STIM1 promotes migration, phagosomal maturation and antigen cross-presentation in dendritic cells

Paula Nunes-Hasler1, Sophia Maschalidi2,3, Carla Lippens4, Cyril Castelbou1, Samuel Bouvet1, Daniele Guido3, Flavien Bermont1, Esen Y. Bassoy1, Nicolas Page4, Doron Merkler4, Stéphanie Hugues4, Denis Martinvalet1, Bénédicte Manoury3,6,7 & Nicolas Demaurex1

Antigen cross-presentation by dendritic cells (DC) stimulates cytotoxic T cell activation to promote immunity to intracellular pathogens, viruses and cancer. Phagocytosed antigens generate potent T cell responses, but the signalling and trafficking pathways regulating their cross-presentation are unclear. Here, we show that ablation of the store-operated-Ca2+-entry regulator STIM1 in mouse myeloid cells impairs cross-presentation and DC migration in vivo and in vitro. Stim1 ablation reduces Ca2+ signals, cross-presentation, and chemotaxis in mouse bone-marrow-derived DCs without altering cell differentiation, maturation or phagocytic capacity. Phagosomal pH homoeostasis and ROS production are unaffected by STIM1 deficiency, but phagosomal proteolysis and leucyl aminopeptidase activity, IRAP recruitment, as well as fusion of phagosomes with endosomes and lysosomes are all impaired. These data suggest that STIM1-dependent Ca2+ signalling promotes the delivery of endolysosomal enzymes to phagosomes to enable efficient cross-presentation.
Dendritic cells (DC) are phagocytic immune cells that link innate and adaptive immunity by processing and presenting ingested antigens. One of the unique functions of DCs is cross-presentation, which is a specific type of antigen presentation that occurs via major histocompatibility complex class I (MHC-I) molecules to activate CD8+ T cells and help generate antigen-specific immunity to intracellular pathogens, viruses and cancer cells. Cross-presentation of antigens acquired through phagocytosis produces more potent T cell responses than soluble antigens, and DCs are particularly involved in phagocytosis and transport of large particles (>500 nm) to draining lymph nodes. However, the precise molecular mechanisms by which cross-presentation of phagocytosed antigens occurs are not well understood. Cross-presentation requires a number of proteins normally located in the endoplasmic reticulum (ER), such as tapasin, calreticulin, ERP57 and the translocon Sec611. DC phagosomes are particularly rich in ER proteins, but the signalling and trafficking mechanisms regulating the relationship between the ER and the phagosome during cross-presentation is controversial.

Ca2+ signalling is linked to a variety of DC functions including differentiation, maturation, migration, cytokine secretion, phagocytic ingestion and antigen presentation. However, most studies have relied on the use of non-specific inhibitors, ionophores and chelators, which can have pleiotropic effects. Stromal interaction molecule (STIM) proteins, which include the two isoforms STIM1 and STIM2 each with multiple splice variants, are ER transmembrane proteins that sense the ER Ca2+ depletion resulting from activation of inositol trisphosphate (InsP3) receptors. They subsequently remodel the ER and promote the formation and expansion of membrane contact sites (MCS) between the ER and plasma membrane (ER–PM MCS), where they directly activate PM-resident Ca2+ channels of the ORAI1 and transient receptor potential (TRPC) families in the process termed store-operated Ca2+-entry (SOCE). Electrophysiological studies suggest that SOCE is the major Ca2+ entry pathway in DCs, and one study suggests that STIM2 is the major isoform regulating DC function in mice. In human peripheral blood monocyte-derived DCs genetic manipulation of ORAI1 and STIM1 suggested that STIM1 is critical for DC maturation, but another study suggests that STIM1 and STIM2 are dispensable for a variety of DC functions in mice.

Although the classic model of cross-presentation postulates that antigens are first partially proteolysed in phagosomes, retrotranslocated from the phagosome to the cytosol where they are further processed by the proteasome, and then reimported into the ER for loading onto ER-resident MHC-I molecules, some studies propose that non-canonical trafficking pathways involving fusion of ERGIC vesicles and recycling endosomes with phagosomes may explain the presence of ER proteins on phagosomes. However, the signalling and targeting mechanisms that control these pathways are unclear. In neutrophils, we previously showed that STIM1 promotes the formation of contact sites between the ER and phagosomes that allow localized Ca2+ signalling, raising the question of whether STIM1 may also affect the association between phagosomes and the ER in DCs.

In the present study, we characterize the consequences of genetic ablation of Stim1 on DC functions including differentiation, maturation, migration, phagocytosis and cross-presentation. Our data establish that STIM1 is the major isoform controlling SOCE in mouse DCs and suggest that STIM1 promotes cross-presentation at least in part by increasing Ca2+-dependent migration. In addition, STIM1 promotes the formation of contact sites between the ER and phagosomes that in turn produce localized Ca2+ signals that may potentiate proteolysis and fusion of phagosomes with endosomes and lysosomes.

**Results**

**Stim1 promotes cross-presentation of phagocytosed antigens.** To determine whether STIM1 promotes cross-presentation, PBS solutions with 0, 0.5, or 1% ovalbumin (OVA)-coated beads (OVAb) were injected into footpads of LysM-Cre; Stim1fl/fl littermates. After 24 h, CD45.1, H2-Kb/OVA(257–264)-reactive CD8α+ T cells (OT-I) labelled with carboxyfluorescein succinimidyl ester (CFSE) were injected retro-orbitally. Draining (DL) and non-draining (NDL) lymph nodes were harvested after 72 h, and the total number of CD45.1+ OT-I cells within the CD8α+ population, as well as the CFSE dilution as a measure of OT-I proliferation, were determined. The full gating strategy is shown in Fig. 1a. STIM1 deficiency dramatically reduced the total number of CD45.1+ OT-I cells within the CD8α+ gate in DL of mice injected with 1 or 0.5% OVAb but not in NDL (Fig. 1a, b), or in lymph nodes from mice injected with PBS (Supplementary Fig. 1a). OT-I proliferation was reduced in a dose-dependent manner (Fig. 1a, b), indicating that effectively, cross-presentation was impaired upon Stim1 ablation in myeloid cells.

We next examined whether cell-specific DC cross-presentation was impaired by co-culturing bone-marrow-derived DCs (BMDC) pulsed with OVAb along with OT-I cells in vitro and measuring OT-I proliferation after 72 h by bromodeoxyuridine (BrdU) incorporation. Western blots confirmed that STIM1 protein expression was reduced by >90% in BMDCs isolated from LysM-Cre; Stim1fl/fl mice (labelled henceforth Stim1−/−) for simplicity as compared to BMDCs isolated from Stim1fl/fl mice (labelled henceforth WT, Supplementary Fig. 1b). Maturation with microbial products Cpg oligodeoxynucleotides and lipopolysaccharide (LPS) increased OT-I proliferation by ∼twofold (Fig. 1c), while Ca2+ chelation with BAPTA-AM or inhibition of InsP3-receptors with Xestospongin C (Xesto) decreased cross-presentation, as did the inhibitors of the NADPH oxidase and the vacular-ATPase, diphenylpropidium iodide (DPI) and concanamycin A (ConA) respectively, applied as positive controls (Fig. 1d). This indicates that cross-presentation is Ca2+-dependent. Stim1 ablation reduced the relative levels of cross-presentation to similar extents in immature and mature cells, an effect that was most pronounced at intermediate DC7 cell ratios (Fig. 1c). Similar results were obtained when measuring OT-I proliferation by CFSE dilution (Supplementary Fig. 1c, d). The Stim1-dependent defect in cross-presentation was specific for processing of phagocytosed antigens, as STIM1-deficient BMDCs were equally efficient at cross-presenting as WT when pulsed with varying doses of SIINFEKL peptide, an OVA(257–264) fragment that does not require proteolytic processing (Fig. 1e, Supplementary Fig. 1e). To confirm that abrogating STIM1 expression reduces cross-presentation in vitro, a CD8α+ DC cell line highly efficient at cross-presentation, DC2141148, was transduced with either control or shRNA directed against Stim1 (shCTR and shStim1 respectively). STIM1 depletion was verified by Western blot (Supplementary Fig. 1b). Similar to primary BMDCs, cross-presentation was reduced by STIM1 depletion in DC2114 cells (Fig. 1f). DC2114 induced nearly fourfold higher levels of OT-I proliferation as compared to BMDCs (Supplementary Fig. 1f), generating large colonies easily discerned by microscopy where the effect of STIM1 knockdown was clearly apparent (Supplementary Fig. 1g). These data indicate that STIM1 promotes the cross-presentation of phagocytosed antigens in vivo and that this effect is recapitulated in vitro in primary or cultured DCs.

