurement of IFN-γ by ELISA.

MHC class II-restricted antigen presentation of OVA antigens was tested by culture of sorted DCs with OVA122-139 peptide or soluble OVA (25–100 μg/mL) in the presence of purified CD4+ T cells (10^6) derived from OT-II TCR transgenic mice. Culture supernatants were collected at 24 h, and IL-2 was measured by ELISA. We assessed MHC class II-restricted presentation of the E602-620 peptide by I-A^d as described (47). Briefly, BMDCs were incubated with 50 μg/mL of GFP-Ex chimeric protein for 3 h. Cells then were stained with Y. Ae antibodies (eBioscience) specific for Ex-derived E602-620 peptide bound with I-A^d molecules.

**Endocytosis and Phagocytosis Assays.** To determine the uptake of soluble antigens by DCs, 10^5 BMDCs were incubated with 50 μg/mL of GFP-Ex chimera protein for 2 h. The cells were washed, and fluorescence was measured by flow cytometry. For phagocytosis assays, fluorescently labeled latex beads (Sigma) were incubated with 10^5 BMDCs for 2 h, washed, and analyzed by flow cytometry. Similarly, FITC-labeled C. rodentium bacteria were incubated at a ratio of 1:100 in 0.5 mL medium for 2 h. Cells were washed and were analyzed by flow cytometry. As a control, cytocolasin D (Cyl D) was preincubated with DCs for 45 min before the addition of GFP-Ex, latex beads, or FITC-labeled C. rodentium.

**Quantitative Real-Time PCR.** Total RNA was prepared using TRIzol reagent (Invitrogen), and real-time PCR was performed as described previously (11). Expression was normalized to GAPDH. Primer sequences were GAPDH forward: 5′-TCAACACGCAA-CTCCCACTCTCCTCA-3′, GAPDH reverse: 5′-ACCTGTGTTG-CTGTAGCCGTATTCA-3′; TIM-4 forward: 5′-GAAITC TCCAGGAAGTCAAC-3′, TIM-4 reverse: 5′-GTTGTGTGTG-GCTCTCTCAG-3′; CD4 forward: 5′-CTCTGTCCTAAAA-GCGGTCTTAC-3′, CD4 reverse: 5′-TGGCGGAGGTITCA AGATGTT-3′; CD36 forward: 5′-GAACCACTGTCCATTCAA- AACTGG-3′, CD36 reverse: 5′-TCTGGTTTCTTGGCCAGTCA-3′; and CD91 forward: 5′-GACAGGTGTGTTGAGCA CAGATG-3′, CD91 reverse: 5′-AGTCGTTTGTCCTCGTCA CTCC-3′.

**TIM-4 Overexpression.** A TIM-4–expressing lentivirus vector was obtained from OriGene. This construct then was used to transfect DC2.4 cells (24) or wild-type BMDCs using a Nucleofector device and kit (Lonza).

**Determination of B16 Melanoma Lung Metastases.** Mice were injected i.v. with 5 × 10^6 B16 melanoma cells suspended in PBS. After 15–17 d of challenge with melanoma cells, mice were killed, lungs were removed, and the number of metastatic nodules was counted as described previously (48).
**Fig. S1.** Vps34\textsuperscript{f/f};CD11c-Cre mice lack Vps34 expression in DCs but not in macrophages or B cells. DCs, macrophages, and B cells from the spleens of Vps34\textsuperscript{f/f} or Vps34\textsuperscript{f/f};CD11c-Cre mice were enriched by magnetic sorting. Proteins were prepared, and immunoblot analysis was performed using antibodies against Vps34 and \(\beta\)-actin. Representative data from two independent experiments are shown.

**Fig. S2.** Lung myeloid cell populations. The percentages of CD11c\textsuperscript{int}CD11b\textsuperscript{int} and CD11b\textsuperscript{hi}CD11c\textsuperscript{−} cells depicted in Fig. 1\textit{F} are shown. Data are pooled from two independent experiments with five mice in each experimental group.
Fig. S3. T, B, and NK cells in Vps34\textsuperscript{ff};CD11c-Cre mice. Shown are the percentage and absolute numbers of CD8\textsuperscript{+} T cells, CD4\textsuperscript{+} T cells, NK cells, and B cells in the spleen of Vps34\textsuperscript{ff} or Vps34\textsuperscript{ff};CD11c-Cre mice. Data are pooled from three experiments with five to seven mice per group. *P < 0.01.

Fig. S4. Effects of IFN-\gamma and LPS on the induction of MHC class II expression by DCs. Total splenic DCs from the indicated mice were purified and were stimulated with or without 15 ng/mL of IFN-\gamma or 1 \mu g/mL of LPS for 16 h. The expression of MHC class II (IA\textsuperscript{b}) was measured by flow cytometry. The mean intensity of MHC class II expression pooled from the results of three separate experiments is shown.
Fig. S5. Capacity of Vps34 mutant DCs to phagocytose particulate matter, soluble matter, and bacteria. BMDCs from Vps34\textsuperscript{fl/fl} or Vps34\textsuperscript{fl/fl};CD11c-Cre mice were incubated with (+) or without (−) fluorescent latex beads, E\textalpha-GFP chimeric protein (50 μg/mL), or FITC-labeled C. rodentium (C. rod, 1:100 ratio) for 2 h or 4 h; then cells were washed and analyzed by flow cytometry. As a control, cells were preincubated with Ccl D (10 μM), a phagocytosis inhibitor, for 45 min. Representative flow cytometry plots from four individual experiments are shown.

