Carbomer-based adjuvant elicits CD8 T-cell immunity by inducing a distinct metabolic state in cross-presenting dendritic cells

Woojong Lee¹, Brock Kingstad-Bakke¹, Brett Paulson²,³, Autumn Larsen⁴, Katherine Overmyer⁵,⁶, Chandranaik B. Marinaik⁴, Kelly Dulli⁴, Randall Toy⁴, Gabriela Vogel⁵, Katherine P. Mueller⁵,⁶, Kelsey Tweed⁴,², Alex J. Walsh⁴,², Jason Russell⁵, Krishanu Saha⁵,⁶, Leticia Reyes¹, Melissa C. Skala²,⁵, John-Demian Sauer⁷, Dmitri M. Shayakhmetov⁸, Joshua Coon²,², Krishnendu Roy⁴, M. Suresh¹*¹

¹ Department of Pathobiological Sciences, University of Wisconsin-Madison, Madison, Wisconsin, United States of America. ² Morgridge Institute for Research, University of Wisconsin-Madison, Madison, Wisconsin, United States of America. ³ Department of Biomolecular Chemistry, University of Wisconsin-Madison, Madison, Wisconsin, United States of America. ⁴ The Wallace H. Coulter Department of Biomedical Engineering at Georgia Institute of Technology and Emory University and The Parker H. Petit Institute for Bioengineering and Biosciences, Center for ImmunoEngineering, Georgia Institute of Technology, Atlanta, Georgia, United States of America. ⁵ Department of Biomedical Engineering, University of Wisconsin-Madison, Madison, Wisconsin, United States of America. ⁶ Wisconsin Institute for Discovery, University of Wisconsin-Madison, Madison, Wisconsin, United States of America. ⁷ Department of Medical Microbiology and Immunology, University of Wisconsin-Madison, Madison, Wisconsin, United States of America. ⁸ Lowance Center for Human Immunology, Emory Vaccine Center, Departments of Pediatrics and Medicine, Emory University School of Medicine, Atlanta, Georgia, United States of America

* sureshm@vetmed.wisc.edu

Abstract

There is a critical need for adjuvants that can safely elicit potent and durable T cell-based immunity to intracellular pathogens. Here, we report that parenteral vaccination with a carbomer-based adjuvant, Adjuplex (ADJ), stimulated robust CD8 T-cell responses to subunit antigens and afforded effective immunity against respiratory challenge with a virus and a systemic intracellular bacterial infection. Studies to understand the metabolic and molecular basis for ADJ’s effect on antigen cross-presentation by dendritic cells (DCs) revealed several unique and distinctive mechanisms. ADJ-stimulated DCs produced IL-1β and IL-18, suggestive of inflammasome activation, but in vivo activation of CD8 T cells was unaffected in caspase 1-deficient mice. Cross-presentation induced by TLR agonists requires a critical switch to anabolic metabolism, but ADJ enhanced cross presentation without this metabolic switch in DCs. Instead, ADJ induced in DCs, an unique metabolic state, typified by dampened oxidative phosphorylation and basal levels of glycolysis. In the absence of increased glycolytic flux, ADJ modulated multiple steps in the cytosolic pathway of cross-presentation by enabling accumulation of degraded antigen, reducing endosomal acidity and promoting antigen localization to early endosomes. Further, by increasing ROS production and lipid peroxidation, ADJ promoted antigen escape from endosomes to the cytosol for degradation by proteasomes into peptides for MHC I loading by TAP-dependent pathways. Furthermore, we found that induction of lipid bodies (LBs) and alterations in LB composition mediated by
ADJ were also critical for DC cross-presentation. Collectively, our model challenges the prevailing metabolic paradigm by suggesting that DCs can perform effective DC cross-presentation, independent of glycolysis to induce robust T cell-dependent protective immunity to intracellular pathogens. These findings have strong implications in the rational development of safe and effective immune adjuvants to potentiate robust T-cell based immunity.

Author summary

An adjuvant is the pharmacological agent that is added to vaccines to boost immune responses. Currently, there are only seven FDA-approved adjuvants for human use, and vaccines based on these adjuvants have mainly been evaluated for elicitation of antibody-based immunity. However, vaccines need to also stimulate T cell-mediated immunity to protect against diseases such as AIDS, TB and Malaria. Hence, there is a critical need to develop adjuvants that stimulate protective T cell immunity. Here, we identified an adjuvant (Adjuplex; ADJ) that safely induces strong T cell immunity and protects against virus and intracellular bacteria. We also found that ADJ stimulated T cell immunity by unique mechanisms that did not include metabolic activation of antigen-presenting dendritic cells. Instead, ADJ induced a low metabolic state and engaged mechanisms including lipid pathways and induction of reactive oxygen species to promote activation of T cells by dendritic cells, following vaccination. These data not only provide new mechanistic insights into the mechanisms driving activation of T cells by ADJ, it provides a blue print for what adjuvants need to do to induce protection against infections that require T cell immunity.

Introduction

Development of effective vaccines remains the central principle for controlling infectious diseases in humans and animals. Typically, vaccines can be classified into the following categories: replicating vaccines (live-attenuated viruses; e.g. smallpox), non-replicating vaccines (subunit [hepatitis B], virus-like particles [human papilloma virus], toxoid [tetanus], and conjugated vaccines [Hemophilus influenzae type B]) [1]. To date, protection afforded by the most effective vaccines is primarily dependent upon the elicitation of antibodies [2]. By contrast, development of vaccines against infections that require T cells for pathogen control, such as HIV, tuberculosis (TB), and malaria, remains a difficult challenge for vaccinologists [3–5]. Live-attenuated vaccines are highly immunogenic and elicit both humoral and cell-mediated immunity, but their use can be contraindicated during pregnancy and in immunocompromised individuals [6–8]. Subunit or inactivated antigens are generally safe, but are poorly immunogenic unless formulated in pharmaceutical agents called adjuvants [9].

CD8 T cell responses to non-replicating subunit proteins requires antigen cross-presentation by dendritic cells (DCs) [10]. Likewise, DC cross-presentation plays a pivotal role in eliciting CD8 T cell responses to intracellular pathogens (e.g. Listeria monocytogenes) and tumor antigens [11–13]. Cross-presentation of MHC I-restricted antigens to CD8 T cells can occur via vacuolar or cytosolic pathways [14]. In the vacuolar pathway, exogenous antigens are internalized into endosomes and digested by resident cathepsins [15]. By contrast, in the cytosolic pathway, internalized antigens localize to the alkaline endosomal compartment, followed by antigen export into cytosol and downstream processing by the proteasomes [16]. Peptides resulting from proteasomal processing are translocated to endoplasmic reticulum (ER) by the
transporter associated with antigen processing-1 (TAP1) complex or to endosomes, and are loaded on to MHC-I molecules [17].

Apart from engaging the appropriate antigen processing cellular machinery, metabolic reprogramming of DCs is an important facet of effective cross-presentation and activation of naïve T cells [18]. DC activation by Toll-like receptor (TLR) agonists triggers a metabolic switch from catabolic metabolism to anabolic metabolism to accommodate increasing cellular demands for executing cellular functions such as production of pro-inflammatory cytokines, upregulation of co-stimulatory molecules, and directed migration to draining lymph nodes [19]. Hence, understanding of DC metabolism is crucial for rationally designed vaccines that can effectively induce robust CD8 T cell responses to subunit protein antigens via mechanisms of DC cross-presentation.

Currently, there are only seven FDA-approved adjuvants for human use, and vaccines based on these adjuvants have mainly been evaluated for elicitation of humoral immunity [20]. There is high level of interest in developing adjuvants that can stimulate potent CD8 and CD4 T-cell responses to subunit antigens. Carbomer (acyrlic acid polymers)-based adjuvants (CBA) are components of several veterinary vaccines, and known to safely elicit potent neutralizing antibodies to malarial and HIV envelope glycoproteins in mice and non-human primates [21–23]. We and others have previously reported that the carbomer-based nano-emulsion adjuvant, Adjuplex (ADJ; Advanced Bioadjuvants) protects against influenza A virus in mice [24,25]. However, it is unknown whether ADJ can stimulate protective T-cell immunity to systemic infections, and the mechanisms underlying the stimulation of protective CD8 T cells immunity by ADJ are yet to be determined. Here, we have systematically explored the mechanisms underpinning the molecular and metabolic basis for the potent activation of protective CD8 T cell immunity by an acrylic acid-based nano-emulsion adjuvant, ADJ.

**Results**

**ADJ elicits T cell-based protective immunity against viral and intracellular bacterial infections in vivo**

We determined whether ADJ promoted the stimulation of systemic antigen-specific CD8 T-cell responses to an experimental subunit antigen, chicken ovalbumin (OVA). As shown in Fig 1A, subcutaneous (SQ) administration of ADJ/OVA formulation potently augmented the activation of OVA-specific CD8+ T cells in spleen, while OVA alone was poorly immunogenic. Next, we evaluated whether ADJ-based vaccine conferred T cell-based protection to pathogens, *Listeria monocytogenes* (LM) or vaccinia virus (VV) in mice [26–28]. Forty days after boost, mice were challenged with either recombinant LM-expressing OVA (LM-OVA) or recombinant VV-expressing OVA (VV-OVA) [29,30]. After LM-OVA or VV-OVA challenge, we enumerated recall OVA-specific CD8 T-cell responses in spleens and lungs, and LM-OVA or VV-OVA burden in various tissues. After challenge, higher numbers of OVA SIINFEKL-specific CD8 T cells were detected in spleens or lungs of ADJ+OVA-vaccinated mice, as compared to those in unvaccinated mice (Fig 1B and 1C). Consistent with potent OVA-specific recall CD8 T-cell responses in ADJ+OVA mice, LM-OVA and VV-OVA burden in tissues of ADJ+OVA group were markedly lower than in unvaccinated controls (Fig 1D and 1E). In order to benchmark ADJ+OVA-induced protection to LM, we compared the effectiveness of this vaccine approach with previously published live-attenuated LM vaccines [31,32]. In our published work, live-attenuated LM vaccines reduced LM burden by ~4 logs in spleen and liver, as compared to unvaccinated controls [31]. Remarkably, in the current study, LM-OVA burden was below the level of detection in spleen and livers of several ADJ/OVA-vaccinated mice compared to ~10^7 CFU of LM-OVA in organs of unvaccinated controls (Fig 1D). Thus,
ADJ-based subunit protein vaccine provided effective systemic protection against Listeria that may be superior to live-attenuated LM vaccines. Together, data in Fig 1 demonstrated that ADJ-based subunit vaccine provided T-cell-based protective immunity against bacterial and viral pathogens, presumably by promoting cross-presentation of antigen to OVA-specific CD8 T cells in vivo.
Carbomer-based nano-emulsion adjuvant ADJ enhances cross-presentation of antigens by DCs in vitro and in vivo

Next, in order to determine the effects of ADJ on antigen-presenting cells, we assessed whether exposure of bone marrow-derived DCs (BMDCs) to ADJ lead to enhanced antigen cross-presentation to CD8 T cells in vitro and in vivo. We evaluated the ability of ADJ-treated DCs to activate naïve CD8 T cells to undergo antigen-driven proliferation by culturing BMDCs with either OVA or ADJ+OVA. Subsequently, BMDCs were co-cultured with carboxyfluorescein succinimidyl ester (CFSE)-labeled naïve OVA SIINFEKL-specific TCR transgenic OT-1 CD8 T cells for 72 hours. BMDCs cultured with media or OVA induced proliferation of a small fraction of OT-1 CD8 T cells (Fig 2A). By contrast, BMDCs cultured with ADJ+OVA stimulated proliferation of >80% of OT-1 CD8 T cells in vitro, as indicated by reduced levels of CFSE fluorescence. To qualitatively assess the magnitude of ADJ-mediated cross presentation of OVA antigen to CD8 T cells in vitro, BMDCs were treated with ADJ+OVA or OVA, and then were evaluated for their capacity to activate SIINFEKL-specific B3Z T cell hybridoma cells using a reporter assay [33]. BMDCs stimulated with ADJ+OVA significantly induced β-galactosidase in B3Z cells compared to OVA only control, suggesting enhanced antigen cross-presentation by ADJ-treated BMDCs (Fig 2B). To further corroborate if ADJ promotes cross-presentation by enhancing the expression levels of SIINFEKL/H-2Kb complexes on the surface of antigen-presenting cells, we treated BMDCs with media, OVA, or ADJ+OVA, and quantified cell surface SIINFEKL/H-2Kb complexes using the 25 D1.16 antibody. Significantly greater percentages of DCs exposed to ADJ+OVA expressed elevated levels of SIINFEKL-bearing H-2Kb molecules, in comparison to DCs exposed to media or OVA only (S1 Fig). Next, to assess whether DCs treated with ADJ possess enhanced cross-priming abilities in vivo, we adoptively transferred DCs pre-treated in vitro with ADJ+OVA, LPS+OVA, or OVA into C57BL/6 mice. The percentages and total numbers of SIINFEKL-specific CD8 T cells were significantly higher in spleens of mice that received DCs treated with ADJ+OVA, as compared to mice that received DCs treated with LPS+OVA or OVA (Fig 2C). In summary, data in Figs 2A, 2B, 2C and S1 strongly suggest that ADJ enhances DC cross-presentation of antigens to CD8 T cells in vitro and in vivo.