**Stim1 ablation impairs the Ca2+-dependent migration of BMDCs.** The decrease in the total number of OT-I cells in the DL
of STIM1-deficient mice, a measure of functional cross-presentation, could result from defective migration of DCs to and from the site of OVAb injection where antigens are captured, to the lymph nodes where DCs encounter OT-I cells. To test whether DC migration to lymph nodes was impaired in vivo, a 1:1 mixture of BMDCs from CD45.2 WT mice expressing cytosolic GFP (WT-GFP) and CD45.2 Stim1−/− BMDCs was co-injected into footpads of CD45.1 host mice together with 0.5% NDL of CD45.2 LysM-Cre; Stim1fl/fl mice as compared to Stim1fl/fl littermates (upper graph). Similarly, OT-I proliferation, as assessed by CFSE dilution, was significantly decreased in STIM1-deficient mice (lower graph). N = 3 pairs of mice.

cross-presentation of OVA antigens to OT-I cells by BMDCs exposed to OVAb (4 h) was quantified as the BrDU incorporation after 72 h of co-culture. Stim1 ablation reduced cross-presentation in vitro, both in immature BMDCs and in cells matured with 1 μg mL−1 LPS or 0.1 μM CpG, at varying OT-I:BMDC (T:DC) ratios. N = 3, in triplicate wells. Ratio 1:1 T:DC in unstimulated WT cells = 1.

Pre-incubation with 40 μM BAPTA-AM or 1 μM Xesto reduced cross-presentation, similar to known inhibitors 0.2 nM ConcA or 10 μM DPI, applied as positive controls. N = 3, in triplicate wells. Control DMSO condition = 1.

Incubation with varying doses of the OVA(257–264) fragment SIINFEKL (1 h) induced similar levels of cross-presentation in WT and STIM1-deficient BMDCs. N = 3, in triplicate wells. Reduced cross-presentation was also observed in DC2114 + shSTIM1, as compared to control (+shCTR) as assessed with BrDU. Phagocytic targets added at 20:1 targets: cells. N = 3, in triplicate wells. Error bars are means ± SEM, p < 0.5, **p < 0.1, ***p < 0.01 using a two-way ANOVA and Sidak’s post test for b, c, and e, and a Student’s t-test for d and f.
**Stim1 ablation impairs global and localized Ca²⁺ signals.** SOCE can be functionally measured in isolated cells as the rate of Ca²⁺ entry after internal Ca²⁺ stores are depleted in the absence of external Ca²⁺. To further characterize the Ca²⁺ signalling defects of STIM1-deficient DCs, we used the SERCA inhibitor thapsigargin (Tg) to passively deplete ER Ca²⁺ stores and assess global SOCE. *Stim1* ablation reduced SOCE by ~70% in both immature and mature BMDCs (Fig. 3a), a defect that could not be accounted for by changes in ORAI1 or STIM2 expression (Supplementary Fig. 3a). STIM1 knockdown also reduced SOCE by 70% in DC 2114 cells (Supplementary Fig. 3b), and Ca²⁺ responses evoked by platelet activating factor (PAF) in BMDCs were markedly impaired regardless of maturation (Fig. 3b, Supplementary Fig. 3c). In addition to global Ca²⁺ signals, localized Ca²⁺ hotspots occur during phagocytosis in macrophages, neutrophils and phagocytic fibroblasts. In fibroblasts, these signals coincide in time and space with the STIM1-mediated tethering of ER cisternae to phagosomes, resecting both ER Ca²⁺ release as well as the opening of phagosomal Ca²⁺ channels. We therefore checked whether periphagosomal Ca²⁺ spots and ER-phagosome MCS (ER–Ph MCS) were also present in DCs. Indeed, periphagosomal Ca²⁺ spots were observed in BMDCs exposed to OVAb for 30 min and were reduced by ~50% upon *Stim1* ablation (Fig. 3c, d). Periphagosomal Ca²⁺ spots were observed even after 90 min of OVAb exposure, although their frequency was reduced and their occurrence was no longer STIM1 dependent (Fig. 3c). SOCE channel inhibition with

**Fig. 2** STIM1 promotes Ca²⁺-dependent migration in vivo and in vitro. a CD45.1 congenic wild-type hosts were injected with a mixture of 1 x 10⁶ wild-type GFP+ (WT-GFP), 1 x 10⁶ Stim1⁻/⁻ BMDCs and 0.5% OVAb. DLs were collected at 24 and 48 h post injection, and the CD45.1⁺ CD45.2⁺ population analysed for GFP expression. The percentage of GFP-expressing WT cells in DLs was strongly and significantly higher at 24 h post injection, whereas the initial 1:1 ratio was nearly recovered at 48 h (Fig. 2a, Supplementary Fig. 2a). BMDM migration was next examined in vitro using transwell chambers and the chemoattractant fMIFL to mimic microbial peptides found at the site of infection, SDF-1 (also known as CXCL12), a chemokine expressed by lymphatic vessels, or CCL21 (also known as Exodus-2 or SLC), the major chemokine eliciting DC homing to lymph nodes (Fig. 2b, Supplementary Fig. 2b). No differences between baseline levels of migration between WT and Stim1⁻/⁻ cells were observed, and surface expression of the SDF-1 and CCL21 receptors CXCR4 and CCR7 was similar in WT and Stim1⁻/⁻ BMDCs matured or not with CpG, LPS, or OVAb (Supplementary Fig. 2c, d). WT cells showed strongly, moderate and no Ca²⁺ transients evoked in response to fMIFL, SDF-1, and CCL21, respectively. STIM1-deficient cells displayed a brief Ca²⁺ elevation characteristic of ER-Ca²⁺ release by InsP₃-generating receptors, with an impaired delayed elevation characteristic of SOCE, resulting in a ~50% reduction of the integrated Ca²⁺ response (Fig. 2c). Interestingly, the size of the migration defect was congruent with the size of the induced Ca²⁺ transients evoked by the same concentration of each chemoattractant (10⁻⁶ M) in STIM1-deficient cells, with fMIFL evoking the largest transients and the greatest migration defect, while CCL21 recruited STIM-deficient cells effectively without evoking Ca²⁺ responses (Fig. 2b, c). Preserved CCL21 signalling explains the delayed migration of STIM1-deficient DCs to lymph nodes and suggests that the CCL21-evoked Ca²⁺ transients reported in DCs might depend on other factors such as prostaglandins. Together, these results imply that impaired Ca²⁺-dependent DC migration contributes to defective functional cross-presentation in vivo by delaying the recruitment of DCs to the site of injection and their subsequent rapid migration to lymph nodes. However, other factors likely to contribute, since DCs eventually accumulate in lymph nodes.

---

**ARTICLE** NATURE COMMUNICATIONS | DOI: 10.1038/s41467-017-01600-6

---

**NATURE COMMUNICATIONS** | 8:1852 | DOI: 10.1038/s41467-017-01600-6 | www.nature.com/naturecommunications
GSK7975A (GSK, 10 µM) added concomitantly with OVAβ, and InsP₃R inhibition with Xesto (1 µM) 20 min after OVAβ addition, reduced hotspot frequencies in WT but not STIM1-deficient cells (Fig. 3c). Although Xesto may affect STIM1 recruitment to phagosomes by preventing ER store depletion, these data suggest that both Ca²⁺ release from phagosomes as well as Ca²⁺ release from recruited ER stores contribute to local Ca²⁺ signals. In addition, in transduced BMDCs, periphagosomal mCherry-STIM1 puncta co-localized with periphagosomal hotspots (Fig. 3c, white arrows). Periphagosomal STIM1 puncta that were not associated with Ca²⁺ hotspots were also observed (Fig. 3c, yellow arrows), but the majority of hotspots co-localized with STIM1 clusters. Immunostainings confirmed that STIM1 puncta were observed near phagosomes (Supplementary Fig. 4a). Similar to neutrophils, ER cisternae closely apposed (<30 nm) to phagosomes were observed in BMDCs exposed to OVAβ for 30 min, as visualized using both classic transmission electron microscopy (Supplementary Fig. 4b) as well as focused-ion-beam scanning.
electron microscopy (FIB-SEM) (Fig. 4a–c). 3D reconstruction of FIB-SEM stacks confirmed that sites of ER–phagosome contact represented sections contiguous with the bulk ER (Fig. 4a). Interestingly, the 3D reconstruction also revealed that the same ER cisterna may display multiple sites of contact with the same phagosome (highlighted in yellow in Fig. 4a and Supplementary Movie 1). Additionally, whereas the phagosomal membrane closely follows the surface of the ingested beads for most of the phagosomal surface, in certain instances the region surrounding these ER–Ph MCS displayed multiple sites of bulging away from the bead that were reminiscent of vesicular trafficking (fusion or fission) activity (Fig. 4b, arrows). Quantification of EM slices revealed that similar to neutrophils, Stim1 ablation significantly reduced the number of ER–Ph MCS in BMDCs (Fig. 4c). Together, these data imply that Stim1 depletion has a major impact on global SOCE in DCs, as well as on localized Ca\textsuperscript{2+} signals mediated through ER–Ph MCS.

