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Inflammasome-Dependent and -Independent IL-18 Production Mediates Immunity to the ISCOMATRIX Adjuvant

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Adjuvants are an essential component of modern vaccines and used for their ability to elicit immunity to coadministered Ags. Many adjuvants in clinical development are particulates, but how they drive innate and adaptive immune responses remains poorly understood. Studies have shown that a number of vaccine adjuvants activate inflammasome pathways in isolated APCs. However, the contribution of inflammasome activation to vaccine-mediated immunity in vivo remains controversial. In this study, we evaluated immune cell responses to the ISCOMATRIX adjuvant (IMX) in mice. Like other particulate vaccine adjuvants, IMX potently activated the NALP3–ASC–Caspase-1 inflammasome in APCs, leading to IL-1β and IL-18 production. The IL-18R pathway, but not IL-1R, was required for early innate and subsequent cellular immune responses to a model IMX vaccine. APCs directly exposed to IMX underwent an endosome-mediated cell-death response, which we propose initiates inflammatory events locally at the injection site. Importantly, both inflammasome-related and -unrelated pathways contributed to IL-18 dependence in vivo following IMX administration. TNF-α provided a physiological priming signal for inflammasome-dependent IL-18 production by APCs, which correlated with reduced vaccine-mediated immune cell responses in TNF-α- or TNFR-deficient mice. Taken together, our findings highlight an important disconnect between the mechanisms of vaccine adjuvant action in vitro versus in vivo. The Journal of Immunology, 2014, 192: 3259–3268.

Vaccines are typically comprised of a purified Ag that is formulated with an adjuvant to enhance its immunogenicity (1). Vaccine adjuvants activate complex signaling networks in a variety of immune cell populations, with select apical signaling events required to develop Ag-specific adaptive immunity. The extensive cross talk between innate and adaptive immune cells further complicates defining essential proinflammatory networks in a variety of immune cell populations, with select apical signaling events required to develop Ag-specific adaptive immunity. The extensive cross talk between innate and adaptive immune cells further complicates defining essential proinflammatory networks in a variety of immune cell populations, with select apical signaling events required to develop Ag-specific adaptive immunity.

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studies have demonstrated the ability of IMX to enhance MHC class I cross-presentation and produce long-lasting Ab and T cell immune responses (20–24). The dependence on the adapter protein MyD88 for cellular immunity to an IMX vaccine remains unresolved (24). Similarly, MyD88 has been implicated in the mechanism of MF59 adjuvanticity in mice (25, 26). MF59 is a classical oil-in-water emulsion that consists of squalene oil, citrate buffer, and two surfactants, with individual components having no immunostimulatory properties. IMX also does not mediate direct immune cell activation in vitro and contains no known TLR agonists, with TLR4- or Toll/IL-1R domain-containing adapter-inducing IFN-β-deficient mice showing normal Ag-specific CD8+ T cell immunity to a model IMX vaccine (24). In this study, we explored the role of MyD88 in IMX vaccine–mediated immunity in mice. In particular, we sought to address if the observed MyD88 dependence reflected its involvement in nucleating the IL-1 or IL-18 pathways and, if so, whether this could be linked to involvement of an inflammasome complex.

Materials and Methods

Mice

Mice used in this study were typically 6–10 wk old. IL-1R−, IL-18R−, IL-1-18-deficient, and TNF-α−/− mice on a C57BL/6 background were obtained from The Jackson Laboratory and maintained in specific pathogen-free conditions at Genentech or CSL Limited. NLRP3−/− ASC−/−, IFP−/− caspase-1/11−/−, TNFR1−/−, TNFR2−/−, TNF-α−/−, and TNFR1/2−/− mice on a C57BL/6 background were bred and maintained at specific pathogen-free conditions at Genentech Additional NALP3−/−/− animals were provided by the University of Lausanne and Institute of Arthritis Research (Lausanne, Switzerland) and maintained at CSL Limited and Ludwig-Maximilians-Universität Munich. All experiments were subject to approval by the institutional ethics committees at Genentech (South San Francisco, CA), CSL Limited (Parkville, VIC, Australia), or Ludwig-Maximilians-Universität (Munich, Germany). All animal work was undertaken in accordance with institutional and national guidelines and conformed to the provisions of the Declaration of Helsinki (as revised in Edinburgh in 2000).

Immunization protocol

IMX was provided by CSL Limited and prepared as described previously (27). The vaccine ag used was an endotoxin-low preparation of OVA (0.007 EU mg−1) generated at Genentech from chicken egg white (specific pathogen-free eggs; Charles River Laboratories), as previously described (24), or low-endotoxin OVA purchased from Hyglos. All prime or boost immunizations were administered s.c. into the scruff of the neck in 100 μl PBS, unless otherwise stated. Each vaccine dose contained 5 μg IMX and 30 μg OVA (15–25 μg/mg). Anti-OVA, Anti-CD8 (clone 53–6.7), and IgG2a) were determined by ELISA in individual samples, as previously described (24). IMX is a registered trademark of ISCOTEC, a CSL Limited company.