ADJ induces IL-1β and IL-18 production in DCs, but deficiency for NLRP3, ASC or caspase 1 did not affect ADJ-mediated cross-presentation

Next, we examined the effects of ADJ on the expression profiles of cytokines, chemokines and canonical cell surface markers of BMDC activation. Compared to resting DCs, ADJ-treated DCs showed statistically significant, yet modest increases in expression of CD40, CD80, CD86, and CCR7; no significant differences in expression were observed for MHC-I and MHC–II (Fig 2D). ADJ-treated DCs produced higher levels of IL-12 (p70), TNF-α, IL-1α, CCL3, CCL4, CXCL1, and RANTES, as compared to untreated DCs; no significant differences in expression were observed for IL-6, IL-10, and IFN-β (Fig 2E). Significantly, ADJ-stimulated DCs also produced elevated levels of IL-1β and IL-18 (Fig 2E), which suggests that ADJ treatment leads to inflammasome activation. Since inflammasome activation has been implicated in modulation of antigen presentation by DCs [34,35], we performed B3Z assays using DCs deficient in NLRP3, ASC, or caspase 1 to interrogate whether inflammasome activation is required for ADJ-induced cross-presentation in BMDCs. Loss of NLRP3, ASC or caspase 1 activity did not affect ADJ-induced cross-presentation by DCs, in vitro (Fig 2F). To validate whether caspase 1 is required for ADJ-aided cross-presentation in vivo, we immunized cohorts of wild type (WT) and caspase 1-deficient (Caspase 1 KO) mice with ADJ+OVA, and quantified OVA SIINFEKL-specific CD8 T cells in spleens using MHC I tetramers at day 8 after immunization.
Caspase 1 deficiency did not significantly affect the accumulation of activated SIINFEKL-specific CD8 T cells in spleens (Fig 2G), suggesting that caspase 1 is not essential for ADJ-driven cross-presentation to CD8 T cells in vivo.

https://doi.org/10.1371/journal.ppat.1009168.g002

Caspase 1 deficiency did not significantly affect the accumulation of activated SIINFEKL-specific CD8 T cells in spleens (Fig 2G), suggesting that caspase 1 is not essential for ADJ-driven cross-presentation to CD8 T cells in vivo.
ADJ modulates antigen processing and subcellular localization in DCs

We next examined whether ADJ affected the dynamics of antigen processing in DCs. First, we determined the effect of ADJ on antigen uptake by culturing DCs with OVA that was labeled with pH-insensitive dye, Alexa Fluor 647. Interestingly, we found that antigen uptake was significantly reduced in DCs treated with ADJ (S2 Fig). This finding was not totally unexpected, as some TLR agonists are known to reduce uptake of soluble antigen, yet increase antigen cross-presentation [36]. Next, we assessed whether ADJ affected antigen processing by treating DCs with DQ-OVA, which emits green fluorescence upon proteolytic degradation and red fluorescence upon subsequent aggregation of digested peptides. We found that ADJ enhanced OVA degradation and/or accumulation of processed OVA, as indicated by an increase of both DQ-green and DQ-red fluorescence at 6 hours (Fig 3A). Hence, ADJ might dampen antigen uptake, but enhances antigen processing and/or accumulation of processed antigen in DCs.

Apart from antigen degradation, trafficking of antigens to less acidic vesicular compartments, such as early endosomes, is critical for efficient cross-presentation [37,38]. Hence, we analyzed the effect of ADJ on the intracellular routing of antigen using pHAB-OVA, which emits green fluorescence under acidic conditions. In ADJ-treated DCs, pHAB-OVA was routed to less acidic compartments in DCs within 30 minutes, as indicated by lower fluorescence of pHAB-OVA compared to media control (S2 Fig). In order to precisely localize antigen in ADJ-treated DCs, we investigated the extent to which OVA antigen was localized to early endosomes or lysosomes, using confocal microscopy. Microscopic images showed that in ADJ-treated DCs, antigen preferentially co-localized with early endosomes (EEA1⁺ve), rather than lysosomes (LAMP1⁺ve) (Fig 3B). Unlike in ADJ-treated DCs, antigen was localized to the acidic lysosomes in DCs that were only treated with OVA (Fig 3B). To further corroborate our findings, we employed a co-localization analysis of confocal microscopy images using Pearson’s correlation coefficients (PCC) [39]. This analysis showed that the PCC of EEA1 with OVA was higher, but the PCC of LAMP1 with OVA was lower in ADJ-treated DCs (S3 Fig). Results indicated that a higher proportion of EEA1⁺ve organelles but not LAMP1⁺ve organelles co-localized with antigen in ADJ-treated cells. Collectively, these findings suggested that ADJ preferentially promotes antigen localization to less acidic early endosomal compartments.

ADJ enhances antigen cross-presentation by inducing ROS production and modulating the pH of the antigen-containing compartment by NOX2-dependent mechanisms

Augmented ROS production reduces endosomal acidity and delays antigen degradation, which in turn enhances antigen cross presentation [40]. Here, we used CellROX reagent and the ROS-Glo H₂O₂ assay to quantify endosomal ROS and H₂O₂ production, respectively in ADJ-treated BMDCs. Remarkably, cellular ROS and H₂O₂ levels rapidly increased within 1 hour after treatment with ADJ (Figs 3C and S4). Next, we asked whether alterations in cellular ROS modulated intracellular acidity in ADJ-treated DCs; Lysotracker has been used for visualizing acidic compartments. Within 1 hour of ADJ treatment, concomitant to ROS induction, there was a substantive reduction of Lysotracker MFI, indicating that ADJ might have induced ROS and reduced intracellular acidity in DCs (Fig 3D). In order to test the importance of ADJ-induced ROS in cross-presentation, we employed a combination of pharmacological and genetic approaches. First, pharmacological inhibition of NADPH-oxidase complex2 (NOX2) assembly with apocynin markedly reduced DC cross-presentation to B3Z cells (Fig 3E). Furthermore, unlike strong B3Z activation in ADJ-treated WT DCs, ADJ failed to augment cross-presentation in DCs deficient for the ROS-inducing NOX2 complex component gp91 (Fig...
Data in Fig 3C, 3D and 3E support the idea that maintenance of a less acidic environment in the endosomes established by NOX2-driven ROS might be critical for ADJ-mediated cross-presentation.
ADJ-induced cross-presentation requires proteasomal processing and TAP1 transporters

MHC-I binding peptides can be generated either by phagosomal residential cathepsins, or by cytosolic proteasomes. In order to dissect the pathways required for cross-presentation of OVA-derived peptides by ADJ-treated DCs, we inhibited proteasomal or lysosomal activities using epoxomycin (proteasomal inhibitor) or leupeptin (general cathepsin inhibitor), respectively. ADJ-driven cross-presentation was effectively abrogated by epoxomycin, but augmented by leupeptin, as compared to vehicle controls (Fig 3F). These findings suggested that ADJ-driven antigen cross-presentation required proteasomal, but not lysosomal activity. Next, we investigated whether ADJ enhanced proteasomal activity in DCs using Cell-Based Proteasome-Glo Assays. Here, we found that the activity of 20S proteasome subunits were not affected by ADJ treatment (S5 Fig). Thus, ADJ-mediated cross-presentation requires cytosolic proteasomes, but ADJ does not enhance the proteolytic activity of proteasomes in DCs.

Peptides generated by cytosolic proteasomes require TAP transporters to access MHC I molecules in ER or ER-Golgi intermediate compartment (ERGIC) [41,42]. We tested the extent to which ADJ-induced DC cross-presentation is dependent upon TAP transporters. ADJ-mediated DC cross-presentation of OVA peptides and antigen recognition by B3Z cells was completely abolished in the absence of TAP1 (Fig 3G), which suggested that ADJ-driven DC cross-presentation required TAP1 for loading peptides on to MHC I. Collectively, our data suggest that ADJ induction of DC cross-presentation requires an endosomes-to-cytosol pathway of cross-presentation.

ADJ-mediated cross-presentation requires endosomal antigen leakage mediated by lipid peroxidation

The cytosolic pathway of cross-presentation requires antigen escape from endosome into cytosol, for subsequent degradation by cytosolic proteasomes [16]. Because we observed that ADJ-induced DC cross-presentation required proteasomes as antigen processing machinery (Fig 3F), we questioned whether ADJ promoted antigen translocation from endosomes into cytosol. Endosomal leakage in DCs was visualized by measuring cellular apoptosis, resulting from release of exogenous horse cytochrome C (cytc) into the cytosol [43]. After 24 hours of treatment with cytc in the presence or absence of ADJ, we found that the percentages of annexin-V positive cells were 4 times higher among DCs exposed to ADJ+cytc, compared to cytc- or ADJ-treated DCs. These data suggested that ADJ likely induced cytc escape from endosomes into cytosol, resulting in DC apoptosis (Fig 3H).

It was recently reported that NOX2-driven ROS can cause endosomal lipid peroxidation and release of antigen from leaky endosomes into the cytosol of DCs [44]. To determine if observed endosomal leakage in ADJ-treated DCs (Fig 3H), was caused by lipid peroxidation, we quantified lipid peroxidation in ADJ-treated DCs using a radiometric dye, BODIPY 581/591 C11, which displays a shift in peak fluorescence emission from red to green upon oxidation by lipid hydroperoxides. Within 1 hour after ADJ treatment, there was a marked shift of BODIPY 581/591 C11 from red to green (Fig 3I). Moreover, pharmacological inhibition of lipid peroxidation using α-tocopherol, a lipid-soluble antioxidant which selectively prevents lipid peroxidation by scavenging free electrons [45], significantly reduced ADJ-driven cross-presentation and activation of B3Z cells (Fig 3I). Thus, our data illustrated that ADJ-mediated cross-presentation required ROS, and likely lipid peroxidation for facilitating endosomal antigen leakage.
ADJ enhances cross-presentation without glycolytic reprogramming of DCs via the Akt-mTORC1-KLF2-HIF-1α axis

Typically, catabolic metabolism in resting DCs is characterized by oxidative phosphorylation (OXPHOS) fueled by fatty acid oxidation (FAO) and limited glycolysis [46–48]. During early DC activation by TLR agonists, DCs augment both aerobic glycolysis and OXPHOS to support the anabolic demands required for expansion of the ER and Golgi apparatus, de novo fatty acid (FA) synthesis, and production of inflammatory cytokines. Further, this early glycolytic reprogramming by TLRs is required for upregulation of co-stimulatory molecules,CCR7 oligomerization, and priming T cells [49,50]. Subsequently, DCs inhibit OXPHOS via nitric oxide (NO) and rely on aerobic glycolysis for their survival, especially after sustained exposure to TLR-agonists [51,52]. Based on the augmented ability of ADJ-treated DCs to activate T cells, we hypothesized that metabolic reprogramming plays a distinctive role in this process.

First, we asked whether ADJ engaged aerobic glycolysis as a key source of carbon for metabolic functions that enhance antigen presentation by DCs via the Akt-mTORC1-HIF-1α signaling axis. LPS stimulation potently triggered phosphorylation of p70S6K and Akt within 60 minutes, but treatment with ADJ failed to do the same (Fig 4A). Prolonged glycolytic reprogramming involves the induction and stabilization of hypoxia-inducible factor (HIF-1α), that in turn trigger production of NO and suppression of OXPHOS [53,54]. Using DCs from HIF-1α luciferase reporter mice [55], we compared ADJ and LPS for HIF-1α induction. Only stimulation with LPS, but not ADJ, induced HIF-1α in DCs (Fig 4B). Kruppel-like factor 2 (KLF2) is a transcription factor that inhibits the expression and transcriptional activity of HIF-1α, and downregulation of KLF2 is associated with engagement of glycolysis in immune cells [56]. As another measure of glycolytic reprogramming, we assessed KLF2 expression levels using BMDCs from KLF2-GFP reporter mice [57]. High levels of KLF2 were detected in unstimulated DCs, and KLF2 expression was significantly downregulated in LPS-stimulated DCs, but not in ADJ-treated DCs (Fig 4C).

To confirm these findings in vivo, we immunized KLF2-GFP mice with OVA, ADJ+OVA or LPS+OVA and examined DC KLF2 expression in vaccine-draining lymph nodes. As shown in Fig 4D, DCs from mice immunized with LPS+OVA, but not ADJ+OVA, showed significant down-regulation of KLF2 expression, as compared to DCs from OVA only mice. Thus, unlike LPS, ADJ failed to engage the Akt-mTORC1-KLF2-HIF-1α signaling pathway in DCs.

To directly investigate the effect of ADJ on DCs’ glycolytic metabolism, we quantified intracellular ATP levels in DCs treated with ADJ or LPS. LPS-treated DCs, but not ADJ-treated DCs contained higher levels of ATP than untreated DCs (Fig 4E). As an index of glycolysis in ADJ- and LPS-treated DCs, we quantified glucose consumption and lactate production in vitro. The extracellular concentrations of glucose and lactate were unaffected by ADJ stimulation, suggesting that ADJ did not alter glucose utilization or lactate production by DCs (Fig 4F and 4G). Further, we did not find significant alteration of the NADH/NAD+ ratio in ADJ-treated DCs, which suggested that ADJ exposure did not cause a cellular redox imbalance (Fig 4H). Lastly, we quantified the functional glycolytic capacity of ADJ-stimulated DCs using the glycolysis stress test. We found that neither the glycolytic capacity nor the glycolytic reserves were altered in ADJ-treated DCs, as compared to those in unstimulated DCs, while LPS up-regulated both glycolytic capacities and reserves in DCs (Fig 4I). Together, data in Fig 4 strongly suggested that ADJ-mediated cross-presentation occurred independent of enhanced aerobic glycolysis.