**Stim1 is dispensable for DC maturation and phagocytosis.** Previous reports suggest that DC differentiation and maturation are dependent on Ca\textsuperscript{2+} signalling\cite{22}. BMDCs differentiated from bone-marrow precursors expressed high levels of CD11c, CD11b and low levels of F4/80, characteristic of conventional-like DCs\cite{23} (Fig. 5a, Supplementary Fig. 5a). In both wild-type and Stim1\textsuperscript{−/−} BMDC cultures, > 90% of cells expressed CD11c and expression of all three markers were similar in both genotypes, as was surface levels of MHC-I, indicating that DC differentiation was unaffected by Stim1 ablation (Fig. 5a). DC\textsuperscript{2114} cells were CD8\textsuperscript{α}+, CD11b\textsuperscript{−}, F4/80\textsuperscript{−} and B220\textsuperscript{−}, (characteristic of CD8\textsuperscript{+} lymphoid-like DCs\cite{23}), and expressed similar levels of CD11c when transduced with either shCTR or shSTIM1 (Supplementary Fig. 5b, c). Exposure of BMDCs to TLR ligands induces DC maturation characterized in part by an upregulation of cell-surface markers such as CD40, CD80, CD86 and MHC-II. When BMDCs were exposed to maturation stimuli CpG or LPS for 18 h the cell-surface expression of CD40, CD80, CD86 and MHC-II was similarly increased in both genotypes (Fig. 5b, Supplementary Fig. 2c). STIM1-silenced DC\textsuperscript{2114} cells were also equally capable of upregulating maturation markers in response to CpG (Supplementary Fig. 5d). We then checked whether acute administration of these TLR ligands evoked Ca\textsuperscript{2+} responses in immature cells. LPS evoked small Ca\textsuperscript{2+} transients in ∼30% of BMDCs and these signals were of lower amplitude in Stim1\textsuperscript{−/−} cells, while CpG did not induce any detectable Ca\textsuperscript{2+} transient in either genotype (Supplementary Fig. 5e).

In neutrophils phagocytic ingestion is Ca\textsuperscript{2+}- and STIM1-dependent\cite{17,24,25}, and phagocytosis is Ca\textsuperscript{2+}-dependent in DCs\cite{8}.
**Fig. 5** Stim1 ablation does not affect BMDC differentiation, maturation, or phagocytic rate. **a** BMDCs express CD11c, CD11b, low levels of F4/80 and MHC-I to similar extents regardless of STIM1 expression. N = 8/5/5 for CD11c/CD11b/F4/80. Values of cellular autofluorescence (−Ab) are shown for comparison. The gating strategy is shown in Supplementary Fig. 5a. **b** Expression of CD40, CD80, CD86 and MHC-II in immature cells or in cells matured with either CpG or LPS was similar in cells expressing or not STIM1. The full gating strategy is shown in Supplementary Fig. 2c. **c** Phagocytosis of YG- Fluresbrite-OVA-coated beads (OVAb) was decreased by loading cells with 40 μM BAPTA but not by exposing cells to the non-specific SOCE blocker LaCl3 (50 μM), and was similar in immature and mature STIM1-deficient cells as compared to cells from wild-type littermates. N = 4/5/3/3 for 30 min/4 h/24 h/BAPTA/La3+. The full gating strategy is shown in Supplementary Fig. 2c. **d** The absence of phagocytic defect in STIM1-deficient cells was confirmed by quantifying phagocytosis by microscopy. Phagocytic targets added at 20:1 targets:cells. N = 3. MFI = mean fluorescence intensity. Black bar = 3 μm. Error bars are means ± SEM, *p < 0.5 using a Student’s t-test.

Stim1 ablation does not alter phagosomal ROS or pH. Several phagosomal maturation steps such as actin shedding, ROS production and fusion with granules or lysosomes are Ca2+-dependent in neutrophils and macrophages. In DCs, lower phagosomal proteolyis, higher phagosomal ROS and reduced phagosomal acidity correlate with higher cross-presentation. In this context, reduced phagosomal ROS production, or increased acidity or proteolytic activity might lead to impaired cross-presentation in STIM1-deficient DCs. We therefore measured intracellular ROS production with dihydroethidium (DHE) in cells exposed to OVAb or zymosan particles (phagocytic targets known to elicit high levels of ROS), with the NADPH oxidase activator phorbol myristate acid (PMA) and inhibitor DPI serving as positive and negative controls. Intracellular ROS levels increased by 10–12-fold upon PMA or zymosan stimulation and by 4–6-fold upon exposure to OVAb, showing the highest level in cells matured with CpG (Fig. 6a). Extracellular ROS levels, measured with Amplex Rex, increased by 4–6-fold upon PMA exposure, whereas OVAb did not produce detectable extracellular ROS (Supplementary Fig. 6a). Stim1 ablation had no significant effect on ROS production in any condition tested (Fig. 6a, Supplementary Fig. 6a). BMDCs were also exposed to OVAb coupled to OxyBurst and Alexa-568, and imaged at 30 min and 90 min after bead addition. The ratio of OxyBurst to Alexa-568 fluorescence increased progressively following particle ingestion and was inhibited by DPI, yet again, no significant differences were detected (Fig. 6b).

We next examined whether phagosomal pH might be affected by Stim1 ablation. Phagosomal pH was measured by exposing BMDCs to either FITC-coupled or to pHrodo-Red- and Alexa-488-coupled zymosan. Zymosan was used instead of OVAb as phagocytic targets added at 20:1 targets:cells. FITC and pHrodo coupling to amine-functionalized beads was unstable. BMDCs were imaged after 30 and 90 min of exposure to zymosan, and pre-treatment with ConC used to document V-ATPase-mediated acidification. Phagosomal pH was...
Fig. 6 Stim1 ablation does not affect ROS production or phagosomal pH. a Intracellular ROS production was measured in immature and CpG or LPS-matured BMDCs loaded with 30 μM DHE, and exposed to OVA-coated beads (OVAb), zymosan (Zym) or 100 nM PMA. N = 4, triplicate wells. b ROS production during phagocytosis (30 and 90 min) was assessed by exposing BMDCs to OVAb coupled to OxyBurst and Alexa-568. In a and b, DPI (10 μM) blocked the DHE or OxyBurst signal, but no differences were detected upon Stim1 ablation. N = 6/4/4 coverslips containing 84/54/58 (WT) or 85/50/53 (Stim1−/−) cells, for 30 min/90 min/DPI. c, d Phagosomal acidification (30 and 90 min) was measured using ratiometric or pseudo-ratiometric imaging by exposing cells to FITC-coupled (c) and pHrodo/Alexa-488-coupled (d) OVA-coated zymosan, respectively. Phagosomal acidification was blocked by Conca (0.2 nM), but there were no differences upon Stim1 ablation. N = 3 coverslips for all conditions, comprising 3218/2826/1713/3533 (WT) or 2722/2608/4438 (Stim1−/−) FITC-phagosomes and 2612/2194/700/746 (WT) or 1740/2218/670/862 (Stim1−/−) pHrodo phagosomes, for 30 min/90 min/30 min/90 min Conca/90 min Conca, respectively. f Histogram of all combined 90 min pHrodo phagosomal pH values for WT and Stim1−/− showing a broad distribution of phagosomal pH ranging from very acidic to very basic (pH 4–9), displaying a roughly bimodal distribution, with an average pH that steadily decreased over the course of the recordings in a ConcA-sensitive manner (Fig. 6c–e). Phagosomes within the same cell had widely differing pH values, indicating that heterogeneity is determined at the level of single phagosomes (Fig. 6f). Regardless of the dye used, the phagosomes of Stim1−/− BMDCs displayed an average pH similar to WT cells (6.57 ± 0.11 vs. 6.68 ± 0.19 and 5.67 ± 0.34 vs. 5.66 ± 0.21 after 90 min for WT and Stim1−/− cells using FITC and pHrodo respectively, Fig. 6c, d). Endosomal pH was additionally assessed by loading cells with Alexa-568 and FITC-coupled dextran. Although a trend for a more alkaline endosomal pH in Stim1−/− cells was apparent, the difference was not significant (Supplementary Fig. 6c).

Stim1 ablation impairs phagosomal proteolysis and fusion. We next measured phagosomal proteolysis, using beads coated with Alexa-568 and DQ-OVA, a protease probe consisting of OVA molecules heavily labelled with self-quenching BODIPY dye, which becomes brightly fluorescent upon OVA hydrolysis. DQ-OVA fluorescence increased progressively, and green fluorescence
leaked from the phagosome into the cytosol, indicating that proteolysis occurred (Fig. 7a). Contrary to macrophages\textsuperscript{25}, phagosomal proteolysis reported by this assay was only partially inhibited by ConcA (Fig. 7a). Stim1 ablation significantly decreased DQ-OVA fluorescence by $\sim$30% at 90 min. This effect tended to persist in the presence of ConcA, although the difference here was not significant ($p = 0.09$) (Fig. 7a).

Pre-loading cells with BAPTA-AM reduced proteolysis in both genotypes to the levels of untreated Stim1\textsuperscript{−/−} cells, indicating that Stim1 mediates Ca\textsuperscript{2+}-dependent proteolysis in BMDCs (Fig. 7a). As an alternative approach, the levels of phagosome-associated OVA was determined by quantifying anti-OVA immunostaining of isolated BMDC phagosomes\textsuperscript{35}. OVA degradation was inhibited in Stim1-deficient cells at 30 and 60 min.