Fig. S6. DCs from CD11c-Cre mice are similar to those from wild-type mice. (A) The frequency of CD8α\textsuperscript{+} DCs in the spleen of Vps34\textsuperscript{fl/fl}, Vps34\textsuperscript{fl/fl};CD11c-Cre, and CD11c-Cre mice. The data shown are pooled from two independent experiments with five mice in each experimental group. *P < 0.01. (B) Expression of TIM-4 on subsets of CD8α\textsuperscript{+} DCs in the spleen of Vps34\textsuperscript{fl/fl}, Vps34\textsuperscript{fl/fl};CD11c-Cre, and CD11c-Cre mice. The results shown are from one experiment representative of three independent experiments. (C) Splenic DCs were purified by FACS from the indicated groups of mice and were cultured for 24 h. Culture supernatants were collected to measure TNFα, IL-6, and IL-10 by CBA. The data shown are representative of three independent experiments with six mice in each experimental group. The error bars indicate the means ± SD of triplicate wells.
Fig. S7. TIM-4 expression and dead cell uptake by DCs in bone marrow chimeras. Mixed bone marrow chimeras were generated in lethally irradiated B6 recipient mice (CD45.2) by transfer of $10^7$ donor bone marrow cells from wild-type B6.SJL mice (CD45.1) and Vps34$^{ff}$;CD11c-Cre mice (CD45.2) mixed at a 1:1 ratio. Mice were analyzed 12 wk later. (A) Spleen cells from the chimeric mice were analyzed for the expression of TIM-4 on CD8$^\alpha^+$ and CD8$^\alpha^-$ DCs among CD45.1$^+$ wild-type and CD45.2$^+$ Vps34$^{ff}$;CD11c-Cre DCs. (B) A dead cell uptake assay was performed as described in Fig. 6C. In this experiment, the dead cells were derived from the spleens of mice coexpressing CD45.1 and CD45.2 (F1 animals from the cross of CD45.1- and CD45.2-expressing mice), to distinguish between Vps34$^{ff}$;CD11c-Cre cells (CD45.2$^+$), wild-type cells (CD45.1$^+$), and CD45.1$^+$CD45.2$^+$ double-positive dead cells. (C) A summary of the experiment in B. Data are pooled from two individual experiments with five mice in each experimental group. *P < 0.01.
Fig. S8. Vps34^ff and Vps34^ff,CD11c-Cre DC cocultures. (A) Wild-type (CD45.1) DCs and Vps34^ff or Vps34^ff,CD11c-Cre (CD45.2) DCs were cultured alone or were cocultured at a ratio of 1:1 at a final density of 4 × 10^5 cells per well in U-bottomed plates. After 24 h, the expression of TIM-4 was measured on the surface of CD45.2^CD8α^ DCs. (B) Wild-type (CD45.1) splenocytes and Vps34^ff or Vps34^ff,CD11c-Cre (CD45.2) DCs were cultured alone or were cocultured at a ratio of 1:1 at a final density of 4 × 10^5 cells per well in U-bottomed plates. After 24 h, the expression of TIM-4 was measured on the surface of CD48α^ DCs. (C) Vps34^ff,CD11c-Cre (CD45.2) splenocytes were cocultured with wild-type (CD45.1) DCs or Vps34^ff,CD11c-Cre (CD45.2) DCs at a 1:1 ratio and at a final density of 4 × 10^5 cells per well in U-bottomed plates. After 24 h, the expression of TIM-4 was measured on the surface of CD8α^ DCs. An experiment representative of two independent experiments is shown. (D) TIM-4 expression was measured on the surface of CD8α^ DCs derived from Vps34^ff or Vps34^ff,CD11c-Cre mice stimulated with recombinant mouse IL-10 (10 ng/mL) for 24 h. An experiment representative of two independent experiments is shown.
Fig. S9. Cellular organelles and transmission electron microscopy of DCs. (A) Electron micrographs of BMDCs generated from Vps34f/f and Vps34f/f;CD11c-Cre mice. The arrows indicate double-membrane autophagosomes in the Vps34f/f DCs. Note the accumulation of ER, Golgi, and numerous electron-dense lysosomal structures in DCs from Vps34f/f;CD11c-Cre mice. Images representative of at least 50 individual cells are shown. (Scale bars, 1 μm.) (B) Splenic DCs derived from Vps34f/f and Vps34f/f;CD11c-Cre mice were purified and stained with anti-CD11c antibodies and MitoTracker red or ER-Tracker green dye according to the manufacturer’s protocol. The mitochondrial and ER staining were measured by flow cytometry. An experiment representative of two independent experiments with six mice in each group is shown. *P < 0.01.

Fig. S10. TIM-4 expression on lung myeloid cells and frequency of CD11b+Gr1+ cells in the spleen. (A) TIM-4 expression was measured on CD11b−CD11chi, CD11bhiCD11c−, and CD11binCD11cin populations of myeloid cells in the lungs of Vps34f/f and Vps34f/f;CD11c-Cre mice as shown in Fig. 1F. Pooled data from two separate experiments with five mice are shown. *P < 0.01. (B) The frequency of CD11b+Gr1+ myeloid-derived suppressor cells is shown. Data from a representative experiment with five mice in each experimental group are shown. NS, not significant.