Critical role for Sec22b-dependent antigen cross-presentation in antitumor immunity

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CD8+ T cells mediate antigen–specific immune responses that can induce rejection of solid tumors. In this process, dendritic cells (DCs) are thought to take up tumor antigens, which are processed into peptides and loaded onto MHC-I molecules, a process called “cross-presentation.” Neither the actual contribution of cross-presentation to antitumor immune responses nor the intracellular pathways involved in vivo are clearly established because of the lack of experimental tools to manipulate this process. To develop such tools, we generated mice bearing a conditional DC-specific mutation in the sec22b gene, a critical regulator of endoplasmic reticulum–phagosome traffic required for cross-presentation. DCs from these mice show impaired cross-presentation ex vivo and defective cross-priming of CD8+ T cell responses in vivo. These mice are also defective for antitumor immune responses and are resistant to treatment with anti-PD-1. We conclude that Sec22b−dependent cross-presentation in DCs is required to initiate CD8+ T cell responses to dead cells and to induce effective antitumor immune responses during anti–PD-1 treatment in mice.

INTRODUCTION

DCs are a specialized population of immune cells that excel in antigen presentation and induce adaptive immune responses (Mellman and Steinman, 2001). Like other cells, DCs can present peptides derived from cytosolic antigens loaded on MHC class I to CD8+ T cells and to both endogenous and exogenous antigens bound to MHC class II molecules for recognition by CD4+ T cells. In addition, DCs can take up exogenous antigens and process and load them onto MHC class I molecules to be presented to CD8+ T cells, a process called antigen “cross-presentation” (the resulting induction of a CD8+ T cell response is referred to as “cross-priming”; Joffre et al., 2012).

Several pathways of antigen cross-presentation that involve membrane trafficking through different intracellular compartments were reported in cultured DCs (Savina et al., 2006, 2009; Jancic et al., 2007; Cebran et al., 2011; Nair-Gupta et al., 2014; Alloatti et al., 2015). One of the described cross-presentation pathways requires transfer of ER resident proteins, including the machinery for MHC class I loading with peptides (TAP1/2 transporters, tapasin, calreticulin, etc.), to the endocytic and phagocytic pathways, a traffic step controlled by the SNARE family member Sec22b (Cebran et al., 2011).

The actual contribution of different antigen cross-presentation pathways to immune responses in vivo remains unclear. The K. Murphy group (Hildner et al., 2008) has shown that certain subsets of cross-presenting DCs (i.e., Batf3-dependent DCs) have a critical role in antiviral immune responses and in the rejection of established solid tumors by CD8+ T cells. Recently, the R. Germain group (Castellino et al., 2006; Eickhoff et al., 2015) showed that CD8+ DCs act as “cellular platforms” to support CD4+ T cell help for CD8+ responses, a role that goes beyond their cross-presentation capacities. In contrast, increasing examples of CD8+ DCs cross-presenting antigen in vivo are being reported (den Haan et al., 2000; Kamphorst et al., 2010). The actual contribution of antigen cross-presentation by DCs to specific immune responses is, therefore, a critical unknown.

This is particularly true in the context of immunotherapies that attempt to harness the immune system to treat cancer, including those using checkpoint inhibitors. Expression of programmed cell death protein-1 (PD-1) on the surface of tumor-specific lymphocytes, and interaction with its corresponding ligands (PD-L1 and PD-L2, respectively) on the tumor- or antigen-presenting target cells is a key immune checkpoint that inhibits T cell function. Seminal studies in mouse models of cancer and diverse clinical studies have
established that mAbs blocking the PD-1/PD-L1 pathway, as well as other checkpoints, such as CTLA-4, can unleash the immune system to fight cancer (Leach et al., 1996; Iwai et al., 2002). These therapies can mediate tumor regression in patients with metastatic melanoma, non–small cell lung cancer and renal cell carcinoma, among others (Hodi et al., 2010; Topalian et al., 2012; Lebbé et al., 2014). In mice, anti-immune, checkpoint-based treatments have been analyzed with success in several tumor models. The Melero laboratory (Sánchez-Paulete et al., 2016) has shown recently that Batf3-dependent DCs actively contribute to rejection of tumors during anti–PD-1 and anti-CD137 immunotherapies.

To define the contribution of antigen cross-presentation to CD8+ T cell responses, we generated a mouse line in which the expression of Sec22b was conditionally depleted in DCs. Reduced Sec22b expression in DCs impairs antigen cross-presentation and cross-priming of cell-associated antigens in vivo. Sec22b-defective mice also failed to mount effective antitumor immune responses, to control the growth of immunogenic tumors, and to respond to anti–PD-1–based immunotherapy. These results show that Sec22b-dependent antigen cross-presentation is required during cross-priming of CD8+ T cell responses with dead cell–derived antigens and for anti-checkpoint-tumor immunotherapy in mice.