Reagents

Ultra-pure LPS (200 ng/ml), poly(deoxyadenylic-deoxythymidylic) acid (dATD) (200 ng/ml), ATP (5 mM), N-acetylcysteine (NAC) (10 mM), and acridine orange were from Sigma-Aldrich or InvivoGen. Alexa Fluor 647–conjugated cholera toxin subunit B and Alexa Fluor 546 Dextran were from Life Technologies. rTNF-α (100 ng/ml) was purchased from PeproTech. Bafilomycin A1 (BafA1; 50 nM), chloroquine (4 μM), Ca-074-Me (20 μM), and Z-FF-FMK (20 μM) were purchased from EMD Chemicals or Sigma-Aldrich. L-leucyl-L-leucine methyl ester (Leu-Leu-OMe) (1 mM) was purchased from Chem-Impex. Silica crystalline particles were ~15 μm and generated in sterile PBS (US Silica). Alum (Invivogen) was purchased from Invivogen and used as described.

Macrophage and dendritic cell culture

Immortalized macrophage (iMø) cell lines from wild-type and immunosuppression-deficient animals were generated as described (28). iMø and primary bone marrow dendritic cells (DCs) were cultured in DMEM supplemented with 100 μg/ml FCS, 1% 1-glutamine, and 10 μg/ml Ciprofloxacin (Life Technologies). Cells were seeded at a density of 1 × 106 cells/96 wells and incubated with 200 ng/ml LPS (InvivoGen) for 2 h. Following 6-h stimulation with the indicated agent, supernatants were collected and analyzed by ELISA (R&D Systems) or Western blot as specified in the text and figure legends.

Peritoneal and thioglycollate-induced macrophages

Thioglycollate-induced peritoneal macrophages (pMø) were generated as previously described (24). Briefly, 1 ml thioglycollate broth was injected into the peritoneal cavity. Peritoneal cells containing >80% F4/80+ macrophages were isolated after 5 d in 10 ml macrophage-serum-free media (Invitrogen). pMø were also isolated directly from naive mice and treated as described in the respective figure legends.

Preparation of lymph node lysates for ELISA and Western blot analyses

Mice were injected with 1.3 μg IMX given i.m. in the hind leg. Six hours after administration, mice were sacrificed, and popliteal lymph node were collected, immediately frozen in liquid nitrogen, and grinded using mortar and pestle. Lymph node material was then lysed, and protein levels were adjusted as determined with Bradford assay (Bio-Rad).

Intracellular IFN-γ cytokine staining for CD8+ T cells and NK cells

On days 0 and 7, mice were administered a prime and boost vaccine comprised of OVA (30 μg) and 5 μg IMX given s.c., as previously described (24). On day 14, splenocytes from vaccinated or PBS-injected mice were cultured ex vivo for 4 h in the presence of brefeldin A1 (5 μg/ml) with SINERKL peptide (OVA 257–264) or an irrelevant peptide (each 1 μg/ml). Splenocytes were then stained with anti-CD3 (clone 17A2), anti-CD8 (clone 2.43), washed, fixed, and permeabilized (using BD Biosciences ImmunoCytokine kit), stained with anti-IFN-γ (clone XMG1.2), CD8+ T cells were analyzed by flow cytometry for the expression of IFN-γ using FACSCalibur, Canto, or LSRII instruments (BD Biosciences). NK cell IFN-γ production was evaluated in the draining and nondraining lymph nodes at the indicated time points after a single dose of 5 μg IMX. Briefly, dissociated lymphocytes were cultured ex vivo for 4 h in the presence of brefeldin A1, washed and surface stained with anti-NK1.1 (clone PK136) and anti-CD49b (clone DX5), then fixed, permeabilized, and stained with IFN-γ, as described above. CD69 expression was determined on freshly isolated nonfixed NK cells using anti-NK1.1 CD49b in combination with 7-amino-actinomycin D to exclude dead cells.

Measurement of APC lysosomal swelling

iMø were seeded at a density of 5 × 104 cells/12 wells. Cells were incubated with 1 μg/ml acridine orange for 15 min and then washed three times. Following 6-h stimulation with 2.5 μg IMX or Leu-Leu-OMe (1 μM), cells were harvested, and loss of emission at 600–650 nm was analyzed using an LSRII (BD Biosciences).

Confocal microscopy

Primary murine DCs or macrophages were seeded onto glass-bottom dishes (MatTek, Ashland, MA) at a density of 5 × 105 cells. Cells were stained with acridine orange for 15 min or with Alexa Fluor 546 Dextran for 1 h prior to treatment with IMX or Alexa Fluor 555–OVA ISCOM. Lysosomal maturation was imaged using confocal microscopy (Leica SP2 AOBIS confocal laser-scanning microscope; Leica Microsystems). To monitor ASC speck formation, thioglycollate-induced macrophages were cultured on Lab-Tek II Chamber slides (Nunc) for 1 h and then incubated with 100 ng/ml LPS alone or together with IMX at the indicated concentrations. Cells were washed briefly with PBS and fixed in 4% paraformaldehyde for 10 min at room temperature. After blocking in PBS with 5% donkey serum for 1 h at room temperature, anti-ASC Abs (clone 8E4; Genentech) were diluted in staining buffer (PBS with 5% donkey serum and 0.1% saponin) at 2.5 μg/ml and incubated with cells for 16 h at 4°C. After three washes with PBS, cells were incubated with Alexa 488–conjugated anti-rat IgG (Invitrogen) for 1 h at room temperature. Slides were mounted with Prolong Gold anti-fade reagent (Life Technologies) and viewed with a Zeiss LSM510 upright confocal microscope, using LSM 510 version 3.2 (Carl Zeiss).