ADJ disengages OXPHOS in DCs by iNOS-independent mechanisms

Because ADJ failed to augment glycolysis (Fig 4), we interrogated whether ADJ engaged OXPHOS as an alternative metabolic pathway. By performing extracellular flux analysis, we
measured alterations in oxygen consumption in real time (Fig 5A). At 24 hours after stimulation, the mitochondrial oxygen consumption rate (OCR) was highest in unstimulated DCs [58] and mitochondrial OCR was lower in LPS-treated DCs. Notably, even baseline OCR for ADJ-treated DCs was markedly lower than in unstimulated DCs, and failed to show detectable increase, following addition of FCCP, a potent mitochondrial un-coupler that disrupts ATP synthesis (Fig 5A). To further evaluate ADJ’s effects on mitochondrial metabolism, we
measured mitochondrial content, membrane potential (Ψm) and mitochondrial superoxide production (mROS). Within 2 hours of ADJ treatment, we observed a drastic reduction in mitochondrial content, Ψm, and mROS, in comparison to both resting and LPS-stimulated DCs (Fig 5B, 5C and 5D). Interestingly, loss of mitochondrial functions persisted over 24 hours in ADJ-treated cells, indicating that ADJ suppressed mitochondrial functions at both early and late stages of stimulation. These data are consistent with a decrease in spare respiratory capacity (Fig 5A) in ADJ-stimulated DCs, and support the inference that ADJ-mediated metabolic programming includes a profound decline in mitochondrial activity.

One of the mechanisms for inhibiting mitochondrial functions in DCs is the induction of NO, which interferes with electron transport chain by blocking oxygen consumption and ATP production [59]. We probed whether impairment of mitochondrial function by ADJ was linked to NO induction in DCs. The cellular levels of inducible nitric oxide (iNOS) (Fig 5E) and extracellular nitrite levels (Fig 5F) in the supernatant of ADJ-treated DCs did not vary, in
comparison to unstimulated DCs, while LPS-stimulated DCs contained elevated levels of cellular iNOS and extracellular nitrite. In summary, these data collectively suggested that ADJ disengaged mitochondrial functions by mechanisms independent of iNOS.

**ADJ-induced intracellular lipid body formation is required DC cross-presentation**

Next, we explored how ADJ might enhance cross-presentation independent of aerobic glycolysis in DCs. The formation of intracellular lipid bodies (LB) was shown to be critical for efficient DC cross-presentation [60,61]. Therefore, we determined whether LBs are also essential for ADJ-mediated cross-presentation. To assess whether ADJ induced intracellular formation of LBs in DCs, unstimulated, LPS-, or ADJ-treated DCs were stained with BODIPY 493/503 which stains neutral lipids. Neutral lipids were barely detected in resting DCs, but ADJ or LPS-stimulated DCs contained abundant levels of neutral lipids, as indicated by an increase in MFI of BODIPY 493/503 in a flow cytometer or as visualized by confocal microscopy (Fig 6A and 6B). To elucidate mechanisms underlying the increased intracellular content of neutral lipids in DCs, we examined whether ADJ altered FA uptake by promoting expression of the scavenger receptor CD36. Intriguingly, ADJ increased the expression of CD36 in ADJ-stimulated DCs, but LPS downregulated CD36 expression in DCs (Fig 6C).

It has been reported that the glucose-derived pentose phosphate pathway (PPP), an offshoot of the glycolytic pathway, is critical for generation of LBs upon TLR-stimulation in DCs [49]. We did not observe increased glycolytic flux in ADJ-stimulated cells to fuel PPP (Fig 5I), but we hypothesized that the NADPH/NADP+ ratio will be altered in ADJ-treated cells because imported FAs need to be activated before incorporation into triglycerides by esterification with coenzyme A, through a reaction catalyzed via fatty acyl-CoA synthetase. As an indirect measure of NADPH/NADP+ ratio, we quantified optical redox ratio (NAD(P)H/NAD(P)H + FAD ratio) in ADJ-stimulated cells using optical multiphoton microscopy [62]. We discovered a significant drop in the redox ratio in ADJ-stimulated cells compared to unstimulated cells (S6 Fig). To further distinguish NADPH/NADP+ from NADH/NAD+ ratio, we used a bioluminescence-based assay and confirmed that ADJ-stimulated cells displayed lower NADPH/NADP+ ratio (Fig 6D). Together, these data suggested that ADJ stimulation likely induced LB formation by utilizing intracellular NADPH and imported FAs, independent of glucose-derived de novo FAs.

Lastly, to examine whether LB formation is required for ADJ-driven cross-presentation, we treated DCs with xanthohumol, a DGAT1/2 inhibitor. Notably, treatment of ADJ-treated DCs with xanthohumol significantly inhibited cross-presentation and activation of B3Z cells by ADJ-treated DCs (Fig 6E). Together, this suggested that LBs might be crucial for ADJ-induced DC cross-presentation.

**ADJ alters intracellular lipidomes in DCs and increases accumulation of 18:2 and 18:3-containing lipids**

The saturation and oxidation status of lipids might modulate cross-presentation by DCs [63–66]. However, how lipid composition governs DC cross-presentation remains unknown. In order to map changes in lipid species in ADJ-treated cells, we employed a discovery lipidomics mass spectrometry approach. With this approach, we identified 446 unique lipid species in ADJ-treated DCs as compared to resting or LPS-treated DCs, and ADJ-treated DCs displayed significant increases in lipids containing acyl-chains with linoleic (18:2) or alpha-linoleic (18:3) acid (Fig 6F), which appear to be constituents of the adjuvant ADJ (Fig 6F, gray bars). Note that media with ADJ also contained an increased abundance of 18:2 and 18:3-containing lipids.
lipids, as compared to media alone. In addition, ADJ treatment also led to increases in ceramides containing alpha-hydroxy fatty acids (Cer[AP] and Cer[AS]) and decreased abundance of plasmanyl-phospholipids. Hence, these lipidomic profiles further highlight the global lipid changes induced by ADJ treatment, characterized by increased abundance of linoleic and alpha-linoleic acyl-chains within phospholipids and triglycerides, likely resulting in changes in membrane fluidity. Together, our data suggested that changes in lipid composition could be critical for ADJ-mediated cross-presentation in DCs.

https://doi.org/10.1371/journal.ppat.1009168.g006

Fig 6. Carbomer-based adjuvant induces intracellular lipid body formation and alters intracellular lipidomes in DCs. (A) Quantification of neutral lipid droplets by flow cytometry using BODIPY 493/503 dye. BMDCs treated with ADJ (1%) for 24 h were stained with BODIPY 493/503 dye and analyzed by flow cytometry. (B) Confocal microscopy of DCs were cultured with ADJ (1%) for 24 h, stained with BODIPY 493/503, and counter-stained with DAPI. Scale bars, 10 μm. (C) FACS analysis of CD36 expression in unstimulated, and ADJ- or LPS-stimulated DCs after 24 h. (D) NADPH/NADH+ ratio in resting, ADJ- or LPS-stimulated DCs. (E) β-galactosidase production by B3Z cells after co-culture with BMDCs pre-treated with OVA ± ADJ in the presence/absence of xanthohumol for 5 h. (F) Heat-map of lipids from media or DCs stimulated with ADJ ± LPS; *P < 0.05 (ANOVA for +ADJ treatment, Tukey post-hoc corrected p-values) and abundance was scaled across samples. Lipids that increased with ADJ treatment were enriched for 18:2 or 18:3 acyl-chains (blue bar). Lipids that were decreased with ADJ treatment contained more plasmanyl-phospholipids (yellow bar). Abbreviations: TG, triglyceride; DG, diglyceride; FA, fatty acid; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; SM, sphingomyelin; Cer [AP], alpha-OH fatty acids with pytosphingo sine; Cer [AS], alpha-OH fatty acids with sphingosine. Data are representative of 2 independent experiments. Error bars show SEM; *P<0.01; **P<0.001; ***P<0.0001 (One-way ANOVA- A, C; Student’s t-test- D-E).
Discussion

In this manuscript, we report that a carbomer-based nano-emulsion adjuvant, ADJ, promoted memory T cell-dependent protective immunity against intracellular pathogens, *Listeria monocytogenes* and vaccinia virus. Consistent with strong induction of CD8 T-cell responses to subunit antigens *in vivo*, ADJ promoted DC cross-presentation *in vitro*. Mechanistic investigations of antigen processing and the metabolic basis for adjuvant action demonstrate that ADJ promoted multiple aspects of antigen cross-presentation in DCs, in the apparent absence of switch from catabolic to anabolic metabolism. This constitutes a novel mechanism because the current axiom suggests that engagement of active aerobic glycolysis in DCs is necessary for their activation and ability to stimulate CD8 T cells [18].

How did ADJ enhance DC cross-presentation and potentiate CD8 T cell immunity *in vivo*? Effective cross-presentation required antigen targeting to less acidic intracellular compartments, slow degradation of antigens by proteases, and translocation of endosomal antigens into cytosol. Intriguingly, we discovered that ADJ-treated DCs contained greater amounts of degraded antigens, compared to untreated DCs, but this was not linked to elevated antigen uptake. Increased antigen cross presentation mediated by ADJ appears to be the result of greater induction of ROS by ADJ that presumably leads to alkalization, attenuated antigen degradation, and accumulation of partially degraded antigens in alkaline endosomes. Thus, one mechanism by which ADJ might enhance cross-presentation is by delaying antigen degradation and promoting partially degraded antigen accumulation in DCs, which in turn sustains antigen presentation by DCs.

Our studies also demonstrated that ADJ-treated DCs utilized an endosomal-to-cytoplasm pathway of cross-presentation, as indicated by augmented endosomal protein leakage into cytosol and abolishment of ADJ-driven cross-presentation by pharmacological inhibition of cytosolic proteasomes. The molecular mechanisms underlying the translocation of endosomal antigens into cytosol during cross-presentation remains controversial. One possible mechanism is explained by the transporter hypothesis, in which exogenous antigens are unfolded by gamma-interferon-inducible lysosomal thiol reductase (GILT) in the phagosome and transported into cytoplasm by ER-associated degradation (ERAD) machinery or chaperone-mediated transport by Hsp90 [67–69]. Another potential mechanism is endosomal membrane disruption, induced by NOX2-dependent ROS production that serves as precursors for lipid peroxides in the endosomes. In our studies, we found that: (1) ADJ strongly induced ROS production; (2) NOX2 deficiency and pharmacological inhibition of NOX2 assembly abolished cross-presentation; (3) antioxidant tocopherol diminished ADJ-driven cross-presentation. Therefore, our data favor the endosomal disruption model, where ADJ-induced ROS might have a dual function in cross-presentation: increase endosomal pH to delay antigen degradation and promote antigen translocation into cytosol by lipid peroxidation.

Only recently, it was discovered that TLR-driven metabolic shift to anabolic metabolism is an integral component of the activation program that is required to activate naïve T cells [18]. While the metabolic basis of how TLR agonists support DC effector functions is well characterized, the exact metabolic roles of many immune adjuvants in dictating DC activation and antigen presentation remain poorly understood. Our data suggest that the two important energy yielding metabolic pathways, glycolysis and OXPHOS, were minimally engaged or inactive in ADJ-treated DCs, which is indicative of a unique cellular state of metabolic quiescence during cross-presentation. How ADJ-treated DCs effectively stimulate T cells *in vivo* without the need to switch to glycolysis or to trigger enhanced mitochondrial metabolism remains unclear. Recently, it was reported that DCs contain intrinsic glycogen, which can be readily catabolized into glucose upon LPS stimulation to fuel intracellular glucose [70]. Unlike FAO, which
requires a substantial number of functional mitochondria, glycogenolysis occurs in the cytoplasm. Because ADJ treatment results in a loss of mitochondrial functions and minimal engagement of glycolysis in DCs, it is plausible that ADJ-treated DCs can catabolize intrinsic glycogen into glucose to sustain their survival in the absence of functional mitochondria. Follow-up studies should evaluate whether ADJ-treated DCs utilize intracellular glycogen reserves during metabolic quiescence and/or whether glycogenolysis is required for ADJ-mediated DC cross-presentation.

Despite a ‘hypometabolic’ state, ADJ-treated DCs displayed effective antigen cross-presentation and stimulated CD8 T cell responses in vivo. However, this leads to the question of why ADJ-stimulated DCs adapt to this particular type of metabolism that is metabolically inefficient, at least in terms of ATP production. A recent study suggests that high levels of glucose repress DC-induced T cell responses by engaging the mTORC1-HIF1α/iNOS pathway in DCs [71]. In their studies, limiting glucose availability to DCs enhanced T cell responses by diminishing competition for glucose from DCs in the immune microenvironment. It is plausible that ADJ drives DCs to a metabolic state that is less dependent upon glucose-driven catabolic pathways, which enables DC outputs tailored for effective cross-presentation to T cells. For instance, conventional processing of exogenous antigens involves acidification of the lysosomal compartment, which is required for efficient generation of peptides for loading into MHC II and that is dependent on activity of ATP-driven proton pumps [72]. During this process, lysosomal V-ATPase complex is assembled by intracellular H+ ions generated by aerobic glycolysis (resulting from oxidation of NADH into NAD+ and H+ ions), leading to an increase in lysosomal acidity. Notably, ADJ did not trigger redox imbalance of NADH/NAD+ ratio in DCs, but only reduced intracellular ATP production. Thus, it is plausible that maintenance of a low metabolic state by reducing a key carbon source for generating ATP and keeping balanced redox ratio might be essential for reducing intracellular acidity by inhibiting lysosomal V-ATPase assembly in DCs.

The divergent functions of inflammasome activation in shaping adaptive immunity have been demonstrated for other vaccine adjuvants, such as Alum and ISCOM [54]. Production of IL-1β and IL-18 suggest that ADJ might activate inflammasomes in DCs. This finding was unexpected, especially because LPS-induced succinate stabilized HIF-1α in macrophages, which is known to be required for inflammasome activation under inflammatory conditions [73]. However, ADJ did not promote stabilized-HIF1α accumulation under normoxia in DCs. It has been also documented that phago-lysosomal destabilization after adjuvant phagocytosis, such as Alum and Carbopol, is an important step in inflammasome activation [74,75]. While we have not directly characterized the intracellular location of ADJ in DCs, ADJ increased lysosomal pH, which in turn may result in lysosomal stabilization. Inflammasome activation has been closely associated with enhanced cross-presentation in multiple studies [34,35], but our studies showed that loss of NLRP3, ASC or caspase-1 did not affect the ability of ADJ to enhance DC cross-presentation in vitro. It is possible that ADJ might stimulate IL-1β by inflammasome-independent mechanisms [76–78] and/or that ADJ modulates DC cross-presentation, by inflammasome-independent mechanisms, at least in vitro. Caspase 1 deficiency did not impair ADJ’s ability to induce DC cross-presentation in vitro and in vivo, indicating that ADJ likely engages other mechanism(s) needed for DC cross-presentation, without caspase-1 activity. This finding is consistent with a previous report, in which induction of OVA-specific antibodies by Carbopol, a polyanionic carbomer, was not significantly affected in caspase 1-deficient mice [74]. Future studies are warranted to determine how and whether ADJ triggers inflammasome activation in DCs and the role of inflammasome activation in engendering protective cell-mediated immunity in vivo.
The causative link between the formation of intracellular LBs and cross-presentation efficiency by IFN-γ and ISCOMs has been recently established [60,61]. Consistent with previous findings, our studies also suggest that ADJ-induced cross-presentation requires intracellular LB formation in DCs. However, how LBs exert their effects on DC cross-presentation remains undetermined. While we have not yet examined whether ADJ-aided LBs directly affect antigen processing, our lipidomic studies highlight the unique composition of lipids within ADJ-treated DCs, which shows accumulations of 18:2 and 18:3 acyl tails and ceramides, presumably due to uptake of ADJ itself. Because ADJ upregulates CD36 expression in DCs, it is conceivable that ADJ enhances the uptake of external lipid without de novo fatty acid synthesis derived from glucose [79]. In line with low metabolic profiles in ADJ-stimulated DCs, the formation of intracellular LBs using external fatty acids could be a more efficient pathway to store intracellular lipids since it requires minimal energy and overall metabolic activities. The global intracellular lipidome modified by ADJ suggests a possible explanation for ADJ-induced cross-presentation, presumably by regulating antigen export to cytosol during cross-presentation. For example, an increase in double-bonds within phospholipids could directly increase membrane fluidity, leading to an increase in endosomal antigen leakage [80,81]. An enrichment in certain ceramides could also contribute to the formation of lipid rafts, which are known to be critical for regulation of endosomal NOX2 assembly [82]. Thus, enrichment of certain lipid classes in the endosomes mediated by ADJ, such as unsaturated phospholipids and ceramides, could be another important rate-limiting step for antigen export to the cytosol, in addition to ROS-driven endosomal disruption.

In summary, we have identified a carbomer-based adjuvant (ADJ) that elicits protective CD8 T cell responses to soluble subunit antigens, protects against viruses and an intracellular bacterial pathogen, and enhances cross-presentation by the cytosolic pathway. The most striking finding is that ADJ-stimulated DCs, which are highly efficient in cross-presenting antigens, exhibited a distinct metabolic state that is characterized by minimum glycolytic activity, low mitochondrial respiration, and intracellular LB formation (S7 Fig). Thus, our model challenges the prevailing metabolic paradigm by suggesting that retaining DCs in a quiescent state is a unique mechanism to regulate efficient DC cross-presentation. Our findings have significant implications in understanding the mechanism of action of adjuvants and development of safe and effective vaccines that elicit potent T cell-based immunity against infectious diseases, such as HIV, TB and malaria.

Methods

Ethics statement

All animal experiments were performed in accordance with the protocol (Protocol number V5308 and V5564) approved by the University of Wisconsin School of Veterinary Medicine Institutional Animal Care and Use Committee (IACUC). The animal committee mandates that institutions and individuals using animals for research, teaching, and/or testing must acknowledge and accept both legal and ethical responsibility for the animals under their care, as specified in the Animal Welfare Act (AWA) and associated Animal Welfare Regulations (AWRs) and Public Health Service (PHS) Policy.

Experimental animals

7-12-week-old C57BL/6J (B6), Gp91 (NOX2) -/- (Stock number, 002365), and TAP1 -/- (Stock number: 002944) were purchased from Jackson Laboratory or obtained from restricted-access SPF mouse breeding colonies at the University of Wisconsin-Madison Breeding Core Facility. Caspase 1-deficient, ODD-LUC mice backcrossed to Albino C57BL/6 background, OT-I mice...
were provided by Drs. J. D. Sauer, Richard Eisenstein, and Jing Zhang (University of Wisconsin-Madison), respectively. KLF2-GFP reporter mice were provided by Dr. Stephen Jameson (University of Minnesota). NLRP3-KO and ASC-KO mice were kindly provided by Drs. Vishva Dixit and Kim Newton (Genentech-Roche, CA); NLRP3/AIM2-dKO mice were kindly provided by Dr. Thirumala-Devi Kanneganti (St. Jude Children’s Hospital, TN).

**Chemicals and reagents**

Adjuplex (endotoxin-free) was purchased from Empirion LLC. Hen egg white ovalbumin grade V (OVA) from chicken egg white (A5503), LPS purified from Escherichia coli O111:B4 (L2630), Leupeptin (L2884), α-Tocopherol (T3251), gelatin from cold water fish skin (G7041), Collagenase B (1108831001), Collagenase D (1108882001), and Cytochrome c from equine heart (C2506) were purchased from Millipore Sigma. Ovalbumin, Alexa Fluor 647 Conjugate (O34784), DQ Ovalbumin (D12053), CellROX Deep Red reagent (C10422), Lysotracker Deep Red reagent (L12492), LiveDead eFlour 780 (A10628), BODIPY 493/503 (D3922), Mito-Tracker Deep Red (M22426), DiOC₆ (D273), MitoSOX Red Mitochondrial Superoxide Indicator (M36008), Protease Inhibitor Tablets (P88666), Pierce Phosphatase Inhibitor Mini Tablets (88667), Thermo Scientific Halt Protease Inhibitor Cocktails (78430), Prolong Gold Antifade (P36934), and DAPI (D1306) were purchased from Thermo Fisher Scientific. pHAb Amine Reactive Dye (G9841) was purchased from Promega. Epoxomycin (10007806) and xanthohumol (15399) were purchased from Cayman Chemicals. Paraformaldehyde was purchased from Electron Microscopy Sciences (15710-S). Chlorophenol red-β-D-galactopyranoside (CPRG, sc-257242) and apocynin (sc-203321) were purchased from Santa Cruz Biotechnology. NP-40 (M158-50ML), Tris (97061-794), Tween-20 (97062-332), Triton X-100 (97063-864), Glycine (0167-1kg), and brain heart infusion (90003-040) were purchased from VWR (Amresco). Glutaraldehyde (O2957-1) and Agar (BP1423-500) were purchased from Fisher Scientific.

**Antibodies**

Hamster anti-CD11c-BV421-conjugated (N418, 565452), Hamster anti-CD11c/PE-Cy7-conjugated (N418, 558079), Rat anti-CD40-BV421-conjugated (3/23, 562846), Hamster anti-CD80-APC-conjugated (16-10A1, 560016), Mouse anti-H-2Kb-FITC-conjugated (AF6-88.5, 562002), Mouse anti-I-A[b]-PE-CF594 conjugated (AF6-120.1, 1:400, 562824) Annexin V-PE-conjugated (556421), Rat Anti-CD4-BUV496-conjugated (GK1.5, 564667) Rat Anti-CD8-BUV395-conjugated (53-6.7, 563786) Rat Anti-CD44-BV510-conjugated (563114), and BV421 Streptavidin (563259) were purchased from BD Biosciences. Rat anti-CD86-PE/Cy7-conjugated (GL-1, 105014) was purchased from Biolegend. Rat anti-CCR7-PE-conjugated (4B12, 12-1971-82), Hamster anti-CD36-Alexa Fluor 700-conjugated (HM36, 56-0362-80), and Biotin Mouse OVA257-264 (SIINFEKL) peptide bound to H-2Kb Monoclonal Antibody (1:50, eBio25-D1.16 (25-D1.16), 13-5743-81) were purchased from eBioscience. APC-conjugated H2-Kb tetramers bearing the ovalbumin peptide SIINFEKL was obtained from NIH Tetramer Core Facility at Emory University. Mouse anti-NOS2 Antibody PE-conjugated (C-11, sc-7271) and Rat anti-mouse LAMP1 antibody (1D4B, sc-19992) were purchased from Santa Cruz Biotechnology. Purified Anti-Mouse CD16 / CD32 (Fc Shield, 70-0161-U500, 2.4G2, 1:100) was purchased from Biolegend. Alexa Fluor 488 goat anti-rabbit IgG (H+L) secondary antibodies (A11008), Alexa Fluor 568 goat anti-rat IgG (H+L) secondary antibodies (A11077), and goat anti-rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, HRP (G-21234) were purchased from Thermo Fisher Scientific. Rabbit anti-EEA1 antibody (C45B10, 3288), Rabbit anti-Phospho-Akt (Ser473, Clone: D9E) antibody, Rabbit anti-Akt polyclonal
antibody (9272S), Rabbit anti-phosphor-p70 S6 kinase (Thr389, Clone: 108D2), and Rabbit polyclonal anti-p70 S6 kinase antibodies (9202S) were purchased from Cell Signaling Technology.

Tissue processing and flow cytometry

Primary monoclonal antibodies for detecting surface markers/tetramers were used for flow cytometry at 1:200 dilution (provided in the resource table) unless stated otherwise. Spleens and lymph nodes were processed into single-cell suspensions by standard procedures. In some experiments, lymph nodes and lungs were digested in 2mg/mL of Collagenase (Collagenase B for lungs; Collagenase D for draining lymph nodes) for 15 minutes at 37°C. Single-cell suspensions were first stained for viability with LiveDead eFlour 780 stain (eBioscience), blocked with FACS buffer (1% BSA in PBS) containing FC block (1:100), and stained with antibodies diluted in Brilliant Stain Buffer (BD Biosciences) or FACS buffer for 30–60 minutes. To detect Kb/SIINFEKL expression, DCs were first stained with biotin-labeled anti-SIINFEKL/H-2Kb monoclonal antibody on ice for 40 minutes. Next, samples were stained with BV421-streptavidin (1:500) on ice for 30 minutes. Intracellular staining of iNOS was performed using Cytofix/Cytoperm fixation/permeabilization kit (BD Biosciences; 555028) as previously described [51]. Samples were acquired with a BD LSR Fortessa (BD Biosciences) and data were analyzed with FlowJo software (TreeStar, Ashland, OR).

BMDC generation and cell culture

Primary cultures of bone marrow-derived DCs were generated as previously described [83,84]. Briefly, femur, tibia, and humerus were flushed using RPMI-1640 medium supplemented with 1% FBS. Bone marrow cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, and 10ng/ml GM-CSF (Peprotech) in 150mm petri dishes. Equal parts of additional media with 10ng/ml of GM-CSF were added on day 3. Loosely attached immature DCs were collected at day 6–7 and subsequently used for the experiment. The B3Z hybridoma was a generous gift from Dr. Bruce Klein (University of Wisconsin-Madison). B3Z cells were maintained in Iscove’s Modified Dulbecco’s media supplemented with 10% FBS, 100 U/ml penicillin G and 100 g/ml streptomycin sulfate. Vero cells were maintained in low-glucose DMEM media, supplemented with 10% FBS, 100 U/ml penicillin G and 100 g/ml streptomycin sulfate.

Vaccination, viral titers, and enumeration of Listeria challenge

C57BL/6J mice were vaccinated by the subcutaneous route at the tail base with 50ul of the vaccine (containing 5% ADJ and 10μg chicken OVA). Mice were boosted after 21 days of the initial vaccination. At > 40 days after vaccination, mice were challenged intranasally with 2 x 10⁶ recombinant vaccinia virus-expressing OVA as previously described [29]. Spleens, lungs, and ovaries were used for viral titers. Tissue samples for viral titers were homogenized in 400ul 1mM pH 8.0 Tris using bead homogenizer. Clarified supernatant was trypsinized in Trypsin-EDTA (0.05%) and titrated in complete RPMI on Vero cells (American Type Culture Collection: ATCC CCL-81). Listeria monocytogenes expressing chicken ovalbumin (LM-OVA) was provided by Dr. Hao Shen (University of Pennsylvania School of Medicine) as previously described [30]. Mice were infected intravenously with 1.7 x 10⁵ CFUs of LM-OVA. To quantify Listeria burdens, tissues were homogenized in GentleMACS C-Tubes via GentleMACS disassociator. Organs were processed in sterile 0.1% Nonidet-P40 + PBS in GentleMACS C Tubes. Serial dilutions of tissue samples were plated on brain heart infusion (BHI) agar plates for 24
hours at 37°C. Vaccinia viral titer and Listeria burden in tissues were normalized by the weight of the tissues.

**In vivo KLF2-GFP detection and DC-T cell priming**

To assess *in vivo* KLF2-GFP expression, cDCs were analyzed in draining lymph nodes 24 hours after footpad injection with 25μl of the vaccine (containing 10μg chicken OVA with or without 10% ADJ or 10μg LPS). For *in vivo* DC-T cell priming, BMDCs were loaded with 1mg of OVA with or without 1% ADJ for 6 hours, washed twice with PBS, and injected intravenously into mice. Spleens were collected on day 8 and the percentages of CD44^HI^ H-2K^b^/SIIN-FEKL tetramer-binding CD8 T cells were quantified by flow cytometry.

**OT-I CD8 T cell *in vitro* proliferation**

OT-I CD8 T cells from the spleen were purified using mouse Pan T cell isolation (Miltenyi biotec) and fluorescently labeled with CellTrace CFSE Cell Proliferation Kit (Thermo Fisher Scientific) according to manufacturer’s instructions. BMDCs (1x10^5^ cells/well) were cultured with OVA or ADJ+OVA on 96 well flat bottom plate (Corning), extensively washed with sterile PBS, and co-cultured with purified CFSE-labeled OT-I CD8 T cells (5x10^5^ cells/well) for 72 hours. After 72 hours, OT-I CD8+ T cell division was measured by CFSE dilution using flow cytometry.

**B3Z activation assay for *in vitro* cross presentation**

The cross-presentation capacity of murine BMDCs was measured using B3Z hybridoma cells, as previously described [33,85]. Briefly, DCs were plated at 1 x 10^5^ cells/well in 96-well round-bottom culture-treated plates (Corning). In some experiments, BMDCs were pre-treated for 1 hour with aponocyn (300μM), leupeptin (50μM), α-tocopherol (100μM), epoxomicin (10μM) or xanthohumol (30μM). Subsequently, BMDCs were cultured with OVA (1mg/ml) with or without ADJ and appropriate chemicals for 5 hours. Next, BMDCs were fixed with 0.025% glutaraldehyde for 2 minutes at room temperature. washed with PBS and cultured with B3Z cells (1 x 10^5^ cells/well) for 18 hours. After 18 hours, B3Z cells were washed and incubated with CPRG substrate (0.15mM) in 200ul of lysis buffer (0.1% NP 40+ PBS) for 18 hours at room temperature. The absorbance at 590nm was measured using a plate reader. Wells containing B3Z cells + BMDCs without OVA served as background control.

**Cytokine detection from cell culture supernatants**

Supernatants from cultures of BMDCs cultured with or without ADJ (1%) were collected at 24 hours. Cytokines in cell culture supernatants were quantified using Bio-Plex Pro Mouse Cytokine 23-plex Assay (Bio-Rad, M60009RD), IFN-β ELISA kit (R&D Systems, DY8234-05), and IL-18 ELISA (Thermo Fisher Scientific, BM618-3) according to manufacturer’s protocol.

**Antigen capture and processing assay**

BMDCs were seeded at 4–5 x 10^5^ cells per well in 96-well culture flat-bottom plate (Corning) for the following assays. For antigen uptake assay, 20ug Alexa Fluor 647-conjugated OVA was mixed with or without ADJ (1%) in 200ul of complete RPMI media. BMDCs were incubated with pre-warmed Alexa Fluor-647 OVA with or without 1% ADJ for 10 or 30 minutes. The pH of OVA-containing endosomal compartment was determined using ovalbumin-conjugated with pHAB-reactive dye, as previously described with some modifications [86,87]. Conjugation of ovalbumin with pHAB-amine reactive dye was performed according to
manufacturer’s instructions. Briefly, OVA was labeled with amine-reactive dye at 1:10 excess molar ratio (ovalbumin:pHAB amine reactive dye). Free-unlabeled dye was removed by PD-10 columns (GE healthcare) and concentrated using Amicon Ultra Centrifugal Filters (Millipore). BMDCs were incubated with pHAB-conjugated OVA with or without 1% ADJ for 30 minutes, and chased for 30 minutes. Antigen degradation studies were performed using DQ-OVA; DCs were incubated with DQ-OVA (20 ug) with or without 1% ADJ for 30 minutes, and chased for 6 hours.

Detection of intracellular ROS, H$_2$O$_2$, lipid peroxidation, and intracellular acidity

ROS and H$_2$O$_2$ productions were measured using CellROX Deep Red reagent and ROS-Glo H$_2$O$_2$ assay, respectively. For ROS production, samples were washed with PBS and incubated with 50nM CellROX Deep Red in PBS for 15 minutes at 37°C. For intracellular H$_2$O$_2$ detection, ROS-Glo H$_2$O$_2$ assay (Promega; G8820) was performed according to manufacturer’s instructions; luciferin activity from added H$_2$O$_2$ substrate was measured by SpectraMax i3x (Molecular Probes). Lipid peroxidation was measured using Image-iT lipid peroxidation kit (Thermo Fisher Scientific; C10445) according to manufacturer’s instructions. For tracking acidic organelles, samples were washed and incubated with 50nM Lysotracker Deep Red in PBS for 15 minutes at 37°C.

Endosomal antigen leakage assay

Cytochrome C release assay was performed as previously described, with minor modifications [43]. Briefly, BMDCs were cultured with 5mg/ml of equine cytochrome C (Sigma Aldrich) with or without 1% ADJ. After 24 hours, cells were stained with Annexin-V and analyzed by flow cytometry.

20S proteasome activity assays

In BMDCs, the activities of proteasomes containing luminogenic substrates (the Suc-LLVY, Z-LRR and Z-nLPLnLD sequence recognized by the 20S proteasome) were measured by Proteasome-Glo Chymotrypsin-Like, Trypsin-Like and Caspase-Like Cell-Based Assays (Promega, G1180), according to manufacturer’s instructions [88].

Immunofluorescence, confocal laser scanning microscopy for co-localization

For co-localization of antigen-containing compartment, DCs were seeded in 24-well plates at a density of 1x 10$^6$ cells/well on 1.5mm coverslips (Warner Instrument) and cultured in complete phenol-red free RPMI 1640 media (Lonza) for 30 minutes at room temperature. DCs were pulsed with Alexa Fluor 647-OVA (40 ug/ml) +/- ADJ (1%) for 15 minutes at 37°C, and chased in complete phenol-red free RPMI media for 20 or 60 minutes at 37°C. Cells were washed with PBS, fixed with 4% paraformaldehyde (Electron Microscopy Services) at room temperature for 20 minutes, permeabilized using 0.2% Triton X-100 and blocked in blocking buffer (10% goat serum, 0.1% cold-fish gelatin, 0.1% tween-20) for 1 hour. Antibodies (rabbit anti-EEA1 (Clone: C45B10, 3288S, 1:50, Cell Signaling technology), rat anti-LAMP1 (Clone: 1D4B, sc-19992, 1:50, Santa Cruz Biotechnology) were diluted in blocking buffer at room temperature for 20 minutes, permeabilized using 0.2% Triton X-100 and blocked in blocking buffer (10% goat serum, 0.1% cold-fish gelatin, 0.1% tween-20) for 1 hour. Antibodies (rabbit anti-EEA1 (Clone: C45B10, 3288S, 1:50, Cell Signaling technology), rat anti-LAMP1 (Clone: 1D4B, sc-19992, 1:50, Santa Cruz Biotechnology) were diluted in blocking buffer and used to detect early endosomes or lysosomes at 4°C overnight. Coverslips were extensively washed with PBS + 0.1% Tween-20 and incubated with Alexa 564-conjugated rat IgG antibody or Alexa 488-conjugated rabbit IgG antibody (Invitrogen) in blocking buffer at room temperature for 30 minutes.
temperature for 1 hour. Samples were counter-stained with DAPI (Invitrogen), washed, mounted with Prolong Gold Anti-fade mountant (Invitrogen) and imaged on a Leica SP8 confocal laser-scanning microscope at 63X objective lens. The degree of co-localization was calculated using LAX-S Software (Leica).

**Metabolism assays**

For real-time analysis of ECAR and OCR, Glycolysis Stress and Mito Stress tests (Agilent: 103020–100 and 103708–100) were performed according to manufacturer’s instructions [89]. BMDCs were analyzed with an XF-96 Extracellular Flux Analyzer (Seahorse Bioscience). 1–2 x 10^5 cells/well were seeded in 96-well tissue-culture treated flat-bottom plates for the metabolic measurements; ATP concentrations, glucose, lactate, NAD+/NADH, and NADP+/NADPH were quantified using CellTiter-Glo, Glucose-Glo, Lactate-Glo, NAD+/NADH-Glo, and NADP+/NADPH-Glo kits (Promega: G9241, J6021, J5021, G9071, and G9081), respectively, according to manufacturer’s instructions. For accurate quantification of metabolites from the cell culture supernatant, dialyzed FBS (Gibco; A3382001) was used in red phenol-free complete RPMI media. Nitrite levels were quantified using Griess’ reagent (Sigma Aldrich; 03553-100ML). 3–4 x 10^5 cells/well were used in 96-well non-treated flat-bottom plates for the following metabolism assays and transferred to 96-well round-bottom plates for staining; mitochondrial contents, membrane potentials, and mitochondrial ROS were measured by treating cells with MitoTracker Deep Red, DiOC<sub>6</sub>, and MitoSOX, respectively in PBS for 30 minutes according to manufacturer’s protocol. To visualize neutral lipids, cells were stained with 500 ng/ml BODIPY 493/503 in PBS for 15 minutes; stained unfixed cells were acquired immediately on a flow cytometer.

**KLF2-GFP and ODD-luc reporter assay**

BMDCs derived from ODD-Luc or KLF2-GFP mice were cultured with ADJ (1%) or LPS (100ng/ml) For KLF2-GFP reporter experiments, samples were washed and immediately acquired on flow cytometer. For ODD-luc DCs, cells were lysed using passive lysis buffer and luciferase activities were measured using Luciferase reporter assay system (Promega; E4030) according to manufacturer’s instructions.

**Quantification of the optical redox ratio by live cell microscopy**

Optical imaging of BMDCs was performed as described previously [62]. BMDCs were seeded at a density of 1 x 10^6/ml in glass bottom dishes 2 hours before imaging. For the time course experiments, 1% ADJ was added immediately before imaging. Cells were imaged in a stage-top incubator maintained at 37˚C with CO2 supplementation. Four locations within each dish were imaged for control and ADJ-treated groups. The optical redox ratio was calculated from intensity images, which were acquired for 60 seconds as previously described [62]. The total number of NADH photons was divided by the sum of the total number of NADH photons and the total number of FAD photons on a single pixel basis. A custom CellProfiler pipeline was used to threshold out nuclear signal and background. The average redox ratio value per cytoplasm was then computed.

**Immunoblot**

BMDCs that were cultured with or without ADJ (1%) or LPS (100ng/ml) were washed with cold PBS and lysed in RIPA buffer (CST) containing protease and phosphatase inhibitor cocktail. Total protein levels in each lysate were estimated using Pierce BCA protein assay kit.
(Thermo Scientific; 23227). Samples containing 30–40 ug protein were loaded and resolved on a 12% SDS-PAGE gel (Genscript), transferred to PVDF membrane using iBlot 2 Gel Transfer (Thermo Fisher Scientific; IB24001), blocked with 5% BSA in TBST for phospho-proteins and 5% milk for total proteins in TBST for 1 hour and probed with primary antibodies (at 4C overnight). Primary rabbit antibodies were used as follows at 1:1000 dilution in 5% BSA in TBST: rabbit monoclonal anti-phospho-Akt (Ser473; Clone: D9E), rabbit polyclonal anti-total Akt, rabbit monoclonal anti-phosphor-p70 S6 kinase (Thr389, Clone: 108D2), and rabbit polyclonal anti-p70 S6 kinase antibodies. Blots were extensively washed with TBST and incubated with goat anti-rabbit IgG (H+L)-HRP antibodies (Thermofisher) diluted in 5% non-fat milk in TBST for 1 hour at room temperature. Protein bands were visualized by ECL prime western blotting detection reagent (GE Healthcare.) Membranes were stripped with mild stripping buffer (0.1% SDS, 0.1% Tween 20, 1.5% Glycine), when necessary.

**Discovery lipidomics by LC-MS**

Samples were spun down and snap frozen in liquid N\textsubscript{2} and stored at -80C until extraction. Media samples were thawed on ice, and 50\muL of media was transferred to microcentrifuge tube; cell pellets were directly extracted in tubes. To perform extraction, 187.5\muL of chilled methanol and 750\muL of chilled methyl tert-butyl ether (MTBE) was added to each tube, followed by the addition of 5mm stainless steel beads. All samples were then bead-beaten in a cold room on a Retsch MM400 mixer mill at a frequency of 25 cycles/sec for 5 minutes to complete cell lysis. This mixture was then vortexed for 1–2 s and quickly centrifuged to remove any stray droplets from the tube openings. Then 187.5\muL of chilled water was added to each tube to separate the hydrophobic and hydrophilic compounds into separate phases. All samples were then vortexed for 30 seconds and then centrifuged at 4C for 2 minutes at a speed of 14000 x g to re-pellet any cell debris. A total of 300\muL of the top-layer of the biphasic extraction was removed from the tube and collected into a low volume, borosilicate glass autosampler vial with tapered insert and dried by vacuum concentrator. All samples were reconstituted with 50\muL of a 9:1 MeOH: Toluene solution for injection.

LC-MS analysis was performed on an Acquity CSH C18 column held at 50˚C (100 mm x 2.1 mm x 1.7 \mu m particle size; Waters) using a Vanquish Binary Pump (400\muL/min flow rate; Thermo Scientific). Mobile phase A consisted of 10mM ammonium acetate and 250\muL/L acetic acid in ACN:H2O (70:30, v/v). Mobile phase B consisted of IPA:ACN (90:10, v/v) with the same additives. Initially, mobile phase B was held at 2% for 2 min and then the following gradient was employed: increase to 30% over 3 min, then to 50% over 1 min, then to 85% over 14 min, and finally to 99% over 1 min where %B was held at 99% for 7 min. The column was then re-equilibrated with mobile phase B at 2% for 1.75 min before the next injection. 10\muL of each extract was injected by a Vanquish Split Sampler HT autosampler (Thermo Scientific) in a randomized order. The LC system was coupled to a Q Exactive HF Orbitrap mass spectrometer (MS) through a heated electrospray ionization (HESI II) source (Thermo Scientific). Source conditions were as follows: HESI II and capillary temperature at 350C, sheath gas flow rate at 25 units, aux gas flow rate at 15 units, sweep gas flow rate at 5 units, spray voltage at 3.5 kV, and S-lens RF at 90.0 units. The MS was operated in a polarity switching mode acquiring positive and negative full MS and MS2 spectra (Top2) within the same injection. Acquisition parameters for full MS scans in both modes were: 30,000 resolution, 1 × 10\textsuperscript{6} automatic gain control (AGC) target, 100 ms ion accumulation time (max IT), and 200 to 2000 m/z scan range. Data dependent (dd-MS2) scans in both modes were then collected at 30,000 resolution, 1 × 10\textsuperscript{6} AGC target, 50 ms max IT, 1.0 m/z isolation window, stepped normalized collision energy (NCE) at 20, 30, 40, with a 10.0 s dynamic exclusion. The resulting LC–MS/MS data
were processed using Compound Discoverer 2.1 (Thermo Scientific) and LipiDex (PMID: 29705063), an in-house-developed software suite. All peaks with a 0.2 min to 23 min retention time and 100 Da to 5000 Da MS1 precursor mass were aggregated into distinct chromatographic profiles (i.e., compound groups) using a 10-ppm mass and 0.5 min retention time tolerance. Profiles not reaching a minimum peak intensity of 1x10^6, a maximum peak-width of 0.35, a signal-to-noise (S/N) ratio of 3, and a 3-fold intensity increase over blanks were excluded from further processing. MS/MS spectra were searched against an in-silico generated lipid spectral library containing 35,000 unique molecular compositions representing 48 distinct lipid classes. Spectral matches with a dot product score greater than 500 and a reverse dot product score greater than 700 were retained for further analysis. Lipid MS/MS spectra which contained no significant interference (<75%) from co-eluting isobaric lipids, eluted within a 3.5 median absolute retention time deviation (M.A.D. RT) of each other, and found within at least 2 processed files were then identified at the individual fatty acid substituent level of structural resolution. If individual fatty acid substituents were unresolved, then identifications were made with the sum of the fatty acid substituents. Lipid quantitation was normalized to cell numbers.

**Quantification and statistics**

All experiments are performed and repeated 2–5 times; data are either pooled or at least representative of 2–5 independent experiments. Data are presented as the mean ± SEM. Student’s two-tailed t-test, Mann-Whitney U test, and one-way ANOVA analyses were used to calculate the statistical significance of differences between groups, and significance was defined at p < 0.05. Statistical differences in measured variables between the experimental and control groups were assessed using Student’s t test and p < 0.05 was considered as statistically significant. Stars are p values in the following ranges: 0–0.001 = '***', 0.01–0.05 = '*', 0.05–0.1 = '•'.

**Supporting information**

S1 Fig. Carbomer-based adjuvant enhances DC presentation of OVA SIINFEKL peptide by H-2Kb MHC I molecules. (A) BMDCs were cultured in media containing OVA (1mg/ml) with or without ADJ (1%) for 6 hours. The cell surface expression of the H-2Kb/SIINFEKL complexes was quantified by staining DCs with 25D1.16 antibodies. Plots are gated on live CD11c+ve cells; Data are representative of 2 independent experiments. Error bars are SEM; *P<0.01; **P<0.001; ***P<0.0001 (One-way ANOVA).

S2 Fig. Carbomer-based adjuvant alters antigen uptake and processing in DCs. (A) Kinetics of antigen uptake by ADJ-treated BMDCs. Cells were cultured with 20ug/ml OVA-Alexa Fluor 647 (pH insensitive dye) with or without 1% ADJ for 10 and 30 minutes. (B) Effects of ADJ on intracellular routing of antigens. BMDCs were cultured with 20ug/ml OVA labeled with the pH sensitive dye (pHAB), with or without 1% ADJ for 30 minutes. Data are representative of 4 independent experiments. Error bars are SEM; *P<0.01; **P<0.001; ***P<0.0001 (Student’s t-test).

S3 Fig. Carbomer-based adjuvant promotes intracellular routing of OVA to early endosomes. BMDCs were pulsed with Alexa Fluor 647-OVA (60 μg/ml) and chased at the indicated time-points to assess EEA1 (A) or LAMP1 (B) co-localization. Pearson’s coefficient was calculated from 10 cells/treatment. Data are representative of 3 independent experiments Error bars
are SEM; *P<0.01; **P<0.001; ***P<0.0001 (Student’s t-test).

(TIF)

S4 Fig. Carbomer-based adjuvant induces intracellular H$_2$O$_2$ production in DCs. BMDCs were treated with 1% ADJ for 1 and 3 h with a luminogenic substrate. H$_2$O$_2$ levels were quantified by ROS-Glo detection solution. Data are representative of 3 independent experiments. Error bars are SEM; *P<0.01; **P<0.001; ***P<0.0001 (Student’s t-test).

(TIF)

S5 Fig. Carbomer-based adjuvants do not affect proteasome activity in DCs. BMDCs were cultured with 1% ADJ for 1 or 3 h and incubated with specific luminogenic proteasome substrates Suc-LLVY (A), Z-LRR (B), and Z-nLPnLD (C) for the chymotrypsin-like, trypsin-like and caspase-like activities, respectively. Following cleavage by the proteasome, the substrate for luciferase was released and the luminescence was detected using plate reader. Data are representative of 2 independent experiments. Error bars are SEM; *P<0.01; **P<0.001; ***P<0.0001 (Student’s t-test).

(TIF)

S6 Fig. Carbomer-based adjuvants reduce optical redox ratio in DCs. Optical redox ratio of unstimulated and ADJ-treated dendritic cells was calculated at the indicated time after stimulation. Representative NAD(P)H intensity (first row), FAD intensity (second row), and optical redox ratio (NAD(P)H/(NAD(P)H+FAD); third row) images of unstimulated and ADJ-treated dendritic cells. Scale bar is 10 μm. Box plots show median (central line), first and third quartiles (lower and upper hinges), the farthest data points that are no further than 1.5* the interquartile range (whiskers), and data points beyond 1.5* the interquartile range from the hinge (dots). Stars compare respective boxes to the first time point of each group (n = 22–75 cells/time point). Data are representative of 2–3 independent experiments. Error bars are SEM; *P<0.01; **P<0.001; ***P<0.0001 (Student’s t-test and one-way ANOVA).

(TIF)

S7 Fig. A schematic illustration demonstrating ADJ-mediated DC cross-presentation of antigens to CD8 T cells.

(TIF)

Acknowledgments

We would like to thank all members of Suresh Laboratory for constructive feedbacks and technical assistance. Special thanks to Drs. Natalie Niemi and David Pagliarini for technical assistance for the use of Seahorse Bioanalyzer. Thanks to Dr. Sathish Kumar for the use of SpectraMax i3x plate reader. We appreciate help by Drs. Gregory Wipez and Gopal Iyer for Bioplex assay and immunofluorescence; Thanks to Zachary Morrow for Listeria challenge experiment. We are thankful to the Emory NIH Tetramer Core Facility for providing MHC-I tetramers. We also wish to acknowledge sincere appreciation for the efforts of the veterinary and animal care staff at UW-Madison.

Author Contributions

Conceptualization: Woojong Lee, M. Suresh.

Data curation: Woojong Lee, Brock Kingstad-Bakke, Brett Paulson, Autumn Larsen, Katherine Overmyer, Chandranaik B. Marinaik, Kelly Dulli, Randall Toy, Gabriela Vogel, Katherine P. Mueller, Kelsey Tweed, Alex J. Walsh, Jason Russell.
Formal analysis: Woojong Lee, Brock Kingstad-Bakke, Brett Paulson, Autumn Larsen, Katherine Overmyer, Chandranaik B. Marinaik, Kelly Dulli, Randall Toy, Gabriela Vogel, Katherine P. Mueller, Kelsey Tweed, Alex J. Walsh, Jason Russell, Leticia Reyes, Melissa C. Skala.

Funding acquisition: M. Suresh.
Investigation: M. Suresh.
Methodology: Woojong Lee.
Project administration: M. Suresh.
Resources: John-Demian Sauer, Dmitry M. Shayakhmetov, M. Suresh.
Supervision: Krishanu Saha, Leticia Reyes, Melissa C. Skala, Joshua Coon, Krishnendu Roy, M. Suresh.
Writing – original draft: Woojong Lee, M. Suresh.
Writing – review & editing: Woojong Lee, Brock Kingstad-Bakke, M. Suresh.

References
1. Pulendran B, Ahmed R. Immunological mechanisms of vaccination. Nat Immunol. 2011; 12(6):509–17. Epub 2011/07/09. https://doi.org/10.1038/ni.2039 PMID: 21739679; PubMed Central PMCID: PMC3253344.
2. Burton DR. Antibodies, viruses and vaccines. Nat Rev Immunol. 2002; 2(9):706–13. Epub 2002/09/05. https://doi.org/10.1038/nri891 PMID: 12209139.
3. Hofy DF. Tuberculosis vaccine development: goals, immunological design, and evaluation. Lancet. 2008; 372(9633):164–75. Epub 2008/07/16. https://doi.org/10.1016/S0140-6736(08)61036-3 PMID: 18629582.
4. Reyes-Sandoval A, Pearson FE, Todryk S, Ewer K. Potency assays for novel T-cell-inducing vaccines against malaria. Curr Opin Mol Ther. 2009; 11(1):72–80. Epub 2009/01/27. PMID: 19169962.
5. Walker BD, Ahmed R, Plotkin S. Moving ahead an HIV vaccine: use both arms to beat HIV. Nat Med. 2011; 17(10):1194–5. Epub 2011/10/13. https://doi.org/10.1038/nm.2529 PMID: 21988996.
6. Kitchener S. Viscerotropic and neurotropic disease following vaccination with the 17D yellow fever vaccine, ARILVAX. Vaccine. 2004; 22(17–18):2103–5. Epub 2004/05/20. https://doi.org/10.1016/j.vaccine.2004.01.026 PMID: 15149765.
7. Lindsey NP, Rabe IB, Miller ER, Fischer M, Staples JE. Adverse event reports following yellow fever vaccination, 2007–13. J Travel Med. 2016; 23(5). Epub 2016/07/06. https://doi.org/10.1093/jtm/taw045 PMID: 27378369.
8. Struchiner CJ, Luz PM, Dourado I, Sato HK, Aguiar SG, Ribeiro JG, et al. Risk of fatal adverse events associated with 17DD yellow fever vaccine. Epidemiol Infect. 2004; 132(5):939–46. Epub 2004/10/12. https://doi.org/10.1017/s0950268804002602 PMID: 15473158; PubMed Central PMCID: PMC2870182.
9. Brito LA, O’Hagan DT. Designing and building the next generation of improved vaccine adjuvants. J Control Release. 2014; 190:563–79. Epub 2014/07/08. https://doi.org/10.1016/j.jconrel.2014.06.027 PMID: 24998942.
10. Bevan MJ. Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. J Exp Med. 1976; 143(5):1283–8. Epub 1976/05/01. https://doi.org/10.1084/jem.143.5.1283 PMID: 1083422; PubMed Central PMCID: PMC219184.
11. Huang AY, Golumbek P, Ahmadzadeh M, Jaffee E, Pardoll D, Levitsky H. Role of bone marrow-derived cells in presenting MHC class I-restricted tumor antigens. Science. 1994; 264(5161):961–5. Epub 1994/05/13. https://doi.org/10.1126/science.7513904 PMID: 7513904.
12. Wolkers MC, Stoetetter G, Vyth-Dreese FA, Schumacher TN. Redundancy of direct priming and cross-priming in tumor-specific CD8+ T cell responses. J Immunol. 2001; 167(7):3577–84. Epub 2001/09/21. https://doi.org/10.4049/jimmunol.167.7.3577 PMID: 11564769.
13. Belz GT, Shortman K, Bevan MJ, Heath WR. CD8 alpha+ dendritic cells selectively present MHC class I-restricted noncytolytic viral and intracellular bacterial antigens in vivo. J Immunol. 2005; 175(1):196–
Carbomer-based adjuvant induces systemic CD8 T-cell immunity by enhancing DC cross-presentation

200. Epub 2005/06/24. https://doi.org/10.4049/jimmunol.175.1.196 PMID: 15972648; PubMed Central PMCID: PMC2778481.

14. Grotzke JE, Sengupta D, Lu Q, Cresswell P. The ongoing saga of the mechanism(s) of MHC class I-restricted cross-presentation. Curr Opin Immunol. 2017; 46:89–96. Epub 2017/05/22. https://doi.org/10.1016/j.coi.2017.03.015 PMID: 28528219; PubMed Central PMCID: PMC5554740.

15. Shen L, Sigal LJ, Boes M, Rock KL. Important role of cathepsin S in generating peptides for TAP-independent MHC class I crosspresentation in vivo. Immunology. 2004; 21(2):155–65. Epub 2004/08/17. https://doi.org/10.1046/j.immunol.2004.07.004 PMID: 15308097.

16. Kovacsics-Bankowski M, Rock KL. A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. Science. 1995; 267(5195):243–6. Epub 1995/01/13. https://doi.org/10.1126/science.7809629 PMID: 7809629.

17. Song R, Harding CV. Roles of proteasomes, transporter for antigen presentation (TAP), and beta 2-microglobulin in the processing of bacterial or particulate antigens via an alternate class I MHC processing pathway. J Immunol. 1996; 156(11):4182–90. Epub 1996/06/01. PMID: 8666786.

18. Pearce EJ, Everts B. Dendritic cell metabolism. Nat Rev Immunol. 2015; 15(1):18–29. Epub 2014/12/24. https://doi.org/10.1038/nr1371 PMID: 25534620; PubMed Central PMCID: PMC495583.

19. Wculek SK, Khoulil SC, Priego E, Heras-Murillo I, Sancho D. Metabolic Control of Dendritic Cell Functions: Digesting Information. Front Immunol. 2019; 10:775. Epub 2019/05/11. https://doi.org/10.3389/fimmu.2019.00775 PMID: 31073300; PubMed Central PMCID: PMC6496459.

20. Del Giudice G, Rappuoli R, Didierlaurent AM. Correlates of adjuvanticity: A review on adjuvants in licensed vaccines. Semin Immunol. 2018; 39:14–21. Epub 2018/05/09. https://doi.org/10.1016/j.smim.2018.05.001 PMID: 29801750.

21. Menon V, Priya RS, Labranche C, Montefiori D, Mahalingam S, Kalyanaraman VS, et al. Characterization of protective immune response elicited by a trimeric envelope protein from an Indian clade C HIV-1 isolate in rhesus macaques. J Med Primatol. 2015; 44(5):275–85. Epub 2015/06/16. https://doi.org/10.1111/jmp.12178 PMID: 26075700.

22. Gupta PK, Mukherjee P, Dhwani S, Pandey AK, Mazumdar S, Gaur D, et al. Production and preclinical evaluation of Plasmodium falciparum MSP-119 and MSP-311 chimeric protein, fMSP-Fu24. Clin Vaccine Immunol. 2014; 21(6):886–97. Epub 2014/05/03. https://doi.org/10.1128/CVI.00179-14 PMID: 24789797; PubMed Central PMCID: PMC4054244.

23. Anlar S, Capan Y, Hincal AA. Physico-chemical and bioadhesive properties of polyacrylic acid polymers. Pharmazie. 1993; 48(4):285–7. Epub 1993/04/01. PMID: 8321879.

24. Gasper DJ, Neidler B, Plisch EH, Rustom H, Carrow E, Imai H, et al. Effective Respiratory CD8 T-Cell Immunity against Influenza Virus Induced by Intranasal Carbomer-Lecithin-Adjuvanted Non-replicating Vaccines. PLoS Pathog. 2016; 12(12):e1006064. Epub 2016/12/21. https://doi.org/10.1371/journal.ppat.1006064 PMID: 27997610; PubMed Central PMCID: PMC5173246 all PLOS Pathogens policies on sharing data and materials.

25. Wegmann F, Moghaddam AE, Schiffler T, Gartlan KH, Powell TJ, Russell RA, et al. The Carbomer-Lecithin Adjuvant Adjuplex Has Potent Immunoactivating Properties and Elicits Protective Adaptive Immunity against Influenza Virus Challenge in Mice. Clin Vaccine Immunol. 2015; 22(9):1004–12. Epub 2015/07/03. https://doi.org/10.1128/CVI.00736-14 PMID: 26135973; PubMed Central PMCID: PMC4550664.

26. Roberts AD, Ordway DJ, Orme IM. Listeria monocytogenes infection in beta 2 microglobulin-deficient mice. Infect Immuno. 1993; 61(3):1113–6. Epub 1993/03/01. https://doi.org/10.1128/IAI.61.3.1113-1116.1993 PMID: 8433263; PubMed Central PMCID: PMC302846.

27. Ladel CH, Fleisch IE, Arnold J, Kaufmann SH. Studies with MHC-deficient knock-out mice reveal impact of both MHC-I- and MHC-II-dependent T cell responses on Listeria monocytogenes infection. J Immunol. 1994; 153(7):3116–22. Epub 1994/10/01. PMID: 7726898.

28. Harty JT, Tvinneim AR, White DW. CD8+ T cell effector mechanisms in resistance to infection. Annu Rev Immunol. 2000; 18:275–308. Epub 2000/06/03. https://doi.org/10.1146/annurev.immunol.18.1.275 PMID: 10837080.

29. McCabe BJ, Irvine KR, Neldner B, Plisch EH, Rustom H, Carrow E, Imai H, et al. Minimal determinant expressed by a recombinant vaccinia virus elicits therapeutic antitumor cytolytic T lymphocyte responses. Cancer Res. 1995; 55(8):1741–7. Epub 1995/04/15. PMID: 7536130; PubMed Central PMCID: PMC2248453.

30. Shen H, Miller JF, Fan X, Kolwycz D, Ahmed R, Harty JT. Compartmentalization of bacterial antigens: differential effects on priming of CD8 T cells and protective immunity. Cell. 1998; 92(4):535–45. Epub 1998/03/10. https://doi.org/10.1016/s0092-8674(00)80946-0 PMID: 9491894.

31. Theisen E, McDougal CE, Nakanishi M, Stevenson DM, Amador-Noguez D, Rosenberg DW, et al. Cyclooxygenase-1 and -2 Play Contrasting Roles in Listeria-Stimulated Immunity. J Immunol. 2018;
Carbomer-based adjuvant induces systemic CD8 T-cell immunity by enhancing DC cross-presentation

200(11):3729–38. Epub 2018/04/22. https://doi.org/10.4049/jimmunol.1700701 PMID: 29678951; PubMed Central PMCID: PMC5964023.

32. Archer KA, Durack J, Portnoy DA. STING-dependent type I IFN production inhibits cell-mediated immunity to Listeria monocytogenes. PLoS Pathog. 2014; 10(1):e1003861. Epub 2014/01/07. https://doi.org/10.1371/journal.ppat.1003861 PMID: 24391507; PubMed Central PMCID: PMC3879373.

33. Karttunen J, Sanderson S, Shastri N. Detection of rare antigen-presenting cells by the lacZ T-cell activation assay suggests an expression cloning strategy for T-cell antigens. Proc Natl Acad Sci U S A. 1992; 89(13):6020–4. Epub 1992/07/01. https://doi.org/10.1073/pnas.89.13.6020 PMID: 1378619; PubMed Central PMCID: PMC402130.

34. Sokolovska A, Becker CE, Ip WK, Rathinam VA, Brudner M, Paquette N, et al. Activation of caspase-1 by the NLRP3 inflammasome regulates the NADPH oxidase NOX2 to control phagosome function. Nat Immunol. 2013; 14(6):543–53. Epub 2013/05/07. https://doi.org/10.1038/ni.2595 PMID: 23644505; PubMed Central PMCID: PMC3708594.

35. Li T, Zehner M, He J, Prochnicki T, Horvath G, Latz E, et al. NLRP3 inflammasome-activating arginine-based liposomes promote antigen presentations in dendritic cells. Int J Nanomedicine. 2019; 14:3503–16. Epub 2019/06/14. https://doi.org/10.2147/IJN.S203739 PMID: 31190807; PubMed Central PMCID: PMC6526778.

36. Tirapu I, Giquel B, Alexopoulos L, Uematsu S, Flavell R, Akira S, et al. PolyI:C-induced reduction in uptake of soluble antigen is independent of dendritic cell activation. Int Immunol. 2009; 21(7):871–9. Epub 2009/06/10. https://doi.org/10.1093/infoimm/npn053 PMID: 19505890; PubMed Central PMCID: PMC2694940.

37. Burgdorf S, Kautz A, Bohnert V, Knolle PA, Kurts C. Distinct pathways of antigen uptake and intracellular routing in CD4 and CD8 T cell activation. Science. 2007; 316(5824):612–6. Epub 2007/04/28. https://doi.org/10.1126/science.1137971 PMID: 17442311.

38. Xie X, Mai J, Xu R, Perez JET, Guevara ML, Shen Q, et al. Porous silicon microparticle potentiate anti-Listeria monocytogenes immunity. PLoS Pathog. 2014; 10(1):e1003861. Epub 2014/01/07. https://doi.org/10.1371/journal.ppat.1003861 PMID: 24391507; PubMed Central PMCID: PMC3879373.

39. Sokolovska A, Becker CE, Ip WK, Rathinam VA, Brudner M, Paquette N, et al. Activation of caspase-1 by the NLRP3 inflammasome regulates the NADPH oxidase NOX2 to control phagosome function. Nat Immunol. 2013; 14(6):543–53. Epub 2013/05/07. https://doi.org/10.1038/ni.2595 PMID: 23644505; PubMed Central PMCID: PMC3708594.

40. Adler J, Parmryd I. Quantifying colocalization by correlation: the Pearson correlation coefficient is superior to the Mander's overlap coefficient. Cytometry A. 2010; 77(8):733–42. Epub 2010/07/24. https://doi.org/10.1002/cyto.a.20896 PMID: 20653013.

41. Savina A, Jancic C, Hugues S, Guermonprez P, Vargas P, Moura IC, et al. NOX2 controls phagosomal pH to regulate antigen processing during crosspresentation by dendritic cells. Cell. 2006; 126(1):205–18. Epub 2006/07/15. https://doi.org/10.1016/j.cell.2006.05.035 PMID: 16839887.

42. Cebrían I, Visentin G, Blanchard N, Jouve M, Bobard A, Moita C, et al. Sec22b regulates phagosomal maturation and antigen crosspresentation by dendritic cells. Cell. 2011; 147(6):1355–68. Epub 2011/12/14. https://doi.org/10.1016/j.cell.2011.11.021 PMID: 22153078.

43. Van Kaer L, Ashton-Rickardt PG, Ploegh HL, Tonegawa S. TAP1 mutant mice are deficient in antigen presentation, surface class I molecules, and CD4+ T cells. Cell. 1992; 71(7):1205–14. Epub 1992/12/14. https://doi.org/10.1016/0092-8674(92)90191-Y PMID: 1379711.

44. Lin ML, Zhan Y, Prioletti A, Prato S, Wu L, Heath WR, et al. Selective suicide of cross-presenting CD8+ dendritic cell populations by cytochrome c injection shows functional heterogeneity within this subset. Proc Natl Acad Sci U S A. 2008; 105(8):3029–34. Epub 2008/02/15. https://doi.org/10.1073/pnas.0712394105 PMID: 18272486; PubMed Central PMCID: PMC2268579.

45. Dingjan I, Verboogen DR, Paardekooper LM, Revelo NH, Sittig SP, Visser LJ, et al. Lipid peroxidation causes endosomal antigen release for cross-presentation. Sci Rep. 2016; 6:22064. Epub 2016/02/26. https://doi.org/10.1038/srep22064 PMID: 26907999; PubMed Central PMCID: PMC4764948.

46. Girotti AW. Lipid hydroperoxide generation, turnover, and effector action in biological systems. J Lipid Res. 1998; 39(8):1529–42. Epub 1998/08/26. PMID: 9717713.

47. Krawczyk CM, Holowka T, Sun J, Blagih J, Amiel E, DeBerardinis RJ, et al. Toll-like receptor-induced changes in glycolytic metabolism regulate dendritic cell activation. Blood. 2010; 115(23):4742–9. Epub 2010/03/31. https://doi.org/10.1182/blood-2009-10-249540 PMID: 20351312; PubMed Central PMCID: PMC2890190.

48. Gotoh K, Morisaki T, Setoyama D, Sasaki K, Yagi M, Iigami K, et al. Mitochondrial p32/C1qbp Is a Critical Regulator of Dendritic Cell Metabolism and Maturation. Cell Rep. 2018; 25(7):1800–15 e4. Epub 2018/11/15. https://doi.org/10.1016/j.celrep.2018.10.057 PMID: 30428349.

49. Zuo H, Wan Y. Metabolic Reprogramming in Mitochondria of Myeloid Cells. Cells. 2019; 8(1). Epub 2019/12/22. https://doi.org/10.3390/cells8010005 PMID: 31861356; PubMed Central PMCID: PMC7017304.
49. Everts B, Amiel E, Huang SC, Smith AM, Chang CH, Lam WY, et al. TLR-driven early glycolytic reprogramming via the kinases TBK1-IKKvarepsilon supports the anabolic demands of dendritic cell activation. Nat Immunol. 2014; 15(4):323–32. Epub 2014/02/25. https://doi.org/10.1038/ni.2833 PMID: 24562310; PubMed Central PMCID: PMC4358322.

50. Guak H, Al Habyan S, Ma EH, Aldossary H, Al-Masri M, Won SY, et al. Glycolytic metabolism is essential for CCR7 oligomerization and dendritic cell migration. Nat Commun. 2018; 9(1):2463. Epub 2018/06/27. https://doi.org/10.1038/s41467-018-04806-6 PMID: 29941886; PubMed Central PMCID: PMC6018630.

51. Everts B, Amiel E, van der Windt GJ, Freitas TC, Chott R, Yarasheski KE, et al. Commitment to glycolysis sustains survival of NO-producing inflammatory dendritic cells. Blood. 2012; 120(7):1422–31. Epub 2012/07/13. https://doi.org/10.1182/blood-2012-03-419747 PMID: 22786679; PubMed Central PMCID: PMC3423780.

52. Blanco-Perez F, Goretzki A, Wolfheimer S, Schulke S. The vaccine adjuvant MPLA activates glycolytic metabolism in mouse mDC by a JNK-dependent activation of mTOR-signaling. Mol Immunol. 2019; 106:159–68. Epub 2019/01/10. https://doi.org/10.1016/j.molimm.2018.12.029 PMID: 30623816.

53. Jantsch J, Chakravortty D, Turza N, Prechtel AT, Buchholz B, Gerlach RG, et al. Hypoxia and hypoxia-inducible factor-1 alpha modulate lipopolysaccharide-induced dendritic cell activation and function. J Immunol. 2008; 180(7):4697–705. Epub 2008/03/21. https://doi.org/10.4049/jimmunol.180.7.4697 PMID: 18351493.

54. Wilson NS, Duewell P, Yang B, Li Y, Marsters S, Koernig S, et al. Inflammasome-dependent and -independent IL-18 production mediates immunity to the ISCOMATRIX adjuvant. J Immunol. 2014; 192(7):3259–68. Epub 2014/03/13. https://doi.org/10.4049/jimmunol.1302011 PMID: 24610009.

55. Safran M, Kim WY, O’Connell F, Flippin L, Gunzler V, Horner JW, et al. Mouse model for noninvasive determination of S1pr1 is required for the establishment of resident memory CD8+ T cells. Nat Immunol. 2013; 14(12):1285–93. Epub 2013/10/29. https://doi.org/10.1038/ni.2745 PMID: 24162775; PubMed Central PMCID: PMC3844557.

56. Everts B, Amiel E, Huang SC, Smith AM, Chang CH, Lam WY, et al. TLR-driven early glycolytic reprogramming via the kinases TBK1-IKKvarepsilon supports the anabolic demands of dendritic cell activation. Nat Immunol. 2014; 15(4):323–32. Epub 2014/02/25. https://doi.org/10.1038/ni.2833 PMID: 24562310; PubMed Central PMCID: PMC4358322.

57. Jantsch J, Chakravortty D, Turza N, Prechtel AT, Buchholz B, Gerlach RG, et al. Hypoxia and hypoxia-inducible factor-1 alpha modulate lipopolysaccharide-induced dendritic cell activation and function. J Immunol. 2008; 180(7):4697–705. Epub 2008/03/21. https://doi.org/10.4049/jimmunol.180.7.4697 PMID: 18351493.

58. Wilson NS, Duewell P, Yang B, Li Y, Marsters S, Koernig S, et al. Inflammasome-dependent and -independent IL-18 production mediates immunity to the ISCOMATRIX adjuvant. J Immunol. 2014; 192(7):3259–68. Epub 2014/03/13. https://doi.org/10.4049/jimmunol.1302011 PMID: 24610009.

59. Safran M, Kim WY, O’Connell F, Flippin L, Gunzler V, Horner JW, et al. Mouse model for noninvasive determination of S1pr1 is required for the establishment of resident memory CD8+ T cells. Nat Immunol. 2013; 14(12):1285–93. Epub 2013/10/29. https://doi.org/10.1038/ni.2745 PMID: 24162775; PubMed Central PMCID: PMC3844557.

60. Bouguerers L, Helft J, Tiwari S, Vargas P, Chang BH, Chan L, et al. A role for lipid bodies in the cross-presentation of phagocytosed antigens by MHC class I in dendritic cells. Immunity. 2009; 31(2):232–44. Epub 2009/08/25. https://doi.org/10.1016/j.immuni.2009.06.022 PMID: 19699172; PubMed Central PMCID: PMC2803012.

61. den Brok MH, Bull C, Wassink M, de Graaf AM, Wagenaars JA, Minderman M, et al. Saponin-based adjuvants induce cross-presentation in dendritic cells by intracellular lipid body formation. Nat Commun. 2016; 7:13324. Epub 2016/11/08. https://doi.org/10.1038/ncomms13324 PMID: 27819292; PubMed Central PMCID: PMC5103066.

62. Walsh AJ, Cook RS, Manning HC, Hicks DJ, Lafontant A, Arteaga CL, et al. Optical metabolic imaging identifies glycolytic levels, subtypes, and early-treatment response in breast cancer. Cancer Res. 2013; 73(20):6164–74. Epub 2013/10/17. https://doi.org/10.1158/0008-5472.CAN-13-0527 PMID: 24130112; PubMed Central PMCID: PMC3801432.

63. Veglia F, Tyurin VA, Mohammadyani D, Blasi M, Dupperet EK, DontiNreddy L, et al. Lipid bodies containing oxidatively truncated lipids block antigen cross-presentation by dendritic cells in cancer. Nat Commun. 2017; 8(1):2122. Epub 2017/12/16. https://doi.org/10.1038/s41467-017-02186-9 PMID: 29242555; PubMed Central PMCID: PMC5730553.

64. Herber DL, Cao W, Nefedova Y, Novitsky SV, Nagaraj S, Tyurin VA, et al. Lipid accumulation and dendritic cell dysfunction in cancer. Nat Med. 2010; 16(8):880–6. Epub 2010/07/14. https://doi.org/10.1038/ nm.2172 PMID: 20622859; PubMed Central PMCID: PMC2917488.
65. Ramakrishnan R, Tyurin VA, Veglia F, Condamine T, Amoscao A, Mohammadyani D, et al. Oxidized lipids block antigen cross-presentation by dendritic cells in cancer. J Immunol. 2014; 192(6):2920–31. Epub 2014/02/21. https://doi.org/10.4049/jimmunol.1302801 PMID: 24554775; PubMed Central PMCID: PMC3998104.

66. Lee JY, Zhao L, Youn HS, Weatherill AR, Tapping R, Feng L, et al. Saturated fatty acid activates but polyunsaturated fatty acid inhibits Toll-like receptor 2 dimerized with Toll-like receptor 6 or 1. J Biol Chem. 2004; 279(17):16971–9. Epub 2004/02/18. https://doi.org/10.1074/jbc.M312990200 PMID: 14966134.

67. Kato Y, Kajiwara C, Ishige I, Mizukami S, Yamazaki C, Eikawa S, et al. HSP70 and HSP90 Differentially Regulate Translocation of Extracellular Antigen to the Cytosol for Cross-Presentation. Autoimmun Dis. 2012; 2012:745962. Epub 2012/10/11. https://doi.org/10.1155/2012/745962 PMID: 23050124; PubMed Central PMCID: PMC3462380.

68. Singh R, Cresswell P. Defective cross-presentation of viral antigens in GILT-free mice. Science. 2010; 328(5984):1394–8. Epub 2010/06/12. https://doi.org/10.1126/science.1189176 PMID: 20538950; PubMed Central PMCID: PMC2925227.

69. Imai J, Hasegawa H, Maruya M, Koyasu S, Yahara I. Exogenous antigens are processed through the endoplasmic reticulum-associated degradation (ERAD) in cross-presentation by dendritic cells. Int Immunol. 2005; 17(1):45–53. Epub 2004/11/18. https://doi.org/10.1093/intimm/dxh184 PMID: 15546887.

70. Thwe PM, Pelgrom LR, Cooper R, Beauchamp S, Reisz JA, D’Alessandro A, et al. Cell-Intrinsic Glycogen Metabolism Supports Early Glycolytic Reprogramming Required for Dendritic Cell Immune Responses. Cell Metab. 2017; 26(3):558–67 e5. Epub 2017/09/07. https://doi.org/10.1016/j.ccmet.2017.08.012 PMID: 28877459; PubMed Central PMCID: PMC5657596.

71. Lawless SJ, Kedia-Mehta N, Walls JF, McGarrigle R, Convery O, Sinclair LV, et al. Glucose represses dendritic cell-induced T cell responses. Nat Commun. 2017; 8:15620. Epub 2017/05/31. https://doi.org/10.1038/ncomms15620 PMID: 28555668; PubMed Central PMCID: PMC5459989.

72. Trombetta ES, Ebersold M, Garrett W, Pypaert M, Mellman I. Activation of lysosomal function during dendritic cell maturation. Science. 2003; 299(5611):1400–3. Epub 2003/03/01. https://doi.org/10.1126/science.1080106 PMID: 12610307.

73. Kanneganti TD. Central roles of NLRs and inflammasomes in viral infection. Nat Rev Immunol. 2010; 10(10):688–98. Epub 2010/09/18. https://doi.org/10.1038/nri2851 PMID: 20847744; PubMed Central PMCID: PMC3909537.

74. Netea MG, Simon A, van de Veerdonk F, Kullberg BJ, Van der Meer JW, Joosten LA. IL-1beta processing in host defense: beyond the inflammasomes. PLoS Pathog. 2010; 6(2):e1000661. Epub 2010/03/03. https://doi.org/10.1371/journal.ppat.1000661 PMID: 20195505; PubMed Central PMCID: PMC2859053.

75. Hornung V, Bauermeister F, Halle A, Samstad EO, Kono H, Rock KL, et al. Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. Nat Immunol. 2008; 9(8):847–56. Epub 2008/07/08. https://doi.org/10.1038/ni.1631 PMID: 18604214; PubMed Central PMCID: PMC2834784.

76. Shaikh SR, Edidin M. Polyunsaturated fatty acids and membrane organization: elucidating mechanisms to balance immunotherapy and susceptibility to infection. Chem Phys Lipids. 2008; 153(1):24–33. Epub 2008/03/19. https://doi.org/10.1016/j.chemphyslip.2008.02.008 PMID: 18346461; PubMed Central PMCID: PMC2442228.
81. Shaikh SR, Edidin M. Polyunsaturated fatty acids, membrane organization, T cells, and antigen presentation. Am J Clin Nutr. 2006; 84(6):1277–89. Epub 2006/12/13. https://doi.org/10.1093/ajcn/84.6.1277 PMID: 17158407.

82. Rao Malla R, Raghu H, Rao JS. Regulation of NADPH oxidase (Nox2) by lipid rafts in breast carcinoma cells. Int J Oncol. 2010; 37(6):1483–93. Epub 2010/11/03. https://doi.org/10.3892/ijo_00000801 PMID: 21042717.

83. Na YR, Jung D, Gu GJ, Seok SH. GM-CSF Grown Bone Marrow Derived Cells Are Composed of Phenotypically Different Dendritic Cells and Macrophages. Mol Cells. 2016; 39(10):734–41. Epub 2016/10/30. https://doi.org/10.14348/molcells.2016.0160 PMID: 27788572; PubMed Central PMCID: PMC5104881.

84. Jin D, Sprent J. GM-CSF Culture Revisited: Preparation of Bulk Populations of Highly Pure Dendritic Cells from Mouse Bone Marrow. J Immunol. 2018; 201(10):3129–39. Epub 2018/10/17. https://doi.org/10.4049/jimmunol.1800031 PMID: 30322963.

85. Ghosh M, Shapiro LH. In vitro Ag Cross-presentation and in vivo Ag Cross-presentation by Dendritic Cells in the Mouse. Bio Protoc. 2012; 2(24):e305. Epub 2012/12/20. https://doi.org/10.21769/bioprotoc.305 PMID: 27030824; PubMed Central PMCID: PMC4809020.

86. Kar S, Colino J, Snapper CM. Distinct Cellular Pathways for Induction of CD4+ T Cell-Dependent Antibody Responses to Antigen Expressed by Intact Bacteria Versus Isolated Soluble Antigen. J Immunol. 2016; 196(10):4204–13. Epub 2016/04/10. https://doi.org/10.4049/jimmunol.1502550 PMID: 27059586.

87. Nath N, Godat B, Zimprich C, Dwight SJ, Corona C, McDougall M, et al. Homogeneous plate based antibody internalization assay using pH sensor fluorescent dye. J Immunol Methods. 2016; 431:11–21. Epub 2016/02/07. https://doi.org/10.1016/j.jim.2016.02.001 PMID: 26951520.

88. Moravec RA, O'Brien MA, Daily WJ, Scurria MA, Bernad L, Riss TL. Cell-based bioluminescent assays for all three proteasome activities in a homogeneous format. Anal Biochem. 2009; 387(2):294–302. Epub 2009/05/21. https://doi.org/10.1016/j.ab.2009.01.016 PMID: 19454251.

89. Pelgrom LR, van der Ham AJ, Everts B. Analysis of TLR-Induced Metabolic Changes in Dendritic Cells Using the Seahorse XF(e)96 Extracellular Flux Analyzer. Methods Mol Biol. 2016; 1390:273–85. Epub 2016/01/25. https://doi.org/10.1007/978-1-4939-3335-8_17 PMID: 26803635.