RESULTS AND DISCUSSION

To investigate the role of Sec22b-dependent cross-presentation in vivo, we generated floxed sec22b knock-in mice and crossed them to CD11c-specific Cre-deleter mice (Caton et al., 2007). We thus obtained mice bearing a selective deletion of the sec22b gene in DCs (Sec22b−/−). As controls, throughout the study, we used littermates expressing the Cre recombinase, and WT alleles of the sec22b gene (Sec22b+/+). Western blot analysis of splenic CD11c+ cells isolated by two populations from spleen, thymus, blood, lungs, and different lymph nodes were affected in Sec22b-defective mice (Fig. S1, A–F). Sec22b depletion did not modify the capacity of splenic CD8+ DCs to respond to different stimuli, such as LPS, IFN-γ, and TNF, as measured by expression of MHC II and CD86 (Fig. S1 G). We also confirmed by Western blot that Sec22b expression was reduced in BMDCs generated from Sec22b−/− mice, as compared with littermates (Fig. 1 D). Sec22b depletion did not affect the percentages of CD11c+CD11b+ cells in culture (Fig. 1 E, left) or LPS-mediated activation, as detected by expression of costimulatory molecules, (CD40, CD80, and CD86 and MHC class II; Fig. 1 E, right). IFN-γ and TNF induced an equivalent augment in the expression of CD86 and MHC II in both Sec22b+/+ and Sec22b−/− mice (Fig. S1 H). Finally, levels of MHC class I expression (both H2-Kb and H2-Dk) were not affected by Sec22b depletion (Fig. 1 F). Therefore, defective Sec22b expression in DCs does not affect their development, survival, or activation capacity in vitro or in vivo.

As a first analysis pertaining to DC function, we assessed the phagocytic and endocytic capacities of BMDCs. Both endocytosis and phagocytosis were similar in bone marrow–derived DCs (BMDCs) generated from Sec22b+/+ or Sec22b−/− mice (Fig. 2, A and B, respectively). To investigate whether Sec22b-defective DCs bear a defect in antigen cross-presentation, we first generated BMDCs from Sec22b−/− and control littermates. As shown in Fig. 2 C, BMDCs from Sec22b-deficient mice bear a partial defect in cross-presentation of soluble and bead-bound OVA, as detected by B3Z activation (top; Kurts et al., 1996), CD69 and CD25 expression (middle), and proliferation of OT-I CD8+ T cells, measured by dilution of carboxyfluorescein succinimidyl ester (CFSE; bottom). The synthetic MHC class I–restricted OVA peptide (SIINFEKL) was presented with equal efficacy by BMDCs generated from Sec22b+/+ and Sec22b−/− mice (Fig. 2 C, left).

In addition, as an alternative source of antigen, we used vaccinia virus (VACV)–OVA–infected RAW macrophages (H2-Kb; RAW-VACV), which transmit virus and infect DCs that can direct present antigens in H2-Kb MHC class I molecules. Alternatively, infected RAW cells were treated with UV light (RAW-VACV-UV) to inactivate the virus, blocking direct infection of DCs and leaving available only the cross-presentation route, as previously described (Iborra et al., 2012). We also used uninfected RAW cells treated with UV (RAW-UV) to test antigen specificity. BMDCs generated from Sec22b+/+ and Sec22b−/− mice were exposed to VACV-infected or control cells for 4 h and used to stimulate preactivated OT-I T cells or CD8+ T cells purified from WT mice previously infected with VACVWT. Production of IFN-γ by OT-I (Fig. 2 D, top) or vaccinia–specific effector CD8+ T cells (Fig. 2 D, bottom) was not affected by Sec22b–depletion in DCs directly infected with the virus (Fig. 2 D, left) but was impaired in DCs co-cultured with RAW-VACV-UV (Fig. 2 D, right). Moreover, MHC class II–restricted antigen presentation, as measured by activation and proliferation of OT-II CD4+ OVA–specific TCR transgenic T cells, was not affected in Sec22b–deficient BMDCs (Fig. 2 E). Therefore, in agreement with our previous results in Sec22b knockdown BMDCs (Cebrian et al., 2011), BMDCs generated from Sec22b–defective mice display impaired cross-presentation, but conventional MHC class I and II antigen presentation is not affected.

Similar to BMDCs, CD11c+ splenic DCs isolated from Sec22b−/− mice also cross-present OVA to CD8+ T
cells less efficiently than do those purified from littermates (Fig. 3 A), whereas MHC class II–restricted presentation to CD4+ T cells is unaffected (Fig. 3 B). To investigate the role of Sec22b depletion in DCs in cross-priming, we first tested whether CD8+ T cells responses were normal in Sec22b−/− mice. We immunizedSec22b+/+ and Sec22b−/− mice with IFA-CpG-SIINFEKL in the footpad and analyzed anti-OVA and anti-SIINFEKL responses by IFN-γ ELISPOT in draining popliteal and inguinal lymph nodes. CD8+ T cells from Sec22b+/+ and Sec22b−/− were equally restimulated with SIINFEKL (Fig. S2 A), whereas the restimulation with OVA was impaired in Sec22b−/− mice. These results indicate that Sec22b−/− mice have a functional CD8+ T cells compartment and that the SIINFEKL–specific repertoire is normal.