Cytokine determinations

IL-1β in supernatants or lysates was measured using OptEIA ELISA (BD Biosciences) according to the manufacturer’s manual. IL-18 was measured using a mouse IL-18 kit (R&D Systems). For IL-18, supernatants were

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concentrated using Amicon Ultra centrifugal filters (3 kDa; Millipore) before analysis.

**Cell viability, lactate dehydrogenase, and caspase-3/7 assays**

Cell viability following IMX (maximum concentration 10 µg/ml) or Alum (maximum concentration 100 µg/ml) treatment was determined in vitro using the CellTiter-Glo cell viability and lactate dehydrogenase (LDH) assays from Promega (as per the manufacturer’s instructions). The CellTiter-Glo cell viability assay was used for the quantitation of ATP in cells and produces a luminescent signal proportional to the amount of ATP contained in cells and present in the supernatant.

**Western blot analysis of cleaved caspase-1, IL-1β, and IL-1β**

Thioglycolate-induced macrophages were incubated with LPS alone or together with IMX for 6 h in DMEM with 0.5% FBS. Culture supernatant were concentrated by Microcon Centrifugal Filters (10 kD; Millipore) and mixed with 4 × SDS sample buffer. Cells were lysed in 1 × SDS sample buffer and boiled at 95°C for 10 min. Samples were then analyzed by SDS-PAGE followed by immunoblotting with the following Abs: anti-caspase-1 (clone 4B4; Genentech), anti-IL-1β (BioVision), anti–IL-1β (R&D Systems), and anti–β-actin (Santa Cruz Biotechnology).

**Mitochondrial superoxide determination**

MitoSOX was used as per the manufacturer’s instructions (Molecular Probes). Briefly, thioglycolate-induced macrophages were labeled with 5 µM MitoSOX for 10 min at 37°C. Cells were then washed three times and cultured for the indicated period with IMX alone or in combination with 10 nM NAC before fixing and analysis by flow cytometry.

**Cloning and production of IL-18BP-Fc fusion**

Human IL-8 BP was PCR amplified from a 107 Human Library mix and cloned into a pRK vector containing murine IgG2A and the DANA mutation. CHO cells were transiently transfected with pRK Hu IL18 BP μIgG2A DANA plasmid using Lipofectamine (Invitrogen, Grand Island, NY). The protein was purified on MabSelect SuRe (GE Healthcare, Piscataway, NJ) and diaлизized into PBS.

**Statistical analysis**

Data represent means ± SEM (in vivo) or ± SD (in vitro) and were analyzed using the Student t test. Statistical analysis with p values of *p < 0.05 and **p < 0.01 were considered significant.

**Results**

**IMX activates the NLRP3 inflammasome pathway in APCs**

IMX administration led to mature IL-1β production in lymph nodes draining from the site of injection (Fig. 1A) (20). The current paradigm for IL-1β production provides that an inflammasome priming event, signal 1, induces the expression of complex components like pro–IL-1β and NLRP3. Priming is then followed by complex formation and activation triggered by signal 2, leading to caspase-1 activation and processing of pro–IL-1β and pro–IL-18 (29). To explore the ability of IMX to provide an efficient signal 2, control or LPS-primed macrophages were cultured together with IMX particles. IL-1β and IL-18 were readily produced by LPS-primed macrophages exposed to IMX, which correlated with the release of IL-1β (p17) and IL-18 (p18) fragments, and processed caspase-1 (Fig. 1B, 1C). Compared with silica and Alum, two well-defined NLRP3 inflammasome activators, IMX induced comparable levels of IL-1β in LPS-primed macrophages (Supplemental Fig. 1A). ASC nucleates most inflammasome complexes and can efficiently self-associate to form large spherical structures, commonly referred to as specks (29). ASC speck formation in response to IMX was similar to that induced by ATP, which activates the NLRP3 inflammasome via the opening of the cation channel of the purinergic P2X7 receptor (Supplemental Fig. 1B, 1C) (30). The dependence on NLRP3, ASC, and caspase-1 for IMX-mediated IL-1β was confirmed using macrophages isolated from mice deficient for each inflammasome component (Fig. 1D, 1E). By contrast, transfected dsDNA (dAdT), which is recognized by the absent in melanoma 2 inflammasome (AIM2), induced normal levels of IL-1β in NLRP3-deficient cells, but was similarly impaired in the absence of ASC or caspase-1/11 (Fig. 1E).

