Saponin-based adjuvants induce cross-presentation in dendritic cells by intracellular lipid body formation

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Saponin-based adjuvants (SBAs) are being used in animal and human (cancer) vaccines, as they induce protective cellular immunity. Their adjuvant potency is a factor of inflammasome activation and enhanced antigen cross-presentation by dendritic cells (DCs), but how antigen cross-presentation is induced is not clear. Here we show that SBAs uniquely induce intracellular lipid bodies (LBs) in the CD11b+ DC subset in vitro and in vivo. Using genetic and pharmacological interference in models for vaccination and in situ tumour ablation, we demonstrate that LB induction is causally related to the saponin-dependent increase in cross-presentation and T-cell activation. These findings link adjuvant activity to LB formation, aid the application of SBAs as a cancer vaccine component, and will stimulate development of new adjuvants enhancing T-cell-mediated immunity.
Adjuvants are diverse compounds that enhance the effectiveness of (cancer) vaccines. For human vaccines, registered adjuvants are limited and include aluminum-based and oil/water-based adjuvants. These adjuvants induce robust antibody responses, but weak cell-mediated immunity, which is crucial for application in anti-cancer vaccines. Saponin-based adjuvants (SBAs) are promising new adjuvants that enhance T-cell-mediated immunity.

Saponins are a large family of amphipathic plant glycosides, structurally sharing a lipophilic triterpene derivative. By purification of the raw plant material, fractions with distinct immunostimulatory properties and safety profiles have been identified. Although most saponins have strong binding affinity for cholesterol, only some stimulate the immune system. Forty nanometer cage-like particles called immune stimulatory complexes (ISCOMs) form when immunostimulative saponin, cholesterol and phospholipid are brought together and dialysed. The physical properties of ISCOM adjuvants contribute to its stability, and reduce the haemolytic effects associated with saponin. SBAs stimulate strong innate and adaptive cellular immunity, and elicit humoral responses of all IgG isotypes with a maximum response occurring uniquely in the CD11b⁺/CD103⁻ DCs (pDCs) or monocyte-derived DCs. cDCs are located in the inflammasome (for example, Al(OH)₃). Dendritic cells (DCs) have a critical role in the induction of T-cell immunity, and elicit humoral responses of all IgG isotypes with a mixed Th1/Th2 balance. By contrast, aluminum-based or oil/water-based adjuvants predominantly drive Th2 responses.

SBAs are now being applied in human vaccines, and several clinical trials have proven safety and efficacy. Next to viral antigens (for example, H1N1), SBAs have been shown to facilitate responses to cancer antigens. We previously demonstrated in our model of in situ tumour ablation that co-injection of SBAs induced superior anti-tumour immunity relative to other adjuvants. This unique effect was accompanied by high SBAs induced superior anti-tumour immunity relative to other adjuvants that trigger the inflammasome (for example, Al(OH)₃). However, this finding does not fully explain why ISCOMs stand out from other adjuvants. Wilson referred to as the ‘cytosolic’ and ‘vacuolar’ pathways. In the cytosolic cross-presentation pathway, internalized proteins are slowly degraded in endosomal compartments by enzymatic digestion at acidic pH. By unknown mechanisms the antigens gain access to the cytosol where they are further degraded in a proteasome dependent manner. Peptides generated this way can then enter the classical MHC-I presentation route. By contrast, cross-presentation through the vacuolar pathway is proteasome independent, but sensitive to blockade of lysosomal proteolysis.

Results
Saponin-based adjuvants induce cross-presentation in DCs. SBAs induce potent immune responses to a broad repertoire of molecular patterns or CD4⁺ T helper cells. Next to activation status, the sub-class of DC is also of importance. DCs can be classified as conventional DCs (cDCs), plasmacytoid DCs (pDCs) or monocyte-derived DCs. cDCs are located in lymphoid and non-lymphoid tissues and can be further classified into two ontogenetically distinct subtypes: the CD8α⁺/CD103⁺ and the CD11b⁺/CD8α⁻/CD103⁻. In vivo studies suggest that cross-presentation is typically performed by the CD8α⁺/CD103⁺ subset of cDCs, however, under specific conditions every other subtype is capable of cross-presentation. For instance, in an inflammatory environment in vivo CD11b⁺/monocyte-derived DCs efficiently cross-present OVA protein expressed by Escherichia coli (ref. 26). In our tumour models, SBAs trigger an unprecedented level of cross-presentation, but how SBAs steer this process is unknown. Lipid body (LB) organelles consist of a phospholipid monolayer that surrounds a core of neutral lipids, such as sterol esters or triacylglycerols. The monolayer contains numerous proteins, many with unknown function. Enzymes of lipid metabolism (for example, diacylglycerol acyltransferase, DGAT), LB membrane proteins (for example, adipose differentiation-related protein, ADRP), and ER proteins (for example, the P47Pase IGTP) have been identified in LB fractions. Ralph Steinman made note of LBs in his seminal 1973 manuscript describing the dendritic cell, but their function in immune cells is only just starting to be explored.

Our data now uncover an essential link between LBs and cross-presentation in the working mechanism of SBA adjuvants occurring uniquely in the CD11b⁺ DC subset. Genetic or pharmacological blockade of LB induction effectively abrogates the SBA-induced antigen cross-presentation, in a vaccination setting as well as in an in situ tumour ablation model. Mechanistic studies suggest that SBAs induce LBs independent of SBA-triggered antigen translocation, and that LBs facilitate the proteosomal route of cross-presentation. These data highlight the importance of lipid body induction in CD11b⁺ DC for SBA vaccine activity, will aid SBA vaccine development, and the design of new adjuvants enhancing T-cell-mediated immunity.
Saponin-based adjuvants induce cross-presentation in dendritic cells. (a,b) Tetramer staining of OVA specific T cells in draining lymph nodes, 10 days following s.c. injection of 20 μg OVA and/or 30 μg ISCOMs (a, vaccination), or 7 days following co-treatment of established B16-OVA melanomas (5–8 mm diameter) with in situ tumour ablation and 30 μg peritumourally injected ISCOMs (b, Cryo-ablation). (c,d) In vitro B32 cross-presentation assays demonstrating a concentration-dependent increase in cross-presentation of OVA, only when Matrix C ISCOMs was added. GM-CSF-cultured BMDCs were exposed to the indicated compounds and 80 μm LacZ production by B3Z (OD595) was measured by the internalization of fluorescent OVA or breakdown of lacZ production in vivo (Supplementary Fig. 1b). The increases in cross-presentation could not be explained by antigen uptake or co-stimulation by SBAs in DCs are antigen uptake or co-stimulation independent. In contrast, a saponin mixture not enriched for immuno-active fractions (crude saponin) did not induce cross-presentation, even at higher concentrations (Fig. 1e). Importantly, SBA treatment did not lead to reduced cell viability or MHC-I levels on the surface of the APCs, as presentation of pulsed peptide was intact in all occasions (Fig. 1e; Supplementary Fig. 3). Finally, we confirmed that within the ISCOMs the saponins are the cross-presentation inducing moiety, and not the ISCOM-stabilizing component cholesterol (Supplementary Fig. 4). Cross-presentation induction in DCs thus is a feature shared by immunoactive saponins, but not by other saponins.