To determine whether the defect in antigen cross-presentation in Sec22b-depleted DCs results in defective cross-priming in vivo, we injected necrotic, OVA-expressing 3T3-RIPK3-OVA cells (Yatim et al., 2015) subcutaneously.
The endogenous CD8^+ T cell response against OVA was analyzed using MHC-I multimers (Yatim et al., 2015) after 9 d (Fig. 3 C). In Sec22b^+/+ mice, the endogenous cytotoxic CD8^+ T cell response was significantly decreased from 2.134 ± 0.2835 to 0.6381 ± 0.1887%, as compared with control littermates (Fig. 3 D for gating strategy; Fig. 3 E). The proportion of migratory and resident DCs in lymph nodes was not affected by Sec22 depletion (Fig. 3 F, left, for gating; middle, for percentages; right, for cell numbers), indicating that the differences in cross-priming capacity are not the consequence of impaired migration capacity of Sec22b^−/− DCs from the periphery. In addition, the number and percentages of the putative cross-presenting subsets CD80^+ resident DCs and CD103^+ migratory DCs were not modified by depletion of Sec22b (Fig. S2, B and C). We conclude that cross-priming of dead cell–associated antigens in vivo requires Sec22b expression in DCs.
The results presented thus far indicate that Sec22b−/− mice represent a suitable model to investigate the contribution of antigen cross-presentation to immune responses. To evaluate the role of cross-presentation in antitumor immunity, we first analyzed the growth of a highly immunogenic OVA-expressing tumor cell line, EG7 (Moore et al., 1988; Helmich and Dutton, 2001). EG7 tumors grew faster in Sec22b−/− mice (Fig. 4, A and B), causing decreased survival (Fig. 4 C), as compared with littermates. That insufficiency to control tumors correlated with impaired OVA-specific CD8+ T cell responses (Fig. 4 D and Fig. S3 A). These results suggest that impaired cross-presentation of tumor antigens by Sec22-deficient DCs causes reduced antitumor immune responses and exacerbated tumor growth.

To address the possible role of Sec22b-dependent antigen cross-presentation in tumor immunotherapy by checkpoint blocking antibodies, we used a less-immunogenic tumor, OVA-secreting MCA101 (Zeelenberg et al., 2008; Sedlik et al., 2014). CD8+ T cells from Sec22b+/+ and Sec22b−/− mice were injected with tumor cells and received or did not receive anti–PD-1 treatment, as described previously (Gubin et al., 2014). As expected, treatment with anti–PD-1 induced tumor rejection in Sec22b+/+ littermates (Fig. 4 E). In contrast, Sec22b−/− mice failed to respond to anti–PD-1 treatment, both in terms of tumor growth and survival (Fig. 4, F and G). Consistently, treatment with anti–PD-1 induced an anti-OVA immune response in littermates, but not in Sec22b−/− mice, when analyzed by T cell restimulation and subsequent secretion of IFN-γ (Fig. 4 H and Fig. S3 B).

To further investigate the mechanism of resistance to anti–PD-1, we measured IFN-γ production and proliferation capacity of CD8+ T cells isolated from tumor (Fig. S3 D). Consistently, treatment with anti–PD-1 induced an anti-OVA immune response in littermates, but not in Sec22b−/− mice, when analyzed by T cell restimulation and subsequent secretion of IFN-γ. DC populations were analyzed by gating on CD11c+ MHC II+ DCs (left), percentages of migratory DCs (migDCs) and resident DCs (resDCs) subpopulations in LN (middle), and cell counting (right). Data shown are means of four independent biological replicates. DC populations were analyzed by gating on CD11c+ MHC II+ DCs (left), percentages of migratory DCs (migDCs) and resident DCs (resDCs) subpopulations in LN (middle), and cell counting (right). Data shown are means of four independent biological replicates. CD8+ T cells from Sec22b+/+ and Sec22b−/− mice were injected with tumor cells and received or did not receive anti–PD-1 treatment, as described previously (Gubin et al., 2014). As expected, treatment with anti–PD-1 induced tumor rejection in Sec22b+/+ littermates (Fig. 4 E). In contrast, Sec22b−/− mice failed to respond to anti–PD-1 treatment, both in terms of tumor growth and survival (Fig. 4, F and G). Consistently, treatment with anti–PD-1 induced an anti-OVA immune response in littermates, but not in Sec22b−/− mice, when analyzed by T cell restimulation and subsequent secretion of IFN-γ (Fig. 4 H and Fig. S3 B).