**Fig. 7** Stim1 promotes phagosomal proteolysis and endomembrane fusion. **a** Proteolysis was measured in BMDCs exposed to DQ-OVA-Alexa-568 beads. Stim1-deficient cells showed lower levels of DQ-OVA fluorescence at 90 min (right panel). Pre-incubation with BAPTA-AM (40 $\mu$M) reduced WT proteolysis to levels similar to Stim1-deficient cells. Lines representing the BAPTA condition are omitted and 90 min points are displaced to the right for clarity. ConcA (0.2 nM) only partially inhibited proteolysis. $N = 5/10/3/5/3$ (coverslips) comprising a total of 236/1050/237/432/200 (WT) or 643/1314/1617/503 (Stim1–/–) cells for 30/90/30 + ConcA/90 + ConcA/90 + BAPTA(min). **b** OVA degradation was measured with anti-OVA immunostainings of isolated phagosomes by flow cytometry. Values are % OVA degradation. Full gating strategy is shown in Supplementary Fig. 7a. Proteolysis was decreased at 30 and 60 min after ingestion. $N = 3$. **c** Phago-lysosome (P-L) fusion was measured by exposing Alexa-488-OVAb to cells loaded with lysosomal FRET acceptor Alexa-594-HA. Colour-coded images (left) show the FRET signal at 90 min for WT and Stim1-deficient cells. P-L fusion indices are matched to the colour-coded bar. P-L fusion was decreased in Stim1-deficient cells as compared to WT at 90 min, whereas BAPTA-AM loading further decreased P-L fusion and eliminated differences between WT and Stim1\textsuperscript{−/−} cells. ConcA decreased P-L fusion to similar levels as BAPTA-loaded cells. $N = 3/4/4/3$ (coverslips) comprising 470/1009/1691/797 (WT) or 643/1314/1617/503 (Stim1\textsuperscript{−/−}) phagosomes for 30/90/90 + ConcA/90 + ConcA/90 + BAPTA (min). **d** Phago-endosome (P-E) fusion was measured by exposing Alexa-488-OVAb to cells loaded with endosomal FRET acceptor Alexa-594-dextran. P-E fusion was decreased in Stim1-deficient cells as compared to WT. Addition of GSK (10 $\mu$M) eliminated differences in P-E fusion. $N = 3$ (coverslips) comprising a total of 213/244/144/146 (WT) or 210/154/233/253 (Stim1\textsuperscript{−/−}) cells for 15/30/15 + GSK/30 + GSK (min). Phagocytic targets added at 201. **e** Leu-AMC fluorescent substrate cleavage was reduced in phagosomes isolated from Stim1-deficient cells but not in whole-cell lysates, $N = 3$. **f** Periphagosomal IRAP, quantified from single confocal slices, was reduced in Stim1-deficient cells. $N = 4$ (coverslips). White bars = 10 $\mu$m. Error bars are means ± SEM. $p < 0.5$, **$p < 0.01$ using a two-way ANOVA and Sidak’s post test for **a–**d** and a Student’s t-test for **e** and **f**
but recovered after 2 h, indicating a partial or delayed OVA degradation defect (Fig. 7b, Supplementary Fig. 7a).

Since phagosome-to-cytosol transfer of peptides is suggested to be favoured by the delivery of ER proteins to phagosomes16, 29, we investigated whether endosome-to-cytosol transfer was influenced by Stim1 ablation as a proxy measure of phagosomal antigen transfer16. BMDCs were loaded with the β-lactamase FRET reporter CCF4 and then exposed to β-lactamase. Upon transfer to the cytosol, β-lactamase cleaved CCF4, reducing the FRET emission in cells incubated at 37 °C with identical kinetics in WT and Stim1-deficient cells (Supplementary Fig. 7b, c). This suggests that defective phagosome-to-cytosol transfer likely cannot account for the decreased DQ-OVA signal and that delivery of the molecular machinery underlying phagosome-to-cytosol transfer may not be dependent on Stim1.

We then tested phago-lysosome fusion. BMDCs were pulsed with the FRET acceptor Alexa-568-HA for 3 h followed by an overnight chase to accumulate the dye in lysosomes30. OVAbcoupled to the FRET donor Alexa-488 were then added for 30 and 90 min. The phago-lysosome-associated FRET signal increased from 0 to 90 min in a ConA and BAPTA-dependent manner, confirming that phago-lysosome fusion is Ca2+-dependent in BMDCs (Fig. 7c). Interestingly, Stim1 ablation reduced phago-lysosome fusion by ~30% at 90 min (Fig. 7c). Since OVA degradation was most strongly affected at earlier time points, we additionally tested whether endosome fusion might be defective, by pre-loading cells for 15 min with Alexa-594-dextran before exposing them to Alexa-488-OVAb and measuring the cellular FRET signal. Indeed, phago–endosome fusion was reduced in Stim1−/− cells at 15 and 30 min, with the effect most pronounced at 15 min (Fig. 7d). Addition of GSK (10 μM) together with OVAb abolished differences between WT and Stim1−/− cells (Fig. 7d). These data suggest that reduced phago–lysosome and phago–endosome fusion contribute to reduced proteolysis in Stim1-deficient cells. Therefore, we tested whether the proteolytic activity of lysosomal or endosomal proteases might be affected. While there was a trend for decreased proteolysis in STIM1-dead cells indicating that Stim1 is the dominant isoform in DCs, and not STIM2 as proposed15. Since double Stim1;Stim2 ablation completely abolishes SOCE in DCs15 however, STIM2 still contributes to Ca2+ signalling in DCs. Similar to Vaeth et al. and, unlike human DCs33, we observe that Stim1 is not required for the upregulation of DC maturation markers in mice, nor does it affect differentiation. Indeed, increased ROS production and upregulation of CCR7 and CXCR4, both events associated with DC maturation are additionally intact. This is in stark contrast to neutrophils derived from similar mouse genetic models, where phagocytosis12, 13 and ROS production25 are both STIM1 dependent. These differences highlight the importance of cell-type dependence of Ca2+ signalling outcomes. Moreover, in contrast to RAW macrophages35 and similar to B cells34, CpG does not induce detectable Ca2+ transients, while LPS induces small and variable Ca2+ transients in only ~30% of cells. Thus, redundant Ca2+-dependent and independent pathways downstream of maturation stimuli appear sufficient to overcome a loss of STIM1 function in DCs.

We expected ROS production to be affected because NADPH oxidase activation is Ca2+- and Stim1 dependent in neutrophils35 and ROS production is required for effective cross-presentation (Fig. 1d and refs 36–38). Surprisingly, ROS production is unaffected in STIM1-deficient DCs suggesting that either sufficient Ca2+-signalling remains, or that oxidase activation is SOCE-independent in DCs. Phagosomal pH is intimately linked to phagosomal ROS production and depends on the delivery of V-ATPase as well as HVCN1 proton channel35, 39, 40. In DCs, phagosomal pH is controversial, with some groups reporting pH levels near 6.5 and others between 7 and 8 within the first 60 min of phagocytosis36, 37. In all previous reports phagosomal pH was measured on cell populations. Here pH measurements are conducted on single phagosomes, using two independent probes, FITC and pHrodo, comparing cells originating from the same mice. No differences in subpopulations or average pH are detected between STIM1 and wild-type cells, but the two dyes yield an average pH differing by nearly 1.0 unit. This likely reflects the lower in situ pKa of pHrodo (6.1 ± 0.4 vs. 7.6 ± 0.3 for FITC) and the increased sensitivity of pHrodo at low pH levels (Supplementary Fig. 6b), which indicates that pHrodo reports a more reliable pH estimate. On the other hand, the differences could also be related to the large variability in phagosomal pH that appears to be regulated at the level of single phagosomes. Shifts in the balance between the peaks of this bimodal, non-Gaussian distribution between experimental conditions may explain why different groups report such disparate values for mean population measurements. Why DC phagosomes display such a large variability remains unclear but phagosomal ROS, which can be highly heterogenous41, and can inhibit V-ATPase recruitment35, is likely involved. Indeed, Hv1−/− neutrophils, which suffer a 50% loss in ROS production, also display a bimodal distribution of phagosomal pH39, and thus it could be interesting to correlate ROS and pH measurements at the level of single phagosomes in future studies in DCs. It is interesting to note that ORAI channels are sensitive to extracellular acidity, being activated at alkaline pH while inhibited at pH < 6.42. The heterogeneity of phagosomal pH observed would thus allow phagosomes with robust ORAI activity to co-exist with phagosomes where ORAI is inhibited, and could explain why only a subset of STIM1-positive periphagosomal puncta coincide with Ca2+- hotspots. The identical pH profiles of STIM1 and wild-type

Discussion

We report here that mice lacking STIM1 in the myeloid lineage fail to effectively cross-present phagocytosed antigens in vivo. The cross-presentation defect is recapitulated in vitro in DCs matured or not with TLR ligands, and is restricted to phagocytosed antigens, pointing to specific defects in phagocytic processing. Both global and localized Ca2+ signals are reduced in STIM1-deficient DCs. Surprisingly, but in line with a recent study15, DC differentiation, maturation and phagocytosis are not affected by Stim1 ablation. Instead, defective cross-presentation is linked to two other impaired DC functions: defective in vivo migration, which correlates with reduced chemotaxis to Ca2+- mobilizing chemokines, and altered phagosomal maturation. STIM1 deficiency reduces SOCE by 70% in both BMDCs and DC16 cells indicating that STIM1 is the dominant isoform in DCs, and not STIM2 as proposed15. Since double Stim1;Stim2 ablation completely abolishes SOCE in DCs15 however, STIM2 still contributes to Ca2+ signalling in DCs. Similar to Vaeth et al. and, unlike human DCs33, we observe that Stim1 is not required for the upregulation of DC maturation markers in mice, nor does it affect differentiation. Indeed, increased ROS production and upregulation of CCR7 and CXCR4, both events associated with DC maturation are additionally intact. This is in stark contrast to neutrophils derived from similar mouse genetic models, where phagocytosis12, 13 and ROS production25 are both STIM1 dependent. These differences highlight the importance of cell-type dependence of Ca2+ signalling outcomes. Moreover, in contrast to RAW macrophages35 and similar to B cells34, CpG does not induce detectable Ca2+ transients, while LPS induces small and variable Ca2+ transients in only ~30% of cells. Thus, redundant Ca2+-dependent and independent pathways downstream of maturation stimuli appear sufficient to overcome a loss of STIM1 function in DCs.