**SBA-induced cross-presentation is co-stimulation independent.** The increases in cross-presentation could not be explained by differential surface expression of the mannose receptor (CD206, involved in uptake of OVA), or MHC-II/II (Fig. 2a), and consistent with literature, SBAs also did not change CD80 or CD86 levels on the in vitro cultured DCs (Fig. 2b)17. Together with the fact that B32 cells react co-stimulation independent, this indicated that maturation status of the DCs is not causing the observed increases in cross-presentation. In line with this, BMDCs from Tlr4+/−, Myd88−/− or Trif−/− mice all had an intact ability to cross-present OVA following ISCOMs treatment (Fig. 2c). Moreover, also the type 1 IFN receptor or the inflammasome component NLRP3 did not affect SBA-aided cross-presentation (Fig. 2c). Antigen uptake or overall antigen processing by GM-CSF DCs, as measured by the internalization of fluorescent OVA or breakdown of DQ-OVA, was not altered following SBA treatment (Fig. 2d).

We conclude that the increases in cross-presentation induced by SBAs in DCs are antigen uptake or co-stimulation independent.

**Endosomal ROS is not involved in cross-presentation by SBAs.** As previous work has implicated a role for reactive oxygen species (ROS) produced by the phagosomal NADPH-oxidase (NOX) complex in cross-presentation41, we set out to determine cellular ROS levels following SBA exposure. Employing two probes for detection of total ROS levels in our DCs (DHR123 and H₂DCFDA), we found that SBA treatment for 5 h (similar to the exposure time in the cross-presentation assays) did not increase ROS levels, while the control PMA did (Fig. 2e). Although ceasing ROS production by co-treatment of the cells with commonly used

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**Figure 1 | Saponin-based adjuvants induce cross-presentation in dendritic cells.** (a) Tetramer staining of OVA specific T cells in draining lymph nodes, 10 days following s.c. injection of 20 μg OVA and/or 30 μg ISCOMs (vaccination), or 7 days following co-treatment of established B16-OVA melanomas (5–8 mm diameter) with in situ tumour ablation and 30 μg peritumourally injected ISCOMs (Cryo-ablation). (c) In vitro B32 cross-presentation assays demonstrating a concentration-dependent increase in cross-presentation of OVA, only when Matrix C ISCOMs was added. GM-CSF-cultured BMDCs were exposed to the indicated compounds and 80 μm LacZ production by B3Z (OD595) was measured by the internalization of fluorescent OVA or breakdown of lacZ production in vivo (Supplementary Fig. 1b). The increases in cross-presentation could not be explained by antigen uptake or co-stimulation by SBAs in DCs are antigen uptake or co-stimulation independent. In contrast, a saponin mixture not enriched for immuno-active fractions (crude saponin) did not induce cross-presentation, even at higher concentrations (Fig. 1e). Importantly, SBA treatment did not lead to reduced cell viability or MHC-I levels on the surface of the APCs, as presentation of pulsed peptide was intact in all occasions (Fig. 1e; Supplementary Fig. 3). Finally, we confirmed that within the ISCOMs the saponins are the cross-presentation inducing moiety, and not the ISCOM-stabilizing component cholesterol (Supplementary Fig. 4). Cross-presentation induction in DCs thus is a feature shared by immunoactive saponins, but not by other saponins.

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Figure 2 | SBA-induced cross-presentation is independent of co-stimulation or ROS. (a) FACS analysis of surface markers CD206 (mannose receptor), MHC-I, and MHC-II. GM-CSF BMDCs were exposed for 5 h to ISCOMs or medium, and processed for FACS staining. Open black lines represent the corresponding isotype controls. Filled grey lines show the medium-treated cells, while the open red lines are the ISCOM treated samples. (b) FACS analysis of DC maturation markers CD80 and CD86 after 18 h stimulation with ISCOMs or 1 μg ml⁻¹ CpG. (c) Bone-marrow of indicated knockout mice was used to generate GM-CSF BMDCs. Cells were exposed for 5 h to medium or ISCOMs, washed and co-cultured α/β with B3Z cells. External peptide pulsing (5 pg ml⁻¹, 30 min) was used to control for viability and/or MHC-I levels. (d) Antigen uptake and processing as analysed by FACS. BMDCs were given 0.25 mg ml⁻¹ OVA coupled to the fluorophore Alexa647, or 1 μg ml⁻¹ DQ-OVA, during the 5 h exposure time to medium or ISCOMs. DQ-OVA will start to fluoresce once degraded. (e) Total cellular reactive oxygen species (ROS) were measured using the DHR123 probe. GM-CSF cultured BMDCs were plated in serum-free medium and pretreated for 10 min with 0.5 or 50 μM dihydrorhodamine 123. Next, ISCOMs or 1 μg ml⁻¹ PMA was added and cells were incubated for 45 min before analysis by FACS. (f) In vitro cross-presentation in the presence of NADPH oxidase inhibitors. Indicated compounds and concentrations were added during the 5 h exposure period to ISCOMs. (g) In vitro cross-presentation using Gp91phox⁻/⁻ bonemarrow DCs. Data in (e) represent single values in titration, whereas the other data show means from triplicates with s.e.m. Data are representative figures from two to three individual experiments. Statistical analyses were performed using two way ANOVA with post hoc Bonferroni tests.
concentrations of the NADPH oxidase inhibitor DPI initially seemed to abrogate the SBA-induced cross-presentation, the peptide control revealed lowered MHC-I expression due to decreased cell viability or down regulation (Fig. 2f). Three alternative NADPH oxidase inhibitors and various ROS scavengers did however not interfere with SBA-induced cross-presentation (Fig. 2f; Supplementary Fig. 5). Finally, SBAs could normally induce cross-presentation in DCs deficient in the ROS-inducing NOX2 complex component Cybb (GP91phox) (Fig. 2g).