To further investigate the mechanism of resistance to anti–PD-1, we measured IFN-γ production and proliferation capacity of CD8+ T cells isolated from spleen (Fig. S3 C) and tumor (Fig. S3 D) of mice bearing MCA101–OVA tumors and treated with anti–PD-1. CD8+ T cells from Sec22b−/− produce IFN-γ and proliferate to the same extent as CD8+ T cells from control mice. Expression of PD-1 in T cells from Sec22b+/+ and Sec22b−/− was similar (Fig. S3 E). The expression of PD-L1 and PD-L2 in CD8+ and CD8+/CD11b+ DCs from spleen was not reduced in Sec22b-deficient DCs (Fig. S3 F). These results show a critical, nonredundant role for Sec22b-dependent antigen cross-presentation in the onset of efficient antitumor immune responses induced by checkpoint blockade.
Although cross-priming of allogeneic antigens in vivo was reported >30 yr ago (Bevan, 1976), the actual contribution of antigen cross-priming (vs. other mechanisms such as cross-dressing (Wakim and Bevan, 2011; Yewdell and Dolan, 2011) or Gap junction–mediated peptide transfer (Neeijssen et al., 2005) to cross-priming is still unclear. In the case of cross-priming, multiple intracellular pathways have been reported in vitro. Each of those pathways is under the control of master regulators that control key intracellular traffic steps specific for each pathway. Here, we developed tools to perform loss of function experiments for Sec22b-dependent antigen cross-priming in vivo. Our results show that Sec22b-dependent antigen cross-priming by DCs has a critical role in the cross-priming of cell-associated antigens and antitumor immune responses in vivo (even though we did not exhaustively investigate all of the cell populations that might express CD11c, notably activated B cells that can help priming CD8+ T cell responses). It is possible that alternative cross-priming pathways independent of Sec22b exist, but their contribution to the priming of CD8+ T cell responses in vivo needs to be addressed. Our results show that the induction of tumor-specific CD8+ T cells by anti–PD-1 requires Sec22b expression in DCs, suggesting that, in addition to acting on effector cytotoxic T cells by releasing tumor-imposed
immunosuppression, anti–PD–1 may also facilitate the priming of CD8+ T cells. Whether Sec22b turns out to be a suitable target to modulate antigen cross-presentation therapeutically will need to be addressed in future studies.

**MATERIALS AND METHODS**

**Compounds and antibodies**

For flow cytometry, the following antibodies were used: anti–CD69-eFluor450 (clone H1.2F3, 48–0691-82; eBioscience), anti–CD25–FITC (clone 7D4, 553072; BD), anti–CD8α-PerCP-Cy5.5 (clone 53–6.7, 45–0081–82; eBioscience), anti–TCR νβ 5.1–PE (clone MR9–4, 553190; BD), anti–CD4–PE–Cy7 (clone RM4–5, 552775; BD), anti–CD19–eFluor450 (clone 1D3, 48–0193; eBioscience), anti–CD3–eFluor450 (clone 17A2, 48–0032–80; eBioscience), anti–CD11c–FITC (clone HL3, 553801; BD), anti–CD11b–PerCP-Cytochrome5.5 (clone M1/70, 45–0112–80; eBioscience), anti–F4–80–FITC (clone BM8, eBioscience #11–4801–81), anti–NKp46–PE (clone 29A1.4, 12–3351–80; eBioscience), anti–MHC I (H–2Kb)–FITC (clone AF6–88.5.5.3, 11–5958–80; eBioscience), anti–MHC II (Iab)–eFluor450 (clone AF120.1, 48–5320–80; eBioscience). For Western blot analysis, the anti–Sec22b antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti–α–actin from Sigma–Aldrich (A2066), anti–gsp/G96 from Enzo Life Sciences (spa850). For phenotypic analysis of immune cell populations CD4, CD8, CD25, CD44, CD62L, TCRβ, and CD3 were used as T cell markers (in different fluorophores, depending on the tissue analyzed and the panel of antibodies used). B220 and CD19 were used as B cell markers. TCR–γδ was used as the γδ T cell marker. NK1.1 and NKp46 were used as natural killer markers. Gr–1 and Ly6G were used as granulocyte/neutrophil markers. CD11c was generally used as a DC marker, with the exception of lungs: alveolar macrophages (CD11c<sup>high</sup>) were discriminated from DCs by analyzing Siglec F expression. CD103, I–Ab, H2–Kb, CD11b, CD8, CD40, and CD86 were used to analyze DCs phenotype (in different fluorophores). F4/80 and Ly6C<sup>c</sup> were used as macroage markers. As for the direct MHC I antigen–presentation analysis, anti–mouse antibodies to CD8α, CD44, and IFN–γ were used as conjugates to PE, FITC, and APC, and were obtained from eBioscience. Anti–PD–L1–PE and anti–PD–L2–APC were purchased from eBioscience.