**IMX mediates rapid lysosomal destabilization in APCs**

Endocytosis of particulate adjuvants can mediate lysosome destabilization, characterized by the release of endosomal enzymes into the cytosol that participate in inflammasome activation (31–33). We have shown that IMX facilitates the translocation of Ags from endosomes into cytosol in APCs and therefore sought to visualize this effect directly (23). DCs preincubated with acridine orange (which stains acidic lysosomes red) and exposed to IMX showed a rapid loss of fluorescence signal (Fig. 2A). The decrease in signal intensity was comparable to that induced by the lysosome-disrupting agent Leu-Leu-OMe (Fig. 2B) (34). Moreover, DCs that phagocytosed fluorochrome-conjugated OVA-containing IMX particles showed lysosome swelling, which was concomitant with the overall loss in fluorescence intensity (Fig. 2C). The translocation of coadministered material from endosomes to the cytosol was confirmed in macrophages that had phagocytosed fluorochrome-labeled dextran particles together with IMX (Supplemental Fig. 1D). To evaluate whether lysosomal acidification was required for IMX to mediate inflammasome activation, IL-1β production was monitored in APCs pretreated with BaF1, a specific inhibitor of the vacuolar-type H+–ATPase. Indeed, BaF1 potently inhibited IL-1β production (Supplemental Fig. 1E). Similar results were obtained with the lysosomotropic agent chloroquine (Supplemental Fig. 1F). The contribution of lysosome-associates proteases to IMX induced inflammasome activation, and IL-1β production was explored using inhibitors of the cysteine proteases cathepsin B and cathepsin L (Supplemental Fig. 1G, 1H), with a specific role for cathepsin B and cathepsin L determined using macrophages from respective gene knockout mice (Fig. 2D). By contrast, activation of the AIM2 inflammasome by dAdT was not affected by cathepsin B or L deficiency. Similarly, cathepsin B and L were required for LPS-primed macrophages to produce IL-18 in response to IMX, again compared with dAdT (Supplemental Fig. 1I).

**IMX induces sterile cell death concomitant with inflammasome activation**

Inflammasome activation can mediate pyroptosis, a form of programmed cell death, which is reportedly dependent on caspase-1 (35). Concomitant with the breach in endosomal integrity, we observed striking morphological changes in macrophages treated with IMX (Supplemental Fig. 2A). These morphological events correlated with a dramatic loss in cellular ATP levels, which was independent of LPS priming, and were not observed with Alum (Fig. 3A). APCs exposed to IMX generated superoxide, which was quenched by preculturing cells with the reactive oxygen species scavenger NAC (Supplemental Fig. 2B). NAC pretreatment of LPS-primed APCs reduced IMX-mediated intracellular ATP depletion and inhibited IL-1β and IL-18 production (Fig. 3B, 3C). Similarly, blocking lysosomal acidification with BaF1 prevented ATP depletion and terminal LDH release from dying cells (Fig. 3D, 3E) (36). Despite IMX mediating caspase-3 and -7 activation in APCs, pharmacological inhibition of caspases, cathepsins, or stress pathways such as JNK or the MAPK p38 failed to rescue macrophage viability (Supplemental Fig. 2C, 2D). Similarly, neither the RIP-1 inhibitor necrostatin-1 nor macrophage RIP3 deficiency prevented this mechanism of sterile cell death (Supplemental Fig. 2E). Notably, IMX-mediated cell death was specific to only certain populations of phagocytic APCs, as illustrated by the selective depletion of the F4/80<sup><sub>high</sub></sup> pMØ in ex vivo cell cultures (Fig. 3F). These findings suggest that phagocytic
capacity and lysosomal environment determine the impact of IMX on lysosomal integrity in APCs. Further, this phenotype may relate to the ability of certain APC populations to rapidly acidify phagosomes following particulate ingestion at the site of IMX injection. Importantly, we did not observe any negative impact of IMX administration on APCs in regional draining lymph nodes; rather, there was a 5-fold increase in the number of CD11c+ cells, which may reflect migratory DCs or inflammatory monocyte-derived DCs trafficking from the injection site (24).

**FIGURE 1.** IMX-induced IL-1β release in vitro is mediated by the NLRP3 inflammasome. (A) Mice were injected s.c. with IMX in the hind leg. After 6 h, the popliteal lymph nodes were analyzed for mature IL-1β by ELISA and Western blot. Data represents pooled lymph nodes from n = 3 mice. (B) Isolated thioglycollate-induced pMØ were untreated or primed with LPS and subsequently incubated with IMX for an additional 6 h. IL-1β and IL-18 were measured by ELISA. (C) Western blot analysis of caspase-1, IL-18, and IL-1β in LPS-primed pMØ cell lysates and cell culture supernatants. Levels of IL-1β and caspase-1 were measured in supernatants (SN) by ELISA (D) or SN and cell lysates (CL) by Western blot analysis (E) from wild-type (WT) and NLRP3−/−, ASC−/−, or caspase (Casp)-1/11−/− LPS-primed macrophages treated with IMX, ATP, or dAdT. Results are shown as the mean ± SD and representative of two or more independent experiments.

**FIGURE 2.** IMX-mediated IL-1β release is dependent on lysosomal maturation and cathepsins. (A) Bone marrow–derived DCs were labeled with acridine orange and left untreated (Ø) or cultured with IMX for 2 h analysis by confocal microscopy. White arrows indicate areas of lysosomal maturation. (B) iMØ were stained with acridine orange and stimulated with IMX or Leu-Leu-OMe for indicated time points. Loss of red fluorescence was monitored by flow cytometry. (C) DCs were incubated with Alexa Fluor 555–conjugated OVA-containing IMX for 6 h, and translocation of OVA into cytosol was evaluated by confocal microscopy. (D) LPS-primed iMØ of wild-type (WT), cathepsin (Cath.) B, and Cath.L−/− mice were stimulated with IMX or transfected with dAdT for 6 h and IL-1β in the supernatant measured by ELISA. The results are representative of three independent experiments and means ± SD as triplicates from one of three independent experiments.
IL-18 signaling is required for NK cell activation induced by IMX in vivo