Altogether these data do not support a significant role for ROS in the induction of cross-presentation by SBAs.

SBAs induce lipid bodies in parallel with cross-presentation. Bougnères et al. previously demonstrated a relationship between cross-presentation in DCs and the lipid body-residing molecule IGTP. This prompted us to examine the presence of LBs in DCs exposed to various non-microbial adjuvants. To this end, we stained cells with the neutral lipid dye Bodipy493/503. Strikingly, SBAs (PC saponin, ISCOMs) appeared to increase the number and the brightness of cytosolic LBs as determined by confocal microscopy (CLSM), always in perfect correlation with cross-presentation levels (Figs 1d and 3a,b). Co-staining for the LB protein ADRP confirmed that the detected lipid aggregates were LBs (Fig. 3c). Other adjuvants like ALPO4, non-immunogenic crude saponin or OVA alone could not elevate the number of LBs (Fig. 3b). A time course demonstrated that the numbers of LBs per cell increased linearly over the first 32 h, which again strongly correlated with cross-presentation levels (Fig. 3d–f). Control experiments confirmed that the saponins, but not the ISCOM particle stabilizing component cholesterol represent the active moiety inducing LBs, and that mice deficient for Tlr4, Nlpr3 or Ifnar had a normal ability to generate LBs following SBAs (Supplementary Fig. 6).

Together, these results suggest that GM-CSF DCs in vitro possess the striking ability to induce LBs following treatment with SBAs, which is tightly correlated with the ability to cross-present soluble antigen.

LB formation and cross-presentation in DCs in vivo. To determine whether in vivo vaccination with SBAs also resulted in LB formation in cross-presenting DCs, we co-injected mice with ISCOMs and OVA. Twelve hours post-injection draining lymph node (DLN) cells were isolated and subjected to CD11c + magnetic bead sorting to isolate all DCs (Fig. 3g). Similarly, DCs were isolated from B16OVA tumour bearing mice after cryo-ablation of the tumour (Fig. 3h). Assaying the isolated DCs for cross-presentation of a soluble antigen, the saponins, which is tightly correlated with the ability to cross-present antigen upon SBA exposure, the Flt3-L generated DCs were incapable of inducing LBs (Fig. 4b), even at longer exposure times.

The BM cultures with Flt3-L or GM-CSF both give rise to a complex set of DC subpopulations each arising from predetermined precursors present in the starting population. The observation that only 45% of the in vitro generated GM-CSF DCs contained LBs prompted us to purify the different DC subsets using flow cytometry, and to test their response to SBAs. First, Flt3-L DCs were sorted based on Naik et al. (Fig. 4c). In the three resulting DC subpopulations no population capable of cross-presenting OVA upon SBA exposure could be detected (Fig. 4d). GM-CSF DCs were sorted based on Heft et al., with modifications (Fig. 4e). Strikingly, only the CD135 (Flt3) negative DCs (MHCII intCD11b+CD115intCD135neg and MHCII intCD11b+CD115negCD135neg subpopulations) could efficiently cross-present OVA in response to SBAs, whereas the CD135 positive DCs (MHCII intCD11b+CD115lowCD135pos subset) could not (Fig. 4f). Since the MHCII intCD11b+CD115hiCD135pos cells are considered multipotent intermediates, we focused on the two differentiated DC populations. Again, SBA-mediated induction of lipid bodies only occurred in the DC subset cross-presenting antigens due to SBAs: at least 80% of the MHCII intCD11b+CD115hiCD115hiCD135pos DCs now contained LBs after 5 h (Fig. 4g). These SBA responsive DCs were previously shown to be derived from monocyctic precursors. As apparently the monocyctic precursors in the Flt3-L culture did not yield SBA responsive DCs, the Flt3-L culture was supplemented with GM-CSF for the last two days. Strikingly, the Flt3-L DCs now acquired full ability to cross-present antigens in response to SBAs (Fig. 4h). Sorting experiments confirmed that CD11b+ (Sirpα+ ) DCs, but not the CD8α+ (CD24+) DCs, were responsible for SBA-induced cross-presentation (Fig. 4i; Supplementary Fig. 8). Similarly, analysis of pDCs, and CD8α+ or CD11b+ cDCs from Flt3-L cultures that were first sorted and then individually exposed to GM-CSF confirmed that only the CD11b+ DCs gained the capacity to respond to SBAs (Fig. 4i). Stainings for the monocyctic marker CD115 showed increased presence of CD115+ cells among the SBA-responsive CD11b+ DCs following GM-CSF exposure (Supplementary Fig. 8).

Collectively, these results define GM-CSF-sensitized CD11b+ DCs, presumably from monocyctic origin, to be the only cells responsive to SBAs.

Only monocyctic CD11b+ DCs respond to SBAs in vivo. Next, we determined whether the selective SBA responsiveness of in vitro cultured DCs also exists in vivo. CD8α+ and CD11b+ DCs sorted from naive spleens based on the markers CD24, Sirpα, CD115 and CD4 (Fig. 5a), could not cross-present OVA upon SBA exposure but efficiently presented externally loaded control peptides (Fig. 5b). This is not unexpected as no CD115 staining was observed on these CD11b+ DCs, indicating the absence of cells from monocytic origin, shown to be responsive to SBA in vitro (Fig. 5a). Therefore, resident and migratory CD8α+, CD103+ or CD11b+ DCs were sorted from naive LNs (Fig. 5c). Subsequent exposure to SBAs demonstrated that LN resident, but especially the LN migratory CD11b+ DCs, were capable of cross-presenting OVA (Fig. 5d). The ability to respond to SBAs coincided with the presence of cells expressing the monocyctic markers CD14 and Ly6C within the resident, but especially the migratory CD11b+ DCs (Supplementary Fig. 9). To evaluate a situation with a high monocytic influx, we analysed mice bearing Flt3-L excreting tumours to provoke expansion of endogenous DCs, including pDCs, in an activated environment. From the tumour-draining LNs we isolated the pDCs...
fraction, a fraction containing all CD8α+ LN-resident DCs, and the CD11bhi DC fraction, and exposed them in vitro to SBAs (Fig. 5e). Clearly, only the CD11bhi DC fraction was able to cross-present OVA upon SBA exposure (Fig. 5f). As expected, these DCs were also the only cells that contained LBs after SBA treatment (Fig. 5g). Additional FACS analysis of MHC-II levels
showed that these cells were highly activated, making it difficult to discriminate migratory from resident CD11b+ cells within this gate (Fig. 5e). As an additional control, we showed that the CD8α+ DC containing fraction was able to cross-present OVA incorporated in nanoparticles, as shown previously (Fig. 5f). Finally, we sorted cells from mice treated by in situ tumour ablation with co-injection of the TLR9 ligand CpG-ODN. This procedure induces the influx of fresh (inflammatory) monocytes into the LNs, but not pDCs like with the Flt3-L excreting tumours. Similar to our former findings, only the CD11bhi DCs could react to SBAs (Supplementary Fig. 10).

Thus, also in vivo monocyte CD11b+ cells uniquely harbor the ability to cross-present soluble antigens and to induce LBs upon exposure to SBAs.

**SBA-induced lipid bodies are critical for cross-presentation.** To investigate whether a causal relationship exists between LBs and cross-presentation, we searched for ways to cease LB formation. Blocking formation of triacylglycerol, fueling the LBs, by inhibiting the rate-limiting enzymes acyl-CoA, diacylglycerol acyltransferase (DGAT) 1 and 2 or by inhibiting acyl-CoA synthetase (ACS) or acetyl-CoA carboxylase (ACC), which are further upstream in lipid synthesis, all abrogated LB formation (Fig. 6a)31,47,48. Importantly, inhibiting LB formation also ended the SBA dependent capacity of DCs to cross-present antigen (Fig. 6b). Viability of the cells and inhibiting LB formation also ended the SBA dependent capacity of DCs to cross-present antigen (Fig. 6b). Viability of the cells and

**Figure 4 | Responsiveness to SBAs in vitro is unique to CD11b+ DCs with monocyotic origin.** (a) In vitro cross-presentation assay using GM-CSF- or Flt3-L-propagated DCs. (b) Confocal images with LB quantifications from Flt3-L-generated BMDCs exposed for 5 h to medium or ISCOMs. (c) Gating strategy of Flt3-L DCs. Sorted populations are gate 1 (pDCs), gate 3 (CD8α+ DCs) and gate 4 (CD11b+ DCs). (d) In vitro cross-presentation assay using the populations as sorted under c. (e) Gating strategy of GM-CSF DCs. Sorted populations are gate 2 (MHCIIhi CD11bhi), gate 3 (MHCIIlo CD11bhi) and gate 4 (MHCIIlo CD11bint). (f) In vitro cross-presentation assay using the populations as sorted under e. (g) CLSM image and LB quantification of populations as sorted under e, after 5 h exposure to ISCOMs or medium. (h) In vitro cross-presentation assay on Flt3-L- or GM-CSF-cultured bonemarrow DCs. In the 10 day Flt3-L cultures, medium was supplemented for the last 48 h with 20 ng ml⁻¹ GM-CSF or medium. (i) In vitro cross-presentation assays on Flt3-L-cultured DCs, receiving GM-CSF for 72 h after the end of culture. In the left panel the entire day 10 cultures were supplemented with GM-CSF for another 72 h, after which the resulting populations were sorted (Supplementary Fig. 8). In the right panel the day 10 cultures were sorted, after which the individual populations were treated with fresh Flt3-L or GM-CSF. All data were reproduced in two to three independent experiments. Statistical analyses were performed using two-way ANOVA with post hoc Bonferroni tests.

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The diminished LB levels, cross-presentation levels were reduced in the *Igtp* −/− cells, confirming the vital role for LBs in SBA-induced cross-presentation (Fig. 6e). This effect was not related to altered MHC-I or CD206 expression by DCs, or OVA processing (Supplementary Fig. 11). To validate the essential role of LBs in the observed processes, we analysed *Adrp* −/− mice also reported to have reduced functional LBs31,49. We confirmed the lower LB contents, and subsequently also found reduced SBA-induced cross-presentation (Supplementary Fig. 12).

Collectively, these results show that LB formation is essential for effective SBA-induced cross-presentation in vitro.

**Endosomal acidification and cytosolic antigen translocation.** Enhanced endosomal antigen translocation to the cytosol has recently been proposed as a possible mechanism for SBA-induced cross-presentation35. Still, the position of LBs relative to cytosolic translocation, and the contribution of each process to SBA-aided cross-presentation is unclear. To assay endosomal translocation, SBA-treated DCs received the mitochondrial protein cytochrome c. Extra-mitochondrial localization of cytochrome c induces apoptosis, so translocation of endosomal cytochrome c to the cytosol will lead to reduced metabolic activity in a MTT assay. This assay, and similar experiments using the plant toxin

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**Figure 5 | Lymph node CD11b+ DCs uniquely possess the ability to respond to SBAs in vivo.** (a) Gating strategy for naive splenocytes. Sorted populations are gates 2 (CD8α+ DCs) and gate 3 (CD11b+ DCs). (b) In vitro cross-presentation assay using the populations as sorted under a. (c) Gating strategy for naive lymph nodes. Lin was defined as B220- and Ly6G-. Sorted populations are gate 4 (LN resident CD8α+ DCs), gate 5 (LN resident CD11b+ DCs), gate 6 (LN migratory CD103 DCs), and gate 7 (LN migratory CD11b+ DCs). (d) In vitro cross-presentation assay using the populations as sorted under c. (e-g) Mice were injected with Flt3-L excreting B16 melanomas to expand endogenous DC pools. After 12 days, pooled lymph nodes were harvested and subjected to FACS sorting. (e) Gating strategy, in which sorted populations are gates 1 to 4. Right panels show additional stainings on populations 1 to 3. (f) In vitro cross-presentation assay using populations as sorted under e. Sorted cells were exposed for 5 h to the indicated compounds (ISCOMs 800 ng ml−1, OVA 300 μg ml−1, PLGA nano-particles corresponding to 30 μg ml−1 OVA). (g) LB quantification in populations as sorted under e. All data were reproduced in two independent experiments. Statistical analyses were performed using two-way ANOVA with post hoc Bonferroni tests.
saporin that inactivates ribosomes when cytosolic,
confirmed that increased cytosolic translocation took place in the presence of SBAs (Fig. 6f). Blockade of endosomal acidification showed that SBA-induced cytosolic translocation and cross-presentation were both dependent on low endosomal pH (Fig. 6g; Supplementary Fig. 13a), while treatment with the proteasome inhibitor epoxomicin demonstrated that cross-presentation also critically depended on the proteasome.
(Supplementary Fig. 13b). Strikingly, blockade of endosomal acidification (and thus endosomal escape) did not lower the numbers of SBA-induced LBs per cell (Fig. 6h). Conversely, pharmacological interference with LB induction also did not abrogate endosomal translocation (Fig. 6i).