**Cell lines and cell culture**

RAW264.7 macrophages (RAW) were grown in DMEM supplemented with 5 mM glutamine, penicillin, streptomycin, β–mercaptoethanol (all from Invitrogen) and 10% heat–inactivated FBS (Sigma–Aldrich). CD8+ T cells were negatively selected using a cocktail of biotin–conjugated antibodies (anti–CD11c, B220, MHC–II, CD4, NK1.1), followed by incubation with streptavidin microbeads (Miltenyi Biotec). Typical yields by FACS staining were >95% pure. Preactivated OT–I cells were obtained by culturing spleen cells from OT–I transgenic mice (C57BL/6–Tg(TcraTcrb)1100Mjb/J) with 10<sup>–8</sup> M of the peptide<sup>25</sup>SIIIFKEKL<sup>26</sup> from OVA for 5 d. At that time, ∼90% of the cells were CD8<sup>+</sup>CD44<sup>hi</sup>. The 3T3–RIPTK3–OVA cells, expressing a nonsecretable form of OVA cells, were obtained from the Matthew Albert laboratory (Yang et al., 2015) and were cultured in DMEM (Thermo Fisher Scientific) supplemented with 10% FBS (BioWest), 0.1 mM nonessential aa, 1 mM sodium pyruvate, 10 mM Hepes, and 50 µM β–mercaptoethanol (all from Thermo Fisher Scientific). Necroptosis was induced by treatment with a specific drug ligand (AP20187, BB homodimerizer; Takara Bio Inc.). EG7 tumor cells were cultured in DMEM supplemented with 10% FBS (Lonza), as well as 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM Glutamax, and 50 µM β–mercaptoethanol (all from Thermo Fisher Scientific). MCA–101 OVA–secreting cells were grown in RPMI–1640 containing 10% heat–inactivated FBS (BioWest), 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM Glutamax, and 50 µM β–mercaptoethanol (all from Thermo Fisher Scientific), as well as hygromycin B 1 mg/ml (Gibco) for selection. All cell lines were tested as mycoplasma–negative by PCR.

**DC activation**

Maturation of BMDCs was induced by a 16–h treatment with 100 ng/ml of ultrapure LPS from *Escherichia coli* 0111:B4 (InvivoGen) or, alternatively, with 100 ng/ml of TNF–α (Protein Service Facility, Inflammation Research Center) or IFN–γ (Invitrogen). DC maturation was controlled by cell–surface expression of costimulatory molecules and MHC class II molecules using specific antibodies. Maturation of splenic CD11c<sup>c</sup> DCs was induced by a 4–h treatment with 100 ng/ml of LPS, TNF, or IFN–γ.

**Virus strains**

The rVACV–OVA and VACV WR strains were gifts from Jonathan W. Yewdell and Jack R. Bennink (National Institutes of Health, Bethesda, MD). Stocks were grown in CV–1 monolayers and used as clarified sonicated cell extracts.

**Primary cell isolation and culture**

BMDCs were produced by culturing the cells for 10 d in GM–CSF–containing medium (as described in Alloatti et al., 2015), with IMDM (Sigma–Aldrich and Thermo Fisher Scientific) containing 10% heat–inactivated FBS (BioWest), 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM Glutamax, and 50 µM β–mercaptoethanol (all from Thermo Fisher Scientific). Supernatant from J558 plasmacytoma cells was used as the granulocyte macrophage colony–stimulating factor source (Winzler et al., 1997). To obtain splenic CD11c<sup>c</sup> DCs, each mouse spleen was injected with 2 ml of a digestion solution with 0.1 mg/ml Liberase (Roche), 0.1 mg/ml DNase I (Roche), 1× PenStrep in RPMI–1640 (both from Thermo Fisher Scientific), following the Roche instructions, and was cut into small pieces and incubated for 25 min at 37°C. Reaction was stopped by addition of complete medium. Spleens were passed through a cell strainer and centrifuged at 200× g.
for 5 min. After red blood cell lysis (Sigma-Aldrich), splenic DCs were isolated by performing one round of negative selection for CD11c+ cells (using EasySep Mouse Pan-DC enrichment kit from STEMCELL Technologies), followed by a round of positive selection using CD11c microbeads (Miltenyi Biotec), according to the manufacturer’s instructions. In all experiments, purity of cells was >90%. For phenotypic characterization of immune cells, thymus and lungs were processed by digestion with Liberase/DNase I as stated above and was subsequently analyzed by flow cytometry.

Animals
C57BL/6J mice and C57BL/6J recombination activating gene 1-deficient OT-I and OT-II TCR. (Vα2, Vβ5.1) transgenic mice were obtained from Charles River, Janvier, and Centre de Distribution, Typage et Archive Animal. Mice were between 5 and 12 wk old. WT C57BL/6J mice for tumor experiments were always obtained from the aforementioned sources.