We expected ROS production to be affected because NADPH oxidase activation is Ca2+ and Stim1 dependent in neutrophils35 and ROS production is required for effective cross-presentation (Fig. 1d and refs 36–38). Surprisingly, ROS production is unaffected in STIM1-deficient DCs suggesting that either sufficient Ca2+-signalling remains, or that oxidase activation is SOCE-independent in DCs. Phagosomal pH is intimately linked to phagosomal ROS production and depends on the delivery of V-ATPase as well as HVCN1 proton channel35, 39, 40. In DCs, phagosomal pH is controversial, with some groups reporting pH levels near 6.5 and others between 7 and 8 within the first 60 min of phagocytosis36, 37. In all previous reports phagosomal pH was measured on cell populations. Here pH measurements are conducted on single phagosomes, using two independent probes, FITC and pHrodo, comparing cells originating from the same mice. No differences in subpopulations or average pH are detected between STIM1 and wild-type cells, but the two dyes yield an average pH differing by nearly 1.0 unit. This likely reflects the lower in situ pKa of pHrodo (6.1 ± 0.4 vs. 7.6 ± 0.3 for FITC) and the increased sensitivity of pHrodo at low pH levels (Supplementary Fig. 6b), which indicates that pHrodo reports a more reliable pH estimate. On the other hand, the differences could also be related to the large variability in phagosomal pH that appears to be regulated at the level of single phagosomes. Shifts in the balance between the peaks of this bimodal, non-Gaussian distribution between experimental conditions may explain why different groups report such disparate values for mean population measurements. Why DC phagosomes display such a large variability remains unclear but phagosomal ROS, which can be highly heterogenous41, and can inhibit V-ATPase recruitment35, is likely involved. Indeed, Hv1−/− neutrophils, which suffer a 50% loss in ROS production, also display a bimodal distribution of phagosomal pH39, and thus it could be interesting to correlate ROS and pH measurements at the level of single phagosomes in future studies in DCs. It is interesting to note that ORAI channels are sensitive to extracellular acidity, being activated at alkaline pH while inhibited at pH < 6.42. The heterogeneity of phagosomal pH observed would thus allow phagosomes with robust ORAI activity to co-exist with phagosomes where ORAI is inhibited, and could explain why only a subset of STIM1-positive periphagosomal puncta coincide with Ca2+ hotspots. The identical pH profiles of STIM1 and wild-type
fusion, decreased global and periphagosomal Ca\textsuperscript{2+} signals, and recent data suggests that different ER proteins such as tapasin, remained to allow effective cytosol transfer. On the other hand, the frequency of periphagosomal Ca\textsuperscript{2+} signals is only half that of neutrophils, as well as compared to WT neutrophils, yet the frequency of periphagosomal Ca\textsuperscript{2+} signals promote local fusion events. It should be noted that decreased cathepsin-dependent proteolysis and reduced phagolysosome fusion correlated with higher, not lower, levels of cross-presentation in DCs. Yet, some proteolysis must occur to generate peptides from whole OVA molecules and inhibiting proteolysis with the broad-spectrum inhibitor leupeptin reduces cross-presentation in some, although not all. Indeed, in the companion study published in this same issue which analyses the function of UNC93B1, a novel STIM1 interactor, Maschalidz et al. not only confirm a role for STIM1 in cross-presentation, but also observe that decreased proteolysis correlates with decreased cross-presentation. Proteolysis has therefore a bipartite effect on antigen processing, with both negative and positive effects on the MHC-I loading depending on its extent. Our data suggest that finely regulated levels of proteolysis must be reached to favour cross-presentation, and that this fine-tuning is achieved at least in part through Ca\textsuperscript{2+}-dependent regulation of endosome fusion and IRAP delivery.

We also considered that antigen export from phagosomes into the cytosol might be affected by STIM1 deletion. Phagosomal export was reported for some but not all antigens, and cytotoxic transfer requires ER proteins such as Sec61, tapasin and TAP. That STIM1 mediates close contacts between the ER and phagosomes argues that at least some ER proteins found on isolated phagosomes are derived from contacts, which may in turn facilitate the delivery of this ER-derived machinery. That the frequency of ER–Ph MCS was fivefold higher in WT DCs as compared to WT neutrophils, yet the frequency of phagolysosomal Ca\textsuperscript{2+} signals is only half that of neutrophils, as well as the observation of phagolysosomal STIM1 puncta without associated Ca\textsuperscript{2+} signals (Fig. 3e), further support the idea that ER–Ph MCS may have roles beyond Ca\textsuperscript{2+} signalling in DCs, and that they could partly mediate specialized DC functions that rely on ER proteins. However, no defects in cytosol transfer were observed. On one hand, ER contacts were only decreased by ~35% upon Stim1 deletion, and potentially, sufficient activity remained to allow effective cytosol transfer. On the other hand, recent data suggests that different ER proteins such as tapasin, MHC-I and Sec61 reach phagosomes through unconventional vesicular trafficking pathways involving the ERGIC, recycling endosome and possibly other compartments. These new findings on alternative trafficking raise the intriguing possibility that endolysosomal trafficking is more generally non-canonical in DCs and that specialized compartments analogous to secondary and tertiary granules in neutrophils might exist in DCs. Indeed, a lysosome-like Rab34-dependent compartment delivers NADPH oxidase in DCs. Since cross-presentation was more strongly impaired than phagolysosome fusion, STIM1 might regulate alternative Ca\textsuperscript{2+}-dependent trafficking pathways in a way that bypasses the need for cytosol transfer. The generation of better tools to manipulate these pathways will be required to gain a deeper understanding of alternative compartments and the signals that regulate their activity.

In summary, we show that STIM1 has an important and selective regulatory role on Ca\textsuperscript{2+}-dependent processes that control phagosome maturation and migration in DCs, thereby impacting the efficiency of cross-presentation. STIM-gated ORAI channels are promising drug targets for treating autoimmune diseases, muscle defects, skin disorders and cancer. In addition, activating ORAI1 through a genetically encoded light-activatable ORAI1 fragment promotes tumour remission in a mouse model of DC cell-based immunotherapy. Together with our data showing that either pharmacological or genetic manipulation of Ca\textsuperscript{2+} signalling can decrease cross-presentation, this suggests that delineating the precise role of SOCE in innate immune cells is highly relevant for the identification of potential risks and benefits associated with the development of SOCE blockers and activators designed for therapeutic use.

Methods

Reagents. The following antibodies (antibody Name/catalogue#/dilution) were purchased from: Biolegend (USA) - PerCP/Cy5.5-anti-CD117 (also known as CCR7)/1:120151/1:100; PE-anti-CD184 (also known as CXCR4)/L276F12/1:6505/1:100; APC-anti-F4/80 (RM8)/123115/1:400; APC-Rat-IgG2a, k Isotype Control/403511/1:100; PE-anti-CD11b/M170/1:10225/1:200; PE-anti-CD68 (GL-1)/105070/1:200; PE-anti-CD34 (5/23)/124621/1:100; anti-CD38 (16-10A1)/104713/1:100; Alexa-647-anti-1-Ab (also known as MHC-II, Kl/15309/1:200; PE-anti-CD8 (53-6.7)/100721/1:100; APC-anti-CD45.1 (also known as PTTPC)/A20/110713/1:100. BD Biosciences (USA): APC-anti-CD11c (HL3)/551119/1:100; anti-CD16/CD32 (2.4G2)/FcBlock/575342/1:200; anti-STIM1/610954/1:100. Millipore (USA): anti-STIM1/AB9870/1:1000. Biosciences (USA): PE-anti-CD11c (N418)/17-0114/81/1:100; anti-CD45.2/104/17-0454/81/1:100. Cell Signalling (USA): anti-STIM2/4917S/1:1000; anti-IRAP/6918/1:250. Sigma-Aldrich (Germany): anti-Orai/08264/1:500. Bio-Rad (USA): anti-mouse-HRP/1706516/1:10000; anti-rabbit-HRP/172101/1:1000. Jackson ImmunoResearch (USA): Alexa-488-anti-mouse/715-455-150/1:800. Recombinant murine A20 (also known as CXCL12). recombinant murine CCL2 (also known as Exodus-2 or XLS), and recombinant murine GM-CSF were obtained from Peprotech (UK). Carboxyfluorescein succinimidyl ester (CFSE) was obtained from eBiosciences. Mouse shSTIM1 (TRC Clone ID: NM_009287-2.7121x1c2a Sequence: CCGGCGCTTCTCCTTTTTGGATGGATAGGGAGAAAGGAAAGGGTTTTT TG) and shCTR (Non-target Control particles SCH002V) Mission shRNA Lentiviral Clones were purchased from Sigma-Aldrich. All lentiviral particles were produced in Lenti-X 293T cells using the Lenti-X HTS Packaging System (Takara, Japan) according to the manufacturer’s instructions. Lentiviral titres were determined using the Lenti-X-p24 Rapid Titre ELISA kit (Trevigen). All cell culture reagents are obtained from ThermoFischer Scientific, and all chemicals were purchased from Sigma-Aldrich unless otherwise stated.

m-Cherry-STIM1 cloned into pENTR1a was purchased from GenScript (USA). m-Cherry-STIM1 was then cloned into the p2K7b lentiviral vector containing the ubiquitin promoter using the Gateway LR cloning (ThermoFisher Scientific, USA) kit. Mouse STIM1 protocol described in the previous paper. OVA-specific (mF)-CFSE was obtained by washing 3.0 μm unlabelled or YG-Fluoresbrite polystyrene microspheres (Polysciences, USA) in 25 mM sodium citrate, 25 mM sodium phosphate buffer, pH 5.0, and incubating beads with 20 mg/ml of endotoxin-free chicken ovalbumin (OVA, InvivoGen, USA) overnight at 4°C on an end-over-end rotor, followed by three washes in sterile PBS (ThermoFisher).