These data show that SBA-triggered antigen translocation, but not SBA-induced LB formation, is critically preceded by endosomal acidification, and imply a role for SBA-induced LBs in facilitating the proteasomal route of cross-presentation.

**LBs are vital for SBA-induced cross-presentation in vivo.** To determine if cross-presentation in vivo, as induced by ISCOM vaccine injection, is LB dependent, we used similar approaches as in *vitro*. Cross-presentation of the ISCOMs/OVA vaccine in Adrp−/− or Igtp−/− mice lacking functional LBs was hampered compared with wild-type mice (Fig. 6j; Supplementary Fig. 14a). Consistently, injecting the DGAT inhibitors Xanthohumol or A922500 significantly reduced cross-presentation (Supplementary Fig. 14b). Importantly, OVA Kβ peptide presentation was intact in all cases, proving equal MHC-I levels and viability of the CD11c+ cells (Fig. 6j; Supplementary Fig. 14). CD11c+ cell percentage or activation status of these DCs was similar in these experiments (Supplementary Fig. 15). Finally, analysis of a mix of LB inhibitors in the *in situ* tumour ablation model confirmed that also here the induction of LBs was essential for the SBA-induced cross-presentation (Fig. 6k).

Collectively, these results uncover a causal connection between SBA-induced LBs and cross-presentation *in vivo*, and link for the first time LB formation in DCs to adjuvant-mediated vaccine efficiency.

**Discussion**

SBAs generate strong Th1-driven cellular and humoral immune responses in various pre-clinical models and in vaccinated cancer patients.12,27–39. How SBAs facilitate immune responses to vaccine antigens remains incompletely understood. We now show that SBAs specifically enhance antigen cross-presentation and the induction of LBs in the monocytic CD11b+ DC subset. Using genetic and pharmacological interference, we demonstrate that LB induction in DCs is causally related to the SBA-induced increase in antigen cross-presentation and subsequent T-cell activation.

Our sorting experiments identify monocytic CD11b+ DCs, as present in GM-CSF DC cultures *in vitro* and in LNs *in vivo*, as the only cells capable of reacting to SBAs by induction of cross-presentation and LB formation. Among all individual DC populations generated in *in vitro* Flt3-L cultures or isolated from naïve spleens no SBA reactive population could be found, coinciding with the absence of migratory monocytic cells.

Interestingly, GM-CSF exposure of the Flt3 DC cultures is required for the monocytic precursors present in these cultures to respond to SBA. This finding is in line with GM-CSF (Csf-2) acting either late in differentiation or activation of monocytic cells. A previous study by Wilson *et al.* showing SBA-induced cross-presentation in the LN migratory DC fraction confirms our results. However, this study also reported significant levels of cross-presentation in CD8+ DCs. This discrepancy with our results could possibly be explained by background cross-presentation of non-formulated OVA antigen by CD8+ DCs in their study. In *vivo* SBA vaccination furthermore will induce inflammation, influencing co-stimulatory signals on DCs and subsequent activation of the OT-I T cells used in the Wilson study. Our B3Z cells react co-stimulation independent which avoids such activation biases. Earlier studies in human DCs demonstrated that SBA vaccination with the cancer antigen NY-ESO-1 induces cross-presentation by myeloid DC, such as MoDC51. Our preliminary data show LB induction in these human moDCs generated in the presence of GM-CSF, linking SBA-induced cross-presentation and LB formation also in the human context.

Thus, SBAs act to induce potent cross-presentation and LB induction in monocytic CD11b+ DCs, a population of DCs distinct from the well-described CD8+ cross-presenting DC. Together with the reported increase of CD11b+ DCs at SBA vaccination sites, this may well explain the efficient cross-priming of CD8+ T cells by SBA vaccines.

Despite the importance of cross-presentation in health and disease, the underlying molecular processes are still poorly understood. Studies so far indicate two main pathways: the ‘cytosolic’ and the ‘vacuolar’ pathway.52 Low proteolytic activity of the endocytic pathway and endosomal stress resulting in ROS production by the NADPH oxidase complex 2 (NOX2), have shown to be imperative for efficient cross-presentation of CD8+ DCs.41 Our cross-presentation studies use a short time of SBA exposure (1 to 5 h), and in this timeframe no role for ROS could be detected. Our translocation studies show that SBAs facilitate endosomal escape, and that this is essential for cross-presentation. However, since not all pore-forming saponins show adjuvant activity, and SBA activity is critically dependent on DCs, other mechanistic elements must be involved as well. Here, the presence of LBs appears to be equally essential for cross-presentation, although LBs seem to be dispensable for endosomal escape of antigens, and vice versa. The observation that both SBA-triggered processes can be uncoupled at the level of the endosome makes it unlikely that LBs alter the phagosomal environment in any direct manner, as also put forward by Bougnères *et al.*

Various types of (metabolic) stress can lead to the induction of LBs. TLR ligands were shown to increase glycolysis that fueled the

**Figure 6 | SBA-induced cross-presentation is dependent on LB formation.** (a) LB quantification as measured by CLSM in cells treated for 5 h with the indicated LB inhibitors: (Xanthohumol: 85 µM, A922500: 40 µM, Triacsin C: 10 µM, TOFA: 120 µM). (b) *In vitro* cross-presentation assays in the presence of the indicated LB inhibitors (inhibited enzymes in brackets). (c,d) Flowcytometry-based analysis of LB content. Wild-type (c), or Igtp−/+ and Igtp−/− (d) BMDCs were incubated for 800 ng ml−1 non-immunoactive saponin, FC saponin, ISCOMs or 250 ng ml−1 IFNγ for 5 h, washed, and stained with Bodipy.493–503 (e) *In vitro* cross-presentation assay using IGTP knockout DCs. (f) Metabolic activity/viability (MTT) assay used to demonstrate cytosolic translocation of indicated toxins. (g) MTT assay showing endosomal acidification is needed for cytosolic translocation. Bafilomycin A1, (at 100 nM) is an inhibitor of endosomal V-ATPase, and thus endosomal acidification. (h) Quantification of LB induction in the presence of ISCOMs and Bafilomycin A1. (i) MTT assay showing that inhibition of LB induction does not abrogate cytosolic translocation. During the exposure to ISCOMs/Cyt C, the LB inhibitors TOFA (120 µM) and A922500 (60 µM) were added. (j) in *in vitro* OT-I cross-presentation assay on isolated LN DCs from OVA protein or peptide-vaccinated wild-type, Igtp−/−, or Adrp−/− mice. (k) B16-OVA tumour bearing mice were treated with cryo-ablation, directly followed by peritumoural injection of 30 µg ISCOMs. A mixture of xanthohumol (500 µg) and A922500 (150 µg) was injected peritumourally, four hrs before ablation and directly after the ablation. 12 h later, draining LN CD11c+ cells were isolated that entered the cross-presentations assays. LacZ content in B3Z cells was analysed after 2 days. All data represent means with s.e.m., except for *d* where data represent mean MFI values normalized to medium values. Similar data were obtained in two to three independent experiments. Statistical analyses were done using ANOVA with post hoc Bonferroni tests.
de novo synthesis of fatty acids in LBs, essential for TLR ligand-induced DC activation. Also other studies have correlated DC activation status with lipid content: When fatty acid synthesis of high-lipid-containing DCs was inhibited, these DCs lost their immunostimulatory phenotype. Likewise, the presence of oxidized lipids in LBs was shown to hamper DC functionality and antigen presentation abilities. This suggests that not only the absolute lipid content of LBs in DCs, but also the composition of the LBs involved can modulate immune responses. In our hands, feeding DCs fatty acids like oleic acid did induce LBs, but not cross-presentation (Supplementary Fig. 16). Finally, the presence of an established tumour (for example, before cryo-ablation) is sufficient to change the LB profile of tumour resident DCs/monocytes. This complicates a solid analysis of LB induction due to SBA administration following ablation. Together, these data imply that not all LBs are functionally equal, and that various LB types may be present at the same time, in the same cell. The fact that SBAs share the induction of LBs with certain TLR ligands places these adjuvants in a peculiar position somewhere in between the microbial and non-microbial adjuvants. Molecular analysis of the ability of SBAs to facilitate cross-presentation specifically in monocyctic CD11b+ DCs via endosomal escape and LB induction will be key to understand SBA adjuvant activity.

IGTP, an IFN-related GTPase associated with LBs in DCs, was shown to be important in cross-presentation of OVA coated on large latex beads. Deletion of IGTP resulted in reduced IFN-induced LB numbers, but it was less established what role IFN had in their cross-presentation assays. As our studies use OVA protein, which in itself is not easily cross-presented, a direct comparison with the use of latex beads is difficult, but one could speculate that the relatively large size of the beads leads to endosomal stress and possibly endosomal rupture. This putative common factor between SBA- and beads induced cross-presentation could implicate that LBs may become particularly important in forced types of cross-presentation.

The data presented here indicate an important role for IGTP and ADRP in the in vivo cross-presentation of soluble antigens after vaccination with SBAs. Whether this implicates direct involvement of these molecules, or that the effects are the indirect result of reduced LB functionaries remains to be determined. To find causal connections at a more detailed level, sub-organelle/molecular studies using purified LBs from differentially treated immune cells are required. An intriguing finding is that GM-CSF-cultured DCs and CD11b+ LN DCs are uniquely able to cross-present antigen upon SBA exposure. It will be important to explore the molecular basis for this DC subset specificity, and how this can be linked to LB formation, antigen cross-presentation and immune activation.

The SBAs used in our study do not contain any known PRR ligand, and consistent with this they fail to upregulate co-stimulatory molecules on DCs in vitro (Fig. 2b and ref. 17). Moreover, cross-presentation was not hampered in TLR4−/− or Myd88−/− cells (Fig. 2c). Analysis of cells deficient in type I IFN or the NLRP3 inflammasome showed that these pathways were not involved either. Interestingly, others repeatedly reported an in vivo dependency of successful SBA vaccination on Myd88, while TLR4 and IL-1R seemed dispensable. This suggests that effective vaccination depends on the in vivo release of endogenous danger signals or the involvement of indirect cytokine signalling. IL-18 signalling is involved in indirect NK cell and CTL activation following vaccination with SBAs, which could partly explain the dependency on MyD88, being an adaptor for the IL18 receptor. Importantly, in this study MyD88 deficiency had no in vivo effect on maturation or vaccine antigen cross-presentation by DCs.

This research connects the initial innate cytokine-cascade with the adaptive cellular response, but leaves room for MyD88/TLR independent mechanisms of DC activation and cross-presentation with an alternative molecular basis.

Cancer vaccines evaluated to date mostly fail to change and overcome the immunosuppressive microenvironment present in many solid tumours. Current strategies using SBAs and cancer antigens (for example, NY-ESO-1) have proven to not only induce antibody responses, but also robust CD8+ T-cell expansion. Nevertheless, vaccinations with single injections of SBA-peptide compositions yielded only modest survival benefit for patients with established tumours. Interestingly, when these vaccines were combined with TLR ligands like CpG-ODN, or followed up by a boosting regimen, beneficial correlates of immunological protection could greatly be improved. We have previously shown that in situ tumour ablation can provide a source of (neo)-antigens for the generation of anti-tumour immune responses, provided that additional immune stimulation is given. SBAs appeared to be superior in creating synergy between the ablation and adjuvant-induced immune effects, inducing long-lasting tumour rejection in immunosuppressed mice. This makes tumour ablation with local co-administration of SBAs a promising treatment strategy to explore further in clinical settings. Future studies will have to show whether combinations of SBAs and TLR ligands are also effective combinations in ablation settings, and it will be appealing to correlate the clinical outcome to the presence and composition of LBs.

Collectively, our data shows that SBA-induced LBs are elementary in the mechanism of SBA adjuvanticity. Understanding the LB-immune connection in SBA vaccination will help to design new adjuvants for improvement of (cancer) vaccines, aiming to enhance cross-presentation and the subsequent induction of potent anti-tumour CTL responses.

Methods

Mice. Igtp−/− and Adrp−/− mice were a kind gift of S. Amigorena (Institut Curie, Paris), and have been described before. Myd88−/−, Tlr4−/−, Traf3−/− and Traf1−/− mice were a kind gift of S. Akira (Osaka University, Japan). Nlrp3−/− (−/−) and ADRP−/− (−/−) littermates were used (for clarity in those experiments referred to as wild type). All experiments without knockout were performed with female wild-type C57Bl/6RcRcsid mice purchased from Charles River (Sulzfeld, Germany), and were used as 7–11 weeks old animals. C57Bl/6 OT-I and DBA/2 OT-II mice were kept in the Nijmegen Animal facility. All mice were maintained on standard lab chow and sterile water, and housed under specific pathogen-free conditions in IVC cages at the Nijmegen Animal facility. All animal experiments were approved by the Animal Experimental Committee of the Radboud UMC, and were performed in accordance with institutional, national and European guidelines.

Cells. Primary cultures of bone-marrow-derived dendritic cells (BMDCs) were generated by culturing total bone marrow cells in complete medium (Roswell Park Memorial Institute (RPMI) medium (Gibco), 10% fetal calf serum (FCS), 1% l-α-aminoglutamine, 0.1% β-mercapto-ethanol and 1% antibiotics/antimycotics (AA, Gibco)), containing 20 mg ml−1 granulocyte-macrophage colony stimulating factor (GM-CSF DCs, culture 7 days), or containing 200 ng ml−1 Fms-related tyrosine kinase 3 ligand (Flt3-L DCs, culture 10 days), as adapted from previously described protocols. GM-CSF and Flt3-L were purchased from Peprotech (Rocky Hill, CT, USA). In some experiments the Flt3-L cultures were supplemented with 20 ng ml−1 GM-CSF for the indicated periods. Ex vivo DCs were isolated from pooled superficial inguinal and axillary lymph nodes, or spleens, according to previously published protocols, as indicated. For some of these experiments, mice were injected before with a B16-F10 melanoma expressing Flt3-L to expand endogenous DC pools, as described before. B16-Flt3-L cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 5% FCS and 1% AA, and 5 × 106 cells were injected s.c. to establish Flt3-L-producing tumours in 9–12 days. Sorting of DC populations was performed after incubation in serum-free medium containing collagenase (Worthington, Lakewood, NJ, USA) and DNase I (Roche) at 37°C, later supplemented with 1 mM EDTA. For isolation of total CD11c+ fractions, a magnetic bead kit was used.
according to manufacturer’s instructions (Clone N418, Miltenyi Biotec, Bergisch Gladbach, Germany). For isolation of DC subsets, total cell suspensions were sorted on a FACSAria system (BD Biosciences), based on the markers as indicated in the figure. Purity (>98%) and additional markers were confirmed using a FACS Cyan system (Beckman Coulter). CD8+ T cells specific for the OVA257–264 SIINFEKL peptide in an H-2Kb MHC-I context, or CD4+ T cells specific for the OVA333-349 peptide in an H-2Dd MHC-II context, were obtained from pooled spleen and lymph nodes of OT-I and OT-II transgenic mice, respectively. Before use, both cell types were purified by magnetic bead sorting (positive selection on CD8, Miltenyi). B3Z cells were cultured in Isocyte’s Modified Dulbecco’s Medium (IMDM) supplemented with 5% FCS, 0.1% β-mercaptoethanol, 50 μg/ml penicillin/streptomycin, 0.5% L-glutamine, 100 μg/ml phenol red-free RPMI 1640 (ATCC) and 10% fetal bovine serum (Greiner Bio-one), 100 U/ml penicillin G and 100 μg/ml streptomycin (Pen/Strep), MEM sodium pyruvate (1 mM), NaHCO3, MEM vitamins, MEM non-essential amino acids (all from Gibco), 20 μM beta-mercaptoethanol, OVA-transfected B16F10 (B16OVA, clone MO5) was cultured in complete medium supplemented with 20 μg ml−1 hygromycin and 1 mg ml−1 G418. B6MECISqGVA cells are transfected with murine B7.1, H-2Kb, and a construct expressing an ER-targeting signal sequence, followed by the OVA257–264 CTL epitope SIINFEKL (original name B6-7.1-sigOVA35). These antibodies were tested and cultured in IMDM with 5% FCS, 0.1% β-mercaptoethanol, 1% antibiotics/antimycotics, 100 μg/ml hygromycin, and 100 μg/ml G418. All cell lines in our study were tested mycoplasma negative.

Antibodies and reagents. For FACS experiments the following antibodies (clone name in brackets) conjugated to various fluorophores were used, together with corresponding isotypes. All antibodies were used in a 1:400 dilution in PBA, unless otherwise stated after the clone name. Anti-CD206 (C066C2; 1:100) (AbD Serotec); anti-CD3e (37.3B; 1:200); anti-CD4 (RM4-5; 1:60); anti-CD8a (53-6.7; 1:100); anti-CD11c (N418; 1:400); anti-CD24 (M2/9; 1:250); anti-CD69 (H1.2F3), anti-CD135 (A2F10.1; 1:100), anti-CD172 (P84), (2E7; 1:800), anti-H-2Kb/Db (28-8-6) (all Biolegend); streptavidin-Alexa488, -555, 12, EMD Millipore (Billerica, MA, USA); acetovanillone (Apocynin), L-ascorbic acid (Sigma-Aldrich), and D-glucose (Invitrogen), and DAPI dye (Sigma, St Louis, MO, USA). Various assays were measured using a photospectrometer (595 nm).

Flow cytometry. Phenotypic characterization of DCs and T cells was performed using standard antibody staining protocols, and analysis was done using a FACS Cyan (Beckman Coulter) or a FACS Verse system (BD). All stainings were preceded by Fc-receptor blocking (anti-mouse IgG1, 1:100, eBiosciences). Typically, 400 ng ml−1 SBAs were added, and 80 μg ml−1 OVA, unless indicated otherwise. After 5 h of incubation at 37°C, cells were washed and cultured for 18 h with 80 × 10^5 B3Z cells. B3Z cells and positive control B6-7.1-sigOVA cells were cultured as described elsewhere27. As a control for cell viability and/or MHC-I expression levels, DCs were washed 30 min before adding the B3Z cells and pulsed with 5 ng ml−1 peptides. Peptides (OVA: SIINFEKL, HPV: RAHYNIVTF, Ad-E1A: GLPVAENLYT) were obtained from Iw, Driftshof (LUMC, Leiden, the Netherlands). In one experiment standard PEGylated PLGA nanoparticles formulated with OVA were used, which were produced in our lab as described elsewhere28. The amount of particles was adjusted so that the wells contained an OVA end-concentration of 30 μg ml−1. The presentation of DCs by SIINFEKL in H-2Kb results in production of β-galactosidase by the B3Z cells, which allows for a high sensitivity of expression. Cells were then washed extensively with PBS (1×) and double quenched (DQ-OVA) which fluoresces once degraded in the endolysosomal system.