Sec22bFlox/Flox (see below) and control mice were originally produced at the Centre d’Immunologie de Marseille (the Malissen group) and were subsequently bred in the animal facility of Institut Curie. CD11c-Cre transgenic mice were obtained from The Jackson Laboratory. All animal procedures were in accordance with the guidelines and regulations of the Institut Curie veterinary department, and all mice used were <6 mo old.

Targeting strategy for generation of Sec22b mice. A genomic fragment encompassing exon 2 and 4 of the sec22b gene was isolated from a BAC clone of B6 origin. Using ET recombination, loxP sites were introduced in the introns flanking the 5’ and 3’ ends of exon 2. The loxP site in the intron found at the 3’ end of exon 2 was abutted to a ftr-neoR-frt cassette. The final targeting construct was abutted to a cassette coding for thymidine kinase and linearized with Pme1.

ES clone selection. JM8.F6 C57BL/6N ES cells (Pettitt et al., 2009) were electroporated with the targeting vector. After selection in G418 and ganciclovir, ES cell clones were screened for proper homologous recombination by Southern blot and PCR analysis. A neomycin-specific probe was used to ensure that adventitious, nonhomologous recombination events had not occurred in the selected ES clones. Properly recombined ES cells were injected into FVB blastocysts. Upon germine transmission, mice were then crossed to flipper mice to delete the ftr-flanked neoR cassette, and the resulting floxed Sec22b allele (official name B6–Sec22bfltm1Ciphe, called here Sec22bflox) was identified by PCR of tail DNA. The pair of primers: sense 5’-ATGGTAAAGACGACCAATAACTTTTC-3’ and antisense 5’-TGGAGTACCTTGAAGCTGAGAA-G-3’ amplified a 600-bp band in the case of the Sec22blox allele and a 900-bp band in the case of the Sec22bfl allele. The excised Sec22bfl allele generated a band of 300 bp (animals were screened for breeding and experiments based on the presence of this 300-bp band). Considering that the phase of the introns flanking exon 2 is asymmetrical, deletion of exon 2 results in an out-of-frame Sec22b allele.

Western blotting
Total cell lysates from BMDCs and splenic DCs were subjected to 4–12% gradient gels (Thermo Fisher Scientific) or 10% and 12% gels and were separated by SDS-PAGE. After transfer to nitrocellulose membranes, they were blocked and incubated with primary antibodies and peroxidase-conjugated secondary antibodies. Bound antibodies were revealed using the BM chemiluminescence blotting substrate (POD) from Roche or GE Healthcare, according to the manufacturers’ directions. The intensity of the bands was quantified by densitometry using Quantity One 4.6.6 software (Bio-Rad Laboratories) and was expressed as arbitrary units.

Antigen uptake assay
Phagocytosis and the endocytic capacity of BMDCs were assessed using 3-µm blue latex beads (Polysciences) and OVA Alexa Fluor 488 conjugate (O34781; Thermo Fisher Scientific), respectively.

Viral infections and virus titration.
Mice were infected intradermally in the ears with 5 × 10⁶ PFU of the required VACV WR strain, as previously described (Iborra et al., 2012).

Antigen presentation assays
Proliferation assay. DCs were incubated with Low Endo soluble OVA from Worthington Biochemical Corporation (LS003061) or with 3-µm beads coated with different ratios of OVA and BSA proteins (OVA 10 mg/ml alone; OVA 2.5 mg/ml–BSA 7.5 mg/ml; OVA 5 mg/ml–BSA 5 mg/ml; and BSA 10 mg/ml alone) or different concentrations of the control minimal peptide (OVA SIINFEKL for cross-presentation and OVA233–249 for MHC II presentation). After 5 h, DCs were washed three times with PBS containing 0.1% (vol/vol) BSA and co-cultured with purified CFSE-OT-I CD8⁺ for 3 d. For monitoring T cell proliferation, diminution of CFSE staining on TCR⁺ CD8⁺ populations was measured by flow cytometry.

T cell activation assay. DCs were incubated for 5 h with different concentrations of Low Endo soluble OVA from Worthington Biochemical Corporation (LS003061) or with 3-µm beads coated with different ratios of OVA and BSA proteins (as mentioned above). Minimal peptide OVA233–249 was used as a control for the capacity of DCs to activate T cells. Next, DCs were washed three times with 0.1% (vol/vol) PBS/BSA, fixed with 0.008% (vol/vol) glutaraldehyde during 10 min at 4°C, washed twice with 0.2 M glycine and once with 0.1% (vol/vol) PBS/BSA, and finally, B3Z hybrid T cells were added. After 16 h, T cell activation was measured by detecting
β-galactosidase activity by OD at 590 nm using chlorophenol red-β-D-galactopyranoside as the substrate for the reaction. The efficiency of antigen presentation on MHC I and MHC II was also evaluated using OT-I (CD8+ and OT-II (CD4+ T) cells, respectively. Where indicated, DCs were fixed with 0.008% of glutaraldehyde before T cell addition. The percentage of CD25+CD69+ T cells was measured by flow cytometry after 16 h of co-culture (MHC I and II–restricted peptides, OVA257–264 and OVA323–339, respectively, were used as controls).