Flow cytometry. Cells were washed once in ice cold FACS buffer (2% BSA, 20 mM EDTA in PBS), blocked with 1:200 FcBlock/FACS buffer 15 min on ice, incubated for 30 min–1 h with the indicated antibodies, and washed with FACS buffer. Fluorescence was analysed using an Accuri C6 flow cytometer and CFlow Plus software (BD Biosciences) unless otherwise indicated.

Mice, cells and transplantation. Mice on a C57Bl/6 background bearing a conditional knockout of the Stim1 gene in the myeloid lineage were generated from LysM-Cre (B6.129P2-Lysm-Cre/J) f (The Jackson Laboratory, USA) and Stim1f"f (B6.Cg-Stim1lemZrao/J) f (a kind gift from Dr. Masatsugu Oh-Hora), strains and genotyped from ear biopsies using a KAPA Genotyping Kit (Sigma) according to the manufacturer’s instructions, and the following primers and PCR conditions: For Stim1, CGATGGCTCACAAGCTCTTGTTTC; AAGCTGTTGTGTCGCTTCACGG; GCCGGCTGGTCGGTAAGATCCATAGTGA; and 94 °C 3 min, 94 °C 7 s, 60 °C 20 s, 72 °C 25 s, 30 cycles, 72 °C 3 min. For LysM-Cre: CCCAGAATGGCAGGATTACGG; CTTGGTGCGTCGAGATTTTCTC; TTA- CAGTGGGCGAGCTGAC; GGTTGAAGCAGTTA; and 95 °C 3 min, 94 °C 5 s, 60 °C 20 s, 72 °C 25 s, 40 cycles, 72 °C 5 min in separate reactions, where 1% DMSO and 1.6% Perfect Match (GE Healthcare, USA) were added to the reaction of the mutant band. Bone marrow was isolated from sex and age-matched 6–16 week-old males and females. All animal manipulations were approved by the Geneva canton’s Direction Générale de la Santé.
Phagocytosis. For flow cytometry-based phagocytosis assays, OVA-coated YG-Fluoresbrite beads were added at a 20:1 ratio in duplicate for the indicated times. Cells were incubated and washed with PBS containing 0.5% OVA and 0.5% BSA. Cell lysates were generated by culturing bone marrow cells isolated from mouse femurs and tibias, where red blood cells had been removed by lysis in ammonium buffer (155 mM NH₄Cl, 10 mM KCl, 0.1 mM EDTA), in DMEM (41965-039) supplemented with 10% endotoxin-free FCS, 50 μM β-mercaptoethanol, 1% sodium pyruvate, 1% penicillin/streptomycin and 20 μg ml−1 murine recombinant GM-CSF. Cells were used between 8 and 13 days of culture and the purity of each culture was determined to be >85% CD11c+ by flow cytometry. The DC2114 cell line, derived from C57BL/6 mice with an H-2Kb haplotype was cultured in IMDM (31980) supplemented with 10% FCS, 50 μM β-mercaptoethanol, and 1% penicillin/streptomycin. Cells were tested for mycoplasma every six months. DC migration was performed by incubating cells in either 1 μg ml−1 LPS or 0.1 μM CpG (ODN 1826, InvivoGen) for 18 h. Seeding cells on coverslips tended to activate them to variable extents, thus unless otherwise indicated cells seeded on coverslips were matured with CpG. Lentivirus transduction was performed by centrifuging cells and viral particles at 5 MΩ in complete medium supplemented with 8 μg ml−1 polybrene at 500 μg ml−1 at 37°C for 1 h. To produce DC2114 cells stably expressing shRNA, cells were cultured in 1 μg ml−1 puromycin as of 2 days after transduction. Splenic OT-I cells were isolated from C57BL/6-Tg[TcraTcrb]1100Mjb/J mice (Jackson) using the CD8− negative selection mouse T cell isolation kit (Miltenyi Biotec, Germany) according to the manufacturer’s instructions. Congenic CD45.1− OT-I cells were obtained by breeding OT-I mice to the congenic CD45.1 strain B6. SJL-Ptpc− Pepc−BoyJ62 (Jackson) and similarly isolating CD8+/− DCs from these mice. Popliteal lymph nodes were harvested 24 h or 48 h post injection and DCs labelled with CFSE were assessed as described above.

In vivo cross-presentation. Three to four-month-old female Ly5-M-Cre+/−, Stim1−/− and otherwise wild-type Stim1−/− littermates were anesthetized with iso-flurane and injected with 50 μl of 1.0% OVA-beads/PBS on the left and 0.5% OVA-beads/PBS on the right footpads, or with only PBS on both footpads as control. Twenty four hours later the mice were retro-orbitally injected with 1 x 10^6 CD45.1+ OT-I cells labelled with 3.5 μg ml−1 polybrene at 500 μg ml−1 at 37°C for 1 h. To produce DC2114 cells stably expressing shRNA, cells were cultured in 1 μg ml−1 puromycin as of 2 days after transduction. Splenic OT-I cells were isolated from C57BL/6-Tg[TcraTcrb]1100Mjb/J mice (Jackson) using the CD8− negative selection mouse T cell isolation kit (Miltenyi Biotec, Germany) according to the manufacturer’s instructions. Congenic CD45.1− OT-I cells were obtained by breeding OT-I mice to the congenic CD45.1 strain B6. SJL-Ptpc− Pepc−BoyJ62 (Jackson) and similarly isolating CD8−/+ cells. Cell purity was verified to be >95% CD8+/−, CD4− by flow cytometry.

Electron microscopy. Classic transmission electron microscopy (TEM) and 3D Focused-ion beam scanning electron microscopy (FIB-SEM) were performed with the help of the Electron Microscopy Core Facility of the University of Geneva. TEM samples were prepared and imaged as described. 3D-FEM was performed on a Helios NanoLab G3 microscope (FEI, Netherlands) with images acquired using the electron beam at 2 kV/0.2 nA, and milling sections with the ion beam at 30 kV/2 nA. Milling conditions were kept constant every 10 nm. Specimens were prepared using a protocol modified from. Cells were fixed in 2.5% glutaraldehyde, 2% PFA in 2 mM CaCl₂, in 0.15 M cacodylate buffer (pH 7.4) (Ca-Caco) for 1 h on ice and washed 5× in cold Ca-Caco. Samples were incubated for 1 h on ice in 2% OsO₄, 1.5% KF/G in Ca-Caco and washed 5× in cold ddH₂O. Samples were then incubated in a freshly prepared and filtered 1% tincarbohydrazide for 20 min at RT, washed 5× in RT ddH₂O, incubated in 2% OsO₄ for 30 min at RT and then incubated in cold 1% uranyl acetate at 4 °C overnight. Samples were then washed 5× in RT ddH₂O and stained using Walton’s lead aspartate method for 30 min at 60 °C, and rinsed 5× in RT ddH₂O. Samples were dehydrated in 2× 7 min steps of cold 20, 50, 70, 90, 100 and 100% anhydrous ethanol, then cold anhydrous acetone for 10 min and RT acetone for 10 min. Samples were then embedded in hard epon resin by infiltrating with 25, 50 and 75% hard epon/acetone at RT for 2 h each, then 100% hard epon overnight. Fresh 100% hard epon was exchanged the next day incubated for 3 h. Epon was once more exchanged before polymerization for 48 h at 60 °C. Cart and microtome sections were cut at 1-2 μm using Walton’s lead aspartate method for 30 min at RT, dehydrated in 2× 5 min steps using 100% epon, embedded in an 1-2 μm epon, and coated with gold and Pelco conductive silver paint (Ted Pella, USA). Samples were sputter-coated with gold for 20 s using a Q150T ES coater (Quorum Technologies, UK).