B3Z and OT-I cross-presentation assays. For the cross-presentation assay, 80 × 10^5 cells (in vitro BMDCs) or 150 × 10^5 ex vivo DCs were pulsed with endotoxin-free chicken egg ovalbumin (OVA, Hyglos GmbH, Germany) in the presence of the indicated adjuvants or inhibitors. Typically, 400 ng ml−1 SBAs were added, and 80 μg ml−1 OVA, unless indicated otherwise. After 5 h of incubation at 37°C, cells were washed and cultured for 18 h with 80 × 10^5 B3Z cells. B3Z cells and positive control B6-7.1-sigOVA cells were cultured as described elsewhere27. As a control for cell viability and/or MHC-I expression levels, DCs were washed 30 min before adding the B3Z cells and pulsed with 5 ng ml−1 peptides. Peptides (OVA: SIINFEKL, HPV: RAHYNIVTF, Ad-E1A: GLPVAENLYT) were obtained from Iw, Driftshof (LUMC, Leiden, the Netherlands). In one experiment standard PEGylated PLGA nanoparticles formulated with OVA were used, which were produced in our lab as described elsewhere28. The amount of particles was adjusted so that the wells contained an OVA end-concentration of 30 μg ml−1. The presentation of DCs by SIINFEKL in H-2Kb results in production of β-galactosidase by the B3Z cells, which allows for a high sensitivity of expression. Cells were then washed extensively with PBS (1×) and double quenched (DQ-OVA) which fluoresces once degraded in the endolysosomal system.

Lipid body staining. Lipid bodies were visualized by CLSM or FACS. For confocal microscopy, 250 × 10^5 DCs were plated for 45 min at 37°C on glass coverslips coated to make 20 pg ml−1 fibronectin (Sigma). After washing with PBS, cells were exposed to the indicated treatment conditions in serum-free medium for 5 h. Typically, 400 ng ml−1 SBAs were used, unless otherwise indicated. Next, cells were fixed in 4% PFA solution for 15 min. For co-staining for ADRP, fixed cells were permeabilized with 0.1% Saponin (Sigma) in PBA and incubated with primary antibodies in PBS plus 0.2% BSA/0.05% saponin for 1 h. Next, cells were washed and incubated with anti-chicken IgY secondary antibodies conjugated to DyLight594 (Abcam; 5 μg ml−1). Bodipy930-933 dye (Invitrogen), and DAPI dye (Sigma, St Louis, MO, USA). Various assays were measured using a photospectrometer (595 nm).

For the OT-I/II cross-presentation assays 25 × 10^5 DCs were plated and treated as indicated above. Purified OT-I or OT-II cells (CD8 or CD4 MACS beads) were labelled with cell-proliferation dyes CFSE or PBSE, according to the manufacturer’s instructions (Invitrogen), and 50 × 10^5 cells were incubated with the DCs for up to 72 h. After one day of incubation, cells were stained with CD90.1, CD8, and the early activation marker CD49d. After 3 days, the late stage activation marker CD44 and the interferon γ (IFN-γ) receptor were expressed. All T-cell assays were performed after double gating on CD8β and CD90.1. Cells were measured using a FACS Cyan (Beckman Coulter) or a FACS Verse system (BD).

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Bodipy signal integrates info from both the number, size and intensity of LBs (validated by Gocze et al.13).

**ROS detection.** The production of total cellular ROS was detected using DH1R23 and H2DCFDA. 150 × 10^6 GM-CSF cultured BMDCs were plated in serum-free medium in 96 wells round bottom plates (Costar). Cells were pretreated for 10 min with 0.5 or 50 μM dihydrodorhamine 123 (DH1R23, Sigma), after which the following compounds were added: 400 ng ml^−1 ISCOMs or 1 ml^−1 phorbol 12-myristate 13-acetate (PMA, Sigma). After 15 or 45 min incubation, DHR staining was directly measured by FACS. Similarly, ROS was detected using CM-H2DCFDA (Invitrogen). For this, cells were washed with HBSS containing magnesium and calcium, after which they were incubated for 45 min with 10 μM H2DCFDA and the above stimuli dissolved in HBSS buffer.

**MTT assays.** GM-DCs were seeded in flat-bottom 96-well plates (Costar) at 170 × 10^3 cells per well and cultured overnight to adhere. Next, they were exposed to the indicated compounds for 5 h (ISCOMs: 800 ng ml^−1, Saponin: 1 nM, Cytochrome C: 2.5 mg ml^−1). After cells were washed and rested o/n, their metabolic activity/viability was measured in a standard MTT assay. In short, 10 μl MTT reagent (4 mg ml^−1) was added to each well after 24 h of incubation, absorbance was measured at 595 nm. Metabolic activity versus controls was calculated as (treatment—blank)/(control—blank) of incubation, absorbance was measured at 595 nm. Metabolic activity versus controls was performed.

**In vivo procedures.** The in vivo effects of ISCOMs on cross-presentation and LB induction were analysed in wild-type, ADRP/IGTP knockouts or their littermates. In vivo effects of ISCOMs or LB inhibitors. LB inhibitors (or vehicle) were added: 400 ng ml^−1 ISCOMs or 50 μg ISCOMs and/or LB inhibitors. LB inhibitors (or vehicle) were injected twice: 4 h before, and together with the OVA/ISCOMs injection. After 15 or 45 min incubation, 100 μl of 0.5% SDS, 4% HCl and 3.5% Milli-Q in isopropanol) was added. After 15 min of incubation, absorbance was measured at 595 nm. Metabolic activity versus controls was calculated as (treatment—blank)/(control—blank) × 100%. Triplicate wells for each concentration were performed.

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Author contributions
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