Direct MHC class I antigen presentation and cross-presentation assay with VACV. DCs were stimulated by co-culture with VACV-OVA–infected RAW cells treated with or without UV irradiation to inactivate the virus. To test DC cross-presenting ability, RAW cells were irradiated with UVC (240 mJ/cm²) either without exposure to VACV-OVA (RAW UV) or after incubation with VACV for 4 h (RAW-VACV-UV). Alternatively, infected RAW cells were left unirradiated (RAW-VACV) to allow direct infection of DCs. 16 h after UV irradiation, RAW cells were co-cultured for 4 h with BMDCs generated from Sec22b+/+ and Sec22b−/− mice. To the co-cultures, we then added CD8+ T cells purified from splenocytes of mice intradermally injected 7 d earlier with WR VACV, or preactivated OT-I cells were added to the cultures for 6 h; then, Brefeldin A (5 µg/ml; Sigma-Aldrich) was added for the last 4 h of culture. Cells were then stained with PE-anti-CD8α and FITC-CD44, fixed in 4% PFA, and incubated with APC-anti-IFN-γ during permeabilization with 0.1% saponin. A mean of 10,000 of each T cell subset was analyzed in each sample. Background activation obtained with CD8+ T cells nonpulsed with any peptide (0–0.3%) was subtracted.

In vivo cross-priming assay with 3T3–RIPK3–OVA necrototic cells
The cross-priming assay was performed as described in (Yatim et al., 2015). In brief, 3T3–RIPK3–OVA cells were harvested and resuspended in complete media at 5 × 10⁶ cell/ml. Dimerizer was added at 1 µM, and cells were incubated for 10 min at 37°C, gently flicking the tube every 2 min. Ice-cold PBS was added, and cells were washed, counted, and resuspended in cold PBS at 10⁷ cells/ml and were kept on ice until injections. 100 µl of cells (10⁶ cells) were injected intradermally in the flanks of the mice. 9 d later, the spleen and the draining lymph node (inguinal lymph node) were harvested, pooled, and stained for surface markers and Kb− MHC I tetramers specific for OVA presented in the context of MHC class I molecules.

Tumor growth experiments
EG7 tumor assay. Sec22b+/+ and Sec22b−/− mice were injected intradermally with 10⁶ EG7 tumor cells in 100 µl of cold PBS. Tumor cells for injection were recovered from log phase in vitro growth and were injected into the right flank skin of recipient mice. Tumors were clearly visible after 7 d and grew progressively, in an encapsulated fashion. Tumor growth was measured each day and followed until d 20 or when the size reached 1,000 mm³. Afterward, mice were euthanized. 10 mice were used for both Sec22b+/+ and Sec22b−/− groups. To measure CD8+ T cell responses, blood samples were collected from mice 7 d after tumor injections, RBCs were lysed three times with RBC lysis buffer (Sigma-Aldrich), and the nonlysed cells were stained with a couple of Kb− MHC I tetramers specific for OVA presented in the context of MHC class I molecules (provided by the Albert group).

MCA101 OVA-secreting tumor assay and immunotherapy. Sec22b+/+ and Sec22b−/− mice were injected intradermally with 5 × 10⁵ MCA-101 OVA-secreting tumor cells in 100 µl of cold PBS. Tumor cells for injection were recovered from log phase in vitro growth and were injected into the right flank skin of recipient mice. Tumors were clearly visible after 4–5 d and grew progressively, in an encapsulated fashion. Tumor growth was measured every 2 d and was followed until d 30 or until the size reached 1,000 mm³. Afterward, mice were euthanized. 10–15 mice were used for each condition. Anti–PD-L1 treatment consisted of five injections of 200 µg of antibody, delivered on d 0, 3, 6, 9, and 12, i.p. Cold PBS was injected to control groups. Mice were bled on d 13 after tumor injections, and RBCs were lysed as described in the previous section. The unrelated protein human serum albumin, the peptide SIINFEKL, and the PMA/ionomycin were given to nonlysed cells and loaded in MHC I by blood antigen–presenting cells. Subsequent restimulation of OVA-specific T cells was analyzed by IFN-γ ELISpot kit, following the manufacturer’s instructions.

IFN-γ production by CD8+ T cells from spleen and tumors obtained from MCA101–OVA–injected mice. Mice were inoculated with MCA101–OVA tumors and received anti–PD-1 treatment as mentioned above. At d 13, mice were sacrificed, and spleens and tumors were collected and processed with Liberase and DNase I, as described. CD8+ T cells from spleens were isolated with EasySep Mouse Naïve CD8+ T Cell isolation kit, following manufacturer’s instruction, and were loaded with Cell Trace Violet (Invitrogen). In addition, total tumor cells were loaded with Cell Trace Violet. 500,000 cells from both spleens and tumors were plated per well in a flat, 96-well plate coated with CD3 (Miltenyi Biotec) at 1 mg/ml with increasing concentrations of CD28 (0, 0.25, 0.5, and 1 mg/ml). After 72 h, plated T cells were treated with Brefeldin A (5 µg/ml; BioLegend) for 3 h to enhance intracellular IFN-γ accumulation. Subsequently, intracellular staining for IFN-γ, as well as CD3, CD4, CD8, and PD-1 staining, was performed.

Analysis of OVA-specific CD8+ T cells in Sec22b+/+ and Sec22b−/− mice. Mice were immunized in the footpad with 50 µl of IFA–CpG (25 µg per mouse)–SIINFEKL (100 µg per mouse). 8 d later, draining popliteal and inguinal lymph nodes
were collected and smashed with a cell strainer and sub-sequently filtered. 500,000 cells were plated per well, and IFN-γ ELISpot analysis was performed as described earlier using OVA and SIINFEKL as antigens for restimulation.

Statistical analysis. All statistical analyses were performed with Prism 7 (GraphPad Software). P-values, as well as statistical tests, are detailed in the figure legends.

Online supplemental material
Fig. S1 shows the phenotypic characterization of immune cell subsets from Sec22b+/+ and Sec22b−/− mice, as well as the expression of costimulatory molecules upon treatment with LPS, TNF and IFN-γ in DCs from the aforementioned mice. It relates to Fig. 1. Fig. S2 comprises information regarding the immunization of Sec22b+/+ and Sec22b−/− mice to analyze endogenous responses, as well as the phenotypic analysis of putative cross-presenting, migratory and resident DCs. It relates to Fig. 3. Fig. S3 includes a correlative analysis of tumor growth and T cell responses and of the different controls regarding the functionality of CD8+ T cells from Sec22b+/+ and Sec22b−/− mice.

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Author contributions: A. Alloatti designed, carried out, and analyzed all experiments, except those detailed below. D.C. Roohluzen assisted with all T cell assays. L. Ioannas performed tumor experiments and assisted with all animal work. J.-M. Carpier assisted with tumor experiments and Western blotting. S. Ioora carried out all vaccinia virus assays. J.G. Magalhães assisted in assay development and the EG7 tumor experiment. P. Kozik assisted with tumor experiments and cross-presentation assays. N. Yatim assisted with necrotic cell assays. N. Yatim and M.L. Albert assisted with experimental design on the RipC3 experiments. D. Sancho conceived the vaccinia virus experiments. S. Amigorena conceived and supervised the study, and A. Alloatti and S. Amigorena wrote the manuscript.

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The authors regret that in the original version of their paper, they mistakenly used the phrase OVA-expressing cells instead of OVA-secreting cells in parts of the text and cited reference Boissonnas et al. (2007. http://dx.doi.org/10.1084/jem.20061890) instead of Zeelenberg et al. (2008. http://dx.doi.org/10.1158/0008-5472.CAN-07-3163) and Sedlik et al. (2014. http://dx.doi.org/10.3402/jev.v3.24646). The Results and discussion paragraph containing the corrected references and full bibliographic information appear below:

"To address the possible role of Sec22b-dependent antigen cross-presentation in tumor immunotherapy by checkpoint blocking antibodies, we used a less-immunogenic tumor, OVA-secreting MCA-101 (Zeelenberg et al., 2008; Sedlik et al., 2014). This tumor is well controlled by antibodies against CTLA-4 or PD-1 (Gubin et al., 2014). Sec22b−/− and Sec22b+/+ mice were injected with tumor cells and received or did not receive anti–PD-1 treatment, as described previously (Gubin et al., 2014). As expected, treatment with anti–PD-1 induced tumor rejection in Sec22b+/+ littermates (Fig. 4 E). In contrast, Sec22b−/− mice failed to respond to anti–PD-1 treatment, both in terms of tumor growth and survival (Fig. 4, F and G). Consistently, treatment with anti–PD-1 induced an anti-OVA immune response in littermates, but not in Sec22b−/− mice, when analyzed by T cell restimulation and subsequent secretion of IFN-γ (Fig. 4 H and Fig. S3 B)."

Sedlik, C., J. Vigneron, L. Torrietti-Dramard, F. Pitoiset, J. Denizeau, C. Chesneau, P. de la Rochere, O. Lantz, C. Thery, and B. Bellier. 2014. Different immunogenicity but similar antitumor efficacy of two DNA vaccines coding for an antigen secreted in different membrane vesicle-associated forms. J. Extracell. Vesicles. 3:24646. http://dx.doi.org/10.3402/jev.v3.24646

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The online HTML and PDF versions of this paper have been corrected. The errors remain only in the print version.