The initial innate immune response can impact the overall magnitude and polarization of T cell immunity (37). Importantly, inflammasome complexes can sense a variety of pathogens, vaccine adjuvants, and pollutants (38–40). However, for the majority of particulate adjuvants, the translation of these findings in animal models remains controversial or unexplored (11). To evaluate the importance of an inflammasome pathway for IMX vaccine immunity, we evaluated innate immune cell activation in the draining lymph node following adjuvant administration (20, 21, 23, 24). Consistent with distinct immunogenic mechanisms, IMX but not Alum led to a marked increase in the early activation marker CD69 by NK cells, which correlated with the production of IFN-γ (Fig. 3A, Supplemental Fig. 3A–C). No significant impact on NK cell IFN-γ production was observed in IL-1R-deficient mice (Fig. 3B). By contrast, mice deficient for IL-18 and the IL-18R mice showed a significant reduction in NK cell activation (Fig. 3C, Supplemental Fig. 3D). This phenotype was verified by infusing control mice with an rIL-18 binding fusion protein (IL-18BP-Fc) prior to IMX administration (Fig. 3D).

Inflammasome-dependent and -independent events contribute to IL-18–dependent NK cell activation

The reduced NK cell response in the absence of IL-18 signaling was consistent with an important role for an inflammasome pathway in sensing IMX in vivo. To explore this, we analyzed NK cell responses in ASC-deficient mice injected with IMX (Fig. 4E, Supplemental Fig. 3E). ASC deficiency led to a reduction in NK cell IFN-γ production and CD69 upregulation; however, the phenotype did not reproduce the extent of inhibition observed in IL-18R-deficient mice. Indeed, IL-18BP-Fc injected into ASC knockout mice further reduced NK cell activation, indicating an ASC-independent mechanism of IL-18 production in vivo (Fig. 4E). Unexpectedly, NLRP3-deficient mice showed no defect in NK cell activation, which diverged dramatically from our in vitro experiments with isolated APCs (Fig. 4F). Mice deficient in the inflammasome adapter NLR family caspase activation and recruitment domain-containing protein 4 (IPAF) treated with IMX similarly exhibited no defect in NK cell activation (Supplemental Fig. 3F). Inflammasome-dependent and -independent functions of ASC have recently been described; we therefore studied the influence of caspase-1 on NK cell activation using caspase-1/11–/– mice (41, 42). Caspase-1/11–deficient mice reproduced the reduced NK cell response in the ASC-deficient background; however, the phenotype was consistently more robust in the absence of IL-18 (Fig. 4G). Taken together, our findings are consistent with IMX mediating the activation of an ASC–caspase-1–dependent inflammatory response to produce IL-18, but that unrelated pathway(s) can further contribute to IL-18 production in mice.

CD8+ T cell and Ab responses induced by a model IMX vaccine require IL-18R signaling

MyD88 was required for CD8+ T cell priming and Ab production in response to an IMX model vaccine (24). We therefore sought to confirm the association between the MyD88 adapter and the IL-18 pathway in adaptive immunity to an IMX vaccine containing OVA (IMX-OVA). Similar to NK cell activation, the frequency of vaccine-induced endogenous OVA-specific CD8+ T cells was significantly impaired in IL-18– and IL-18R–, but not IL-1R–deficient mice (Fig. 5A, 5B, Supplemental Fig. 3G). A partial effect on CD8+ T cell priming, which did not reach statistical significance, was observed in mice deficient for ASC mice (Fig. 5C). Moreover, no significant difference in the Ag-specific CD8+ T cell response was observed in NLRP3- or IPAF-deficient mice (Fig. 5D, Supplemental Fig. 3H). Our findings support that CTL priming by the IMX-OVA vaccine was dependent on an intact IL-18R–MyD88 signaling axis; however, we could not substantiate any involvement of the NALP3 inflammasome pathway. Interestingly, vaccine-generated IgG1 Ab titers were unaffected by the absence of IL-18 signaling; however, IgG2c titers (equivalent to IgG2a) were dramatically reduced (Fig. 5E). Consistent with our findings for cellular

FIGURE 3. IMX induces rapid loss of intracellular ATP and selective APC cell death. (A) pMΦ were isolated and cultured for 16 h with titration of IMX or Alum. Viability was monitored as ATP depletion. (B) ATP depletion in pMΦ treated with IMX with or without NAC pretreatment for 30 min. (C) Untreated (Ø) or LPS-primed pMΦ were incubated with IMX for 4 h, with or without prior NAC treatment. IL-1β and IL-18 levels in the supernatant were measured by ELISA. (D and E) pMΦ were cultured with IMX in presence or absence of BafA1. ATP depletion (D) or LDH release (E) was monitored after 16 h. (F) selective depletion of the F4/80+ pMΦ population ex vivo culture with IMX. Results are representative of two or more independent experiments. Error bars show means ± SD as triplicates from one representative experiment. cmax, maximum concentration; FSC, forward light scatter; SSC, side scatter.
IL-18R

immunity, NLRP3-deficient mice developed normal IgG1 and IgG2c Ab titers in response to vaccination (Supplemental Fig. 3I). TNF-α has been reported to prime inflammasome complexes in vitro (43). Having previously detected low but significant amounts of IL-18 for in vivo immunity remains controversial (7, 8, 32). Thus, TNF-α contributes to IMX activity in vivo.