In vitro cross-presentation. BMDCs were seeded at 20,000 cells per well and DC2114 at 10,000 cells per well in black/clear-bottom 96-well plates (Greiner, for BrDU-based assays) or in round-bottom 96-well plates (Corning, for CFSE assays), and allowed to adhere for 24 h. For 3:1 and 10:1 T cell/DC ratios, BMDCs were diluted accordingly. Cells were exposed to either OVA+B or 4H or SIINFEKL peptide (4μM) for 1 h for the given concentration, before they were washed in complete medium, and resuspended in 50 μl sterile PBS. After 72 h, the mice were sacrificed and popliteal draining and axillary/brachial non-draining lymph nodes were harvested in FACS buffer containing 2% FCS. Lymph nodes were mechanically crushed onto 40 μm cell strainers and the cellular filtrate blocked and stained as described above with anti-CD86/PE-Cy7 anti-CD45.1-APC antibodies. The total number of CD45.1+ cells as well as their CFSE fluorescence was determined for 50,000 CD86+ cells. % Proliferation is defined at the percentage of cells showing diluted levels of CFSE fluorescence as compared to un.injected controls.

In vitro migration. BMDCs isolated from Ly5-M-Cre+/− and from Ubiquitin-eGFP mice (C57BL/6-Tg[Ubc-GFP]) (Jackson), were washed and resuspended in PBS containing 0.5% OVA at 2 x 10^6 cells per ml. In total, 50 μl of cell/ bead mixture were injected into left and right footpads of congenic B6 CD45.1− mice. Popliteal lymph nodes were harvested 24 h or 48 h post injection and DCs were recovered from LN after digestion in an enzymatic mixture containing collagenase D (1 mg ml−1) and DNase I (10 μg ml−1) (Roche) in HBSS. Total LN cells were incubated with 0.5% (v/v) Ficoll for 10 min at 4 °C. Cells were pelleted, washed with 4 μM Fluo-8-AM (AAT Bioquest) for 30 min at 37 °C, 30 min at RT and 2.5 μM BAPTA-AM for the last 10 min, in modified Ringer’s containing 500 μM sulfinpyrazone. Simultaneous excitation at 488 and 543 nm and emission collection in two separate channels for green (Fluo8) and red (mCherry) was used. For quantification of periphagosomal Ca²⁺, hotspots, images were averaged over 6 s at intervals between 20 and 30 s. For quantification of periphagosomal Ca²⁺, Xestospongin C (1 μM) was applied 20 min after exposure of BMDCs to OVA+B and hotspots imaged at 30 min, while GSK9275A (a gift from Dr. Martin Lori˘cner, University of Bern, 10 μM) was applied concurrently with OVA+B and images taken after 30 min.

Western blotting. Cells were washed with ice cold PBS and lysates prepared as described. Total protein was quantified using a BCA assay (ThermoFisher) according to the manufacturer’s instructions. 50 μg per lane were loaded for ORAI1 gels, while 30 μg per lane were loaded for all others. 4-20% Mini-Page TGX Pre-cast gels (BioRad) and iBlot PVDF kits (ThermoFisher) were used for SDS-PAGE and transfer respectively. All antibodies were diluted in 3% milk/1% Tween/BSA, primary antibodies were incubated overnight and secondary antibodies incubated for 1 h. Membranes were visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore) and a X-ray gel imaging system (Syngene, UK).
**Phagosome isolation for leucyl aminopeptidase activity.** A 1.5 × 10^7 BMDCs were exposed to 3 × 10^9 magnetic 3 μm OVA-coated beads (Polysciences) for 15 min on ice. Warm medium was added and cells were incubated for 20 min at 37 °C. Cells were then returned to ice and washed twice in cold PBS and once in cold IEB buffer containing protease inhibitor cocktail (PI, Sigma) (IEB: 10 mM HEPES, 1 mM EGTA, 25 mM KCL, 250 mM sucrose). Cells were then centrifuged, and washed once in IEB/PI and counted. Cells were then resuspended in IEB/PI at a concentration of 4 × 10^6 cells mL^-1^, and passed 20x through a 20 μm clearance ball-bearing homogenizer. Homogenates were then transferred to a magnet and washed once in IEB/PI, once in IEB/PI + 4 mM MgCl2 + 10 mM Na-ATP, and were then incubated on an end-over-end rotor for 15 min at 4 °C. Homogenates were washed one final time on a magnet and resuspended in lysis buffer (20 mM Tris; 150 mM NaCl; 1% NP40) without protease inhibitors. Lysates were incubated on ice for 30 min prior to quantification using a BCA kit (ThermoFisher).

**Phagolysosome and phago-endosome fusion assay.** Phagolysosome fusion was measured using a protocol modified from 33. 3 μm Polybead Amino Microspheres (Polysciences) were labelled with Alexa-488-SE and coated with OVA as described in 1. Twenty four minutes prior to the assay the cells were pulse labelled with 20 μg mL^-1^ Alexa-594-HA (ThermoFisher) in complete medium for 3 h, washed, and followed by a chase period of 24 h to allow the dye to accumulate in lysosomes. Alexa488-OVA beads were added to cells at 201 in complete medium, were washed in modified Ringer’s after 30 min, and imaged using alternate 555/590, 488/530 nm Ex/Em illumination 30 and/or 90 min after addition. When applicable cells were pre-loaded for 30 min with 40 μM BAPTA-AM, and washed prior to stimulus addition and 0.2 nM ConA added just prior to beads. The P-L fusion index is computed as the ratio of the FRET (488/590)/Alexa-488 (488/530) signal minus the basal (external bead) FRET signal, normalized to the total average cellular Alexa-594-HA fluorescence. Phagolysosome fusion assays were performed in a similar manner except that 1 μg mL^-1^ Alexa-594-dextran of 10,000 MW (ThermoFisher) was added 15 min prior to beads, cells were washed 3x in complete medium prior to Alexa488-OVA bead addition and external beads were washed in modified Ringer’s after 15 min. Where applicable, GSK7977A (10 μM) was added concurrently with beads.

**Image analysis and statistics.** All fluorescence image analyses were performed using ImageJ (NIH) on maximum projections of at least 5 × 15 μm z-stacks per condition, except for IRAP quantification which were performed on a single confocal plane from each stack, and which were taken with a 100× objective. Semi-automated segmentation based on a single visually determined threshold for the pH/ROS/proteolysis insensitive wavelength did not fully separate all closely approximated phagosomes. Cells with >1 phagosome were excluded from the analysis to avoid any under-sampling bias of phagosomal membranes. MICS frequency was defined as the number of MICS (also called ER junctions) detected divided by the total number of phagosomes analysed, multiplied by 100 to obtain the average number of ER junctions per 100 phagosomes. Animal studies were not conducted blind, and at least N = 4 animals were tested for each condition, and, within each genotype, were randomly assigned to control and experimental groups. All statistical analyses were performed using Prism 6.0 software (GraphPad). Pairwise comparisons were made using a Mann-Whitney test for experiments based on phagocytosis, as phagocytic events do not follow a Gaussian distribution. A two-sided Student’s-t test was used for all other pairwise comparisons, and, where F-test showed significantly different variances, Welch’s correction was applied. A Two-way ANOVA with Sidak’s multiple comparisons test was used for repeated measures. N = number of independent experiments, at least three independent experiments were performed for all conditions.

**Data availability.** The data that support the findings of this study are available from the corresponding author upon reasonable request.
References

1. Ackerman, A. L. & Cresswell, P. Cellular mechanisms governing cross-presentation of exogenous antigens. Nat. Immunol. 5, 678–684 (2004).

2. Manolova, V. et al. Nanoparticles target distinct dendritic cell populations according to their size. Eur. J. Immunol. 38, 1404–1413 (2008).

3. Gagnon, E. et al. Endoplasmic reticulum-mediated phagocytosis is a mechanism of entry into macrophages. Cell 110, 119–131 (2002).

4. Campbell-Valois, F. X. et al. Quantitative proteomics reveals that only a subset of the endoplasmic reticulum contributes to the phagosome. Mol. Cell. Biol. 11, M111 01378 (2012).

5. Guermonprez, P. et al. ER-phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells. Nature 425, 397–402 (2003).

6. Touret, N., Paroisi, P. & Grinstein, S. The nature of the phagosome membrane: endoplasmic reticulum versus plasmalemma. J. Leukoc. Biol. 77, 878–885 (2005).

7. Zehner, M. et al. The translocon protein Sec61 mediates antigen transport from endosomes in the cytosol for cross-presentation to CD8(+) T cells. Immunity 42, 850–863 (2015).

8. Nair-Gupta, P. et al. TLR signals induce phagosomal MHC-I delivery from the endosomal recycling compartment to allow cross-presentation. Cell 158, 506–521 (2014).

9. Demaurex, N. & Nunes, P. The role of STIM and Orai proteins in phagocytic immune cells. Am. J. Physiol. Cell Physiol. 310, C496–C508 (2016).

10. Hogan, P. G. & Rao, A. Store-operated calcium entry: mechanisms and modulation. Biochem. Biophys. Res. Commun. 460, 40–49 (2015).

11. Shen, W. W. & Demaurex, N. Morphological and functional aspects of STIM1-dependent assembly and disassembly of store-operated calcium entry complexes. Biochem. Soc. Trans. 40, 112–118 (2012).

12. Hsu, S. et al. Fundamental Ca2+ signaling mechanisms in mouse dendritic cells: CRAC is the major Ca2+ entry pathway. J. Immunol. 166, 6126–6133 (2001).

13. Bandypadhyay, B. C., Pingle, S. C. & Ahern, G. P. Store-operated Ca(2+) signaling in dendritic cells occurs independently of STIM1. J. Leukoc. Biol. 89, 57–62 (2011).

14. Felix, R. et al. The Orai-1 and STIM1 complex controls human dendritic cell maturation. PLoS ONE 8, e61595 (2013).

15. Vaeth, M. et al. Ca2+ Signaling but not store-operated Ca2+ entry is required according to their size. Annu. Rev. Immunol. 31, 443–473 (2013).

16. Saveanu, L. et al. IRAP identifies an endosomal compartment required for MHC class I cross-presentation. Science 325, 213–217 (2009).

17. Blum, J. S., Wearsch, P. A. & Cresswell, P. Pathways of antigen processing. Annu. Rev. Immunol. 31, 443–473 (2013).

18. Rao, S., Liu, X., Freedman, B. D. & Behrens, E. M. Spleen tyrosine kinase (Syk)-dependent calcium signals mediate efficient Cpg-induced exocytosis of tumor necrosis factor alpha (TNFalpha) in innate immune cells. J. Biol. Chem. 288, 12448–12458 (2013).

19. Kremlitzka, M., Macsik-Valent, B. & Erdei, A. Syk is indispensable for CpG-induced activation and differentiation of human B cells. Cell. Mol. Life Sci. 72, 2223–2236 (2015).

20. Nunes, P. & Dinauer, M. C. Regulation of the NADPH oxidase and associated ion fluxes during phagocytosis. Traffic 14, 1118–1131 (2013).

21. Rybicka, J. M., Balce, D. R., Chaudhuri, S., Allan, E. R. & Yates, R. M. Phagosomal proteolysis in dendritic cells is modulated by NADPH oxidase in a pH-independent manner. EMBO J. 31, 932–944 (2012).

22. Savina, A. et al. The small GTPase Rac2 controls phagosomal alkalinization and antigen crosspresentation selectively in CD8(+) dendritic cells. Immunity 30, 544–555 (2009).

23. Savina, A. et al. NOX2 controls phagosomal pH to regulate antigen processing during crosspresentation by dendritic cells. Cell 126, 205–218 (2006).

24. El Chemaly, A., Nunes, P., Jimaja, W., Castelbou, C. & Demaurex, N. Hv1 proton channels differentially regulate the pH of neutrophil and macrophage phagosomes by sustaining the production of phagosomal ROS that inhibit the delivery of vacuolar ATPases. J. Leukoc. Biol. 95, 827–839 (2014).

25. Jankowski, A., Scott, C. C. & Grinstein, S. Determinants of the phagosomal pH in neutrophils. J. Biol. Chem. 277, 6059–6066 (2002).

26. Tili, A., Dupre-Crochet, S., Erdar, M. & Nusse, O. Kinetic analysis of phagosomal production of reactive oxygen species. Free Radic. Biol. Med. 50, 1444–147 (2011).

27. Tsujikawa, H. et al. Identification of key amino acid residues responsible for internal and external pH sensitivity of Orai1/STIM1 channels. Sci. Rep. 5, 16747 (2015).

28. Allootti, A. et al. Toll-like receptor 4 engagement on dendritic cells restrains phago-lysosome fusion and promotes cross-presentation of antigens. Immunity 43, 1087–1100 (2015).

29. Singh, R. & Cresswell, P. Defective cross-presentation of viral antigens in GILT− deficient mice. Science 328, 1394–1398 (2010).

30. Kurotaki, T. et al. Efficient cross-presentation by heat shock protein 90-epitope complex-loaded dendritic cells via an endosomal pathway. J. Immunol. 179, 1803–1813 (2007).

31. Blattner, T. W. et al. Flagellar receptor antigen targeting potentiates cross-presentation by human blood and lymphoid tissue BDCA-3+ dendritic cells. Blood 120, 5163–5172 (2012).

32. Accapezzato, D. et al. Chloroquine enhances human CD8+ T-cell responses against soluble antigens in vivo. J. Exp. Med. 202, 817–828 (2005).

33. Jancic, C. et al. Rab27a regulates phagosomal pH and NADPH oxidase redox signaling to promote cross-presentation. Nat. Cell Biol. 9, 367–374 (2007).

34. Maschelaki S. et al. UNC93B1 interacts with the calcium sensor STIM1 for efficient antigen cross-presentation in dendritic cells. Nat. Commun. doi:10.1038/ncomms10601 (2017).

35. Oliveira, C. C. & van Hall, T. Importance of TAP-independent processing pathways. Mol. Immunol. 55, 113–116 (2013).

36. Kauffman, U. et al. Selective Orai1 inhibition ameliorates autoimmune central nervous system inflammation by suppressing effector but not regulatory T cell function. J. Immunol. 196, 573–585 (2016).

37. Endo, Y. et al. Dominant mutations in ORAI1 cause tubular aggregate disease. Cell 162, 828–839 (2015).

38. Ingham, L. & Murrell, D. G. Taming the sphinx: mechanisms of cellular sphingolipid homeostasis. Annu. Rev. Biochem. 81, 495–539 (2012).

39. Maschelaki S. et al. UNC93B1 interacts with the calcium sensor STIM1 for efficient antigen cross-presentation in dendritic cells. Nat. Commun. doi:10.1038/ncomms10601 (2017).

40. Van Den Berghe, M. et al. Orai1 calcium channel orchestrates skin homeostasis. Proc. Natl Acad. Sci. USA 110, E4839–E4848 (2013).

41. Mocita, F. et al. Targeting Stim and Orai proteins as an alternative approach in cancer therapy. Curr. Med. Chem. 23, 3450–3461 (2016).

42. Olson, D. K., Fröhlich, F., Farese, R. V. & Walther, T. C. Targeting Stim and Orai proteins as an alternative approach in cancer therapy. Cell Metab. 23, 3450–3461 (2016).
57. Luik, R. M., Wu, M. M., Buchanan, J. & Lewis, R. S. The elementary unit of store-operated Ca2+ entry: local activation of CRAC channels by STIM1 at ER-plasma membrane junctions. *J. Cell Biol.* 174, 815–825 (2006).

58. Suter, D. M. et al. Rapid generation of stable transgenic embryonic stem cell lines using modular lentivectors. *Stem Cells* 24, 615–623 (2006).

59. Clausen, B. E., Burkhardt, C., Reith, W., Renkawitz, R. & Forster, I. Conditional gene targeting in macrophages and granulocytes using LysMcre mice. *Transgenic Res.* 8, 265–277 (1999).

60. Oh-Hora, M. et al. Dual functions for the endoplasmic reticulum calcium sensors STIM1 and STIM2 in T cell activation and tolerance. *Nat. Immunol.* 9, 432–443 (2008).

61. Hogquist, K. A. et al. T cell receptor antagonist peptides induce positive selection. *Cell* 76, 17–27 (1994).

62. Shen, F. W. et al. Cloning of Ly-5 cDNA. *Proc. Natl Acad. Sci. USA* 82, 7360–7363 (1985).

63. Schaefer, B. C., Schaefer, M. L., Kappler, J. W., Marrack, P. & Kedl, R. M. Observation of antigen-dependent CD8+ T-cell/dendritic cell interactions in vivo. *Cell. Immunol.* 214, 110–122 (2001).

64. Deerinck, T. J., Bushong, E., Thor, A. & Ellisman, M. H. NCMIR methods for 3D EM: a new protocol for preparation of biological specimens for serial block face scanning electron microscopy. *National Center for Microscopy and Imaging Research* https://www.ncmir.ucsd.edu/sbem-protocol/ (2010).

65. Nunes P., Guido D. & Demaurex N. Measuring phagosome ph by ratiometric fluorescence microscopy. *J. Vis. Exp.* 106, e53402 (2015).

66. Sepulveda, F. E. et al. Critical role for asparagine endopeptidase in endocytic Toll-like receptor signaling in dendritic cells. *Immunity* 31, 737–748 (2009).

67. Babdor, J. et al. IRAP+ endosomes restrict TLR9 activation and signaling. *Nat. Immunol.* 18, 509–518 (2017).

**Acknowledgements**

We are grateful to the bioimaging core facility and the electron microscopy core facility (Geneva Medical Centre) and to Drs. Maud Frieden, Claes Wollheim and Isabelle Dunand-Sauther for their valuable advice as well as to Nina Crado-Santos and Christian Vesin for technical assistance with immunostainings and animal experiments, respectively. This work was funded by the Swiss National Foundation [grant number 31003A-149566 and CRSII3_160782 (to N.D.)]; COST Action BM1406, and a Young Investigator Subsidy from The Sir Jules Thorn Overseas Charitable Trust (to P.N.-H.).

**Author contributions**

P.N.-H. designed the study, designed and performed experiments, analysed and interpreted data, and wrote the manuscript. N.D. supervised study design and data interpretation, and edited the manuscript. B.M., S.M., C.L., S.H., designed and performed experiments, analysed and interpreted data. D.Me. and D.Ma. supervised and contributed research tools. D.Ma. also helped with the study design and data interpretation. S.B., D.G., and F.B. performed experiments and analysed data. C.C., E.Y.B., N.P. performed experiments.

**Additional information**

Supplementary Information accompanies this paper at doi:10.1038/s41467-017-01600-6.

**Competing interests:** The authors have no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

**Publisher’s note:** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.