TNF-α primes the NALP3–ASC inflammasome in vitro and contributes to IMX activity in mice

TNF-α has been reported to prime inflammasome complexes in vitro (43). Having previously detected low but significant amounts of TNF-α in the draining lymph node following IMX administration, we investigated whether TNF-α provided a priming signal for IMX-mediated inflammasome activation (24). TNF-α–primed macrophages stimulated with IMX rapidly formed ASC specks (Supplemental Fig. 3c). Speck formation correlated with IL-18 production, but only low amounts of IL-18 were generated, as compared with LPS-primed APCs (Fig. 6A). ASC and NALP3 inflammasome components were required for LPS- or TNF-α–primed macrophages to produce IL-18 in response to IMX in vivo (Fig. 6B). To explore the contribution of TNFR1 versus TNFR2 for TNF-α–mediated inflammasome priming, macrophages from wild-type, single-, or double-deficient mice were primed with TNF-α or LPS and compared for their ability to activate procaspase-1 and process pro–IL-1β and pro–IL-18. Similar to LPS-primed macrophages, TNF-α did not change basal pro–IL-18 protein expression; however, both stimuli led to an increase in the level of NALP3 and pro–IL-1β (Fig. 6C). LPS-mediated procaspase-1 activation was unaffected by TNFR deficiency, whereas TNFR1 and TNFR2 both contributed to maximal TNF-α–mediated inflammasome activation (Fig. 6C). To translate this in vivo, we analyzed IFN-γ production by NK cells in TNFR1/2- or TNF-α–deficient mice. Similar to the ASC-null background (Fig. 4E), the NK cell response was partially compromised in the absence of TNFR1/2 signaling, as compared with IL-18–deficient mice (Fig. 6D, Supplemental Fig. 3K). Finally, TNF-α–deficient mice showed a reduction in CD8+ T cell priming in response to IMX-OVA vaccination (Fig. 6E).

Discussion

In this study, we have identified IMX as a potent activator of the NLRP3 inflammasome in APCs in vitro, exemplified by processing and secretion of IL-1β and IL-18. This places IMX in line with other particulate adjuvants, such as aluminum salts, which have been shown to activate the NLRP3 inflammasome pathway in LPS-primed APCs (25, 44). Like inert particulates, uric acid, silica, and cholesterol crystals, IMX induced a lysosomal stress response that led to the leakage of lysosomal enzymes into the cytosol (33, 45–47). With IMX, endosomal stress resulted in the rapid loss of intracellular ATP, caspase activation, and cell death in distinct APC populations. This endosome-mediated cell-death pathway was not observed with aluminum salts, supporting distinct immunogenic mechanisms for these two adjuvants. Multiple studies have shown NLRP3 inflammasome activation in response to sterile particulates and adjuvants in vitro. However, the involvement of the NALP3 inflammasome and its key effector molecules IL-1β and IL-18 for in vivo immunity remains controversial (7, 8, 44, 48). MyD88 was previously shown to be required for the induction of optimal innate and adaptive immune responses to a model IMX vaccine (24). MyD88 has a well-documented role in TLR signaling; MyD88 also functions as the apical adapter in the IL-1R and IL-18R pathways (49, 50). IMX-mediated NK cell activation and vaccine-induced cytotoxic T cells and B cell immunity were severely

FIGURE 4. IL-18 is required for the innate NK cell response to IMX in vivo. (A) Mice were s.c. injected with IMX or Alum. Representative profiles show the percent of CD161+CD49b+ NK cells producing IFN-γ in the brachial draining lymph nodes after 24 h as compared with naive mice injected with PBS (n = 5/group). (B) NK cell IFN-γ production in response to IMX in the regional draining lymph node is shown for wild-type (WT; n = 11) and IL-1R−/−(n = 4) mice relative to IMX-treated WT lymph nodes (n = 4). (C) Relative (Rel.) NK cell IFN-γ response to s.c. IMX was evaluated in IL-18−/−and IL-18R−/−mice (n = 7 and n = 4) compared with WT (n = 11) or naive control animals (n = 4). (D) WT mice (n = 8) were pretreated with IL-18BP-Fc and 2 h later injected with IMX. The percentage of IFN-γ+ NK cells was analyzed in draining lymph nodes and expressed relative to WT mice receiving IMX only (n = 9). Naive mice were used as negative controls (n = 8). (E) WT, ASC−/−, IL-18R−/− mice, or IL-18R−/− mice pretreated with IL-18BP-Fc (all n = 5) were injected with IMX. The percentage of IFN-γ+ NK cells was determined in the draining lymph node. (F) Percentage of IFN-γ+ NK cells in the draining lymph node of WT (n = 3) or NLRP3−/−(n = 7) mice injected with IMX. (G) Percentage of IFN-γ+ NK cells in the draining lymph node is shown for WT (n = 5), caspase-1/11−/− (n = 4), or IL-18−/−(n = 5) mice injected with IMX. Results from pooled individual experiments (n = 7) with the indicated number of mice per group. All data are expressed relative to WT mice injected with IMX. *p < 0.05, **p < 0.01.
pathway in isolated APCs, and its detection in the draining lymph
node following IMX administration led us to examine its involve-
mament in innate and adaptive immune cell responses (43). TNF-
α might contribute to inflammasome-independent stimulatory effects.
For example, TNF-α has been shown to induce expression of the
high-affinity receptor subunit of the IL-18R complex, which there-
fore might function to further amplify the immunostimulatory
effects of IMX (54).

The complex immune cell interactions that result from adjuvant
administration have provided a substantial challenge to unraveling
key initiating events that drive immunity to vaccine Ags. We have
previously shown that neutrophils and monocytes rapidly infiltrate
the vaccination site following IMX administration, which corre-
lated with the production of chemokines like MCP-1 and M-CSF
(24). Importantly, the inflammatory response was very transient
and localized to the site of injection and regional draining lymph
node, although sufficient to deliver Ag and activate APCs to trigger
adaptive immune responses. Our current paradigm is that the in-
nate immune response at the vaccination site is triggered, at least
in part, by an endosomal-mediated cell death mechanism, which
selectively impacts tissue-resident APCs that initially ingest IMX
particles and coadministered vaccine Ags. Dying APCs trigger the
production of chemokines and cytokines and would provide an
ideal source of cell-associated Ag for responding inflammatory
monocytes to capture and transport to the draining lymph nodes.
Subsequent priming of vaccine Ag-specific CD8+ T cell and Ab
responses in regional draining lymph nodes is dependent on CD11c-
expressing populations of DCs, including MHC class I Ag presen-
tation by migratory DC or monocyte-derived DCs trafficking from
the injection site (24). The MyD88/IL-18R pathway is required
for innate and adaptive immune cell responses; however, it was
not necessary for the ability of IMX to significantly enhance (up to
1000-fold) the MHC class I cross-presentation by DCs (summa-
rized in Supplemental Fig. 4) (24).

Despite the defined involvement of the inflammasome for IL-18
production, NALP3-independent IL-18 production was sufficient
to drive innate and adaptive immunity to an IMX vaccine in mice.
A key question therefore centers on the apparent ASC/caspase-1–
independent processing of pro–IL-18 in vivo. Unlike pro–IL-1β,
pro–IL-18 is constitutively expressed by a variety of immune
and nonimmune cell types, including macrophages and neutrophils
(50). Therefore, APC death at the vaccination site may provide a source
of pro–IL-18, together with an activation signal for neutrophils
that respond to APC death. Neutrophils produce protease-rich extra-
cellular structures in response to pathogenic insults (55). This
response, termed neutrophil extracellular nets, is proposed to limit
the spread of cellular debris. Neutrophils express enzymes capable

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**FIGURE 5.** The IL-18 pathway is crucial for IMX-OVA vaccine–induced adaptive immunity. (A–D) Wild-type (WT) (n = 23, n = 8, and n = 11, respectively), IL-18R<sup>-/-</sup>(n = 16), IL-18R<sup>-/-</sup>(n = 6), IL-1R<sup>-/-</sup>(n = 6) (B), ASC<sup>-/-</sup>(n = 5) (C), or NLRP3<sup>-/-</sup>(n = 5) (D) mice were vaccinated on days 0 and 7 with IMX-OVA vaccine, and the OVA-specific CD8<sup>+</sup> T cell response was evaluated on day 14 in the spleen, as previously described (24). The magnitude of the CD8<sup>+</sup> T cell response is shown relative to the treated WT cohort (n = 2/cohort). (E) OVA-specific serum Ab titers (IgG1 and IgG2c) were evaluated on day 28 in WT (n = 13) or IL-18<sup>-/-</sup> (n = 12) mice with serum dilutions of 1:900. Results are representative of two or more independent experiments. Pooled data with indicated mice per group shown as mean + SEM. *p < 0.05, **p < 0.01.
of processing pro–IL-18, such as proteinase-3 (56–59). Thus, an amount of mature IL-18 could be generated extracellularly at the local vaccination site by inflammasome-independent mechanisms. An equally tractable hypothesis is that proteases released directly from phagocytes undergoing IMX-induced cell death may generate mature IL-18 (summarized in Supplemental Fig. 4). To support this, it has recently been shown that activated caspase-8, like caspase-1, can process pro–IL-18 in vitro without the need for ASC and caspase-1 (60). Exploring the role of caspase-8 in IMX-mediated immunity would be aided by conditional knockout mice, which overcome the embryonic lethality of caspase-8 deficiency (61, 62).

We reveal a detailed mechanistic understanding of the apical inflammatory events mediated by the IMX adjuvant in mice. We propose that instead of direct recognition of IMX by a classical danger-associated molecular pattern or pathogen-associated molecular pattern receptor, IMX is sensed by tissue-resident APCs at the injection site. The resultant sterile inflammatory response leads

**FIGURE 6.** TNF-α may provide a physiological inflammasome priming signal in vivo. (A) IL-1β and IL-18 production by LPS or TNF-α–preprimed pMΦ incubated with IMX for 6 h. (B) IL-18 production by TNF-α–preprimed pMΦ from wild-type (WT), ASC−/−, and NLRP3−/− mice treated with IMX for 6 h. (C) NLRP3, caspase-1, pro–IL-18/IL-18, and pro–IL-1β/IL-1β protein levels in the supernatant (SN) and cell lysate (CL) or LPS or TNF-α–primed pMΦ from WT, TNFR1−/−, TNFR2−/−, or TNFR1/2−/− mice stimulated with IMX for 6 h assessed by Western blot. (D) WT (n = 5), TNFR1/2−/− (n = 5), TNF-α−/− (n = 5), or IL-18R−/− (n = 5) mice were injected with IMX. NK cell IFN-γ was evaluated in the draining lymph nodes ex vivo after 16 h and expressed relative to vaccinated WT mice. (E) Relative (Rel.) OVA–specific CD8+ T cell response in the spleen (day 14) of WT (n = 18) and TNF-α−/− (n = 15) mice following prime-boost (days 0 and 7) vaccination with IMX-OVA. Results in (A)–(C) are representative data from one of two or more independent experiments. (D and E) Pooled data from two or more experiments with indicated mice per group, shown as mean + SEM relative to treated WT cohort. **p < 0.01.
to the recruitment of monocytes and neutrophils, culminating in IL-18-driven immunogenecity to coadministered vaccine Ags.

Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 1 (Related to Figure 1):

(A) pMθ were primed overnight with LPS prior to treatment with ISCOMATRIX (IMX), Silica or alum (100 µg/ml). IL1β in supernatants was measured by ELISA. (B) pMθ were treated with LPS, ISCOMATRIX, the combination of LPS and ISCOMATRIX or left untreated. ASC speck formation was evaluated by confocal microscopy. (C) ASC speck formation of ISCOM- or ATP-treated iMθ quantified per high power field. (D) iMθ were loaded with Alexa555-Dextran and subsequently stimulated with ISCOMATRIX for 6 h. ISCOMATRIX-induced lysosomal translocation of Dextran was evaluated by confocal microscopy. (E-H) LPS-primed iMθ were stimulated with indicated concentrations of ISCOMATRIX for 6 h in presence or absence of (E) bafilomycin A1, (F) chloroquine, (G) the cathepsin B inhibitor Ca-074-Me or (H) the cathepsin L inhibitor Z-FF-FMK. IL-1β in supernatants was measured by ELISA. Mean + SD of one out of three independent experiments is shown. (I) LPS-primed immortalized macrophages of wild-type, cathepsin B/L double-deficient mice were stimulated with IMX or transfected dAdT for 6 h and IL-1β and IL-18 in the supernatant measured by ELISA. The results are representative of two independent experiments and means + SD as triplicates.
Supplemental Figure 2 (Related to Figure 2):

(A) pMΦ were incubated with ISCOMATRIX (IMX) for 4 h or left untreated. Morphological changes were monitored by bright field microscopy. (B) Macrophage ROS production induced by ISCOMATRIX was monitored with the mitochondrial superoxide indicator MitoSOX. Control cells were pre-incubated for 30 min with 10 mM of NAC prior to ISCOMATRIX exposure. (C) pMΦ were treated with LPS, ISCOMATRIX or a combination of LPS and ISCOMATRIX. Caspase-3/7 activity was monitored after 8 h using the caspase-Glo-3/7 assay (arbitrary fluorescence units (AU) are shown). (D) Macrophages were treated with ISCOMATRIX with or without pre-incubation with inhibitors of pan-caspases (Z-VAD), cathepsin B (Ca-074-ME), JNK (SP600125), p38-MAPK (SB203580), caspase-8 (Z-IETD-FMK), cysteine proteases (E64) and aspartic proteases (pepstatin A). ATP levels were determined after 8 h by the CellTiter Glo assay. (E) Wild-type macrophages were pre-incubated for 30 min with Necrostatin-1 (NEC-1) or compared to macrophages isolated from RIP3−/− mice. The dose response to ISCOMATRIX was evaluated using the ATP CellTiterGlo assay after 6 h. Representative experiments from two or more independent experiments performed as triplicates are shown.
Supplemental Figure 3:

(A) Representative profiles showing the gating strategy to evaluate IFNγ-production by CD161⁺CD49b⁺ NK cells 24 h after s.c. ISCOMATRIX (IMX) administration. (B and C) Mice were injected with IMX or alum s.c.. Representative profiles show the percent of NK cells expressing CD69 (B) or producing IFNγ (C) in the brachial draining lymph nodes after 24 h, as compared to naïve mice injected with PBS. The histogram summarizes the NK cell IFNγ-response in ISCOMATRIX-treated animals, relative to the alum treated and naïve cohorts (n=5/group). (D) Representative profiles of CD69 up-regulation by NK cells isolated from the draining lymph node of untreated (naïve), wild type, IL18⁻/⁻ and IL-18R⁻/⁻ mice injected s.c. with ISCOMATRIX. ((E) CD69 up-regulation by NK cells isolated after 16 h from WT (n=5), ASC⁻/⁻ (n=5) or IL18R⁻/⁻ (n=4) mice injected with ISCOMATRIX. Data is shown relative to naïve WT animals (n=1). (F) IFNγ⁺ NK cells in the draining lymph node of WT (n=5) or IPAF⁻/⁻ (n=4) mice injected with ISCOMATRIX. Data is shown relative to treated WT animals (n=1). (G) Representative profiles showing the gating strategy to evaluate IFNγ-production by CD3⁺CD8⁺ T cells in mice vaccinated on days 0, 7 with IMX:OVA vaccine. The OVA-specific CD8⁺ T cell response was evaluated on day 14 in the spleen, and shown compared to a naïve control. (H) WT (n=8) or IPAF⁻/⁻ (n=4) mice were vaccinated on days 0, 7 with IMX:OVA vaccine, and the OVA-specific CD8⁺ T cell response was evaluated on day 14 in the spleen. The magnitude of the CD8⁺ T cell response is shown relative to the treated WT cohort (n=2/cohort of WT mice). (I) OVA-specific serum antibody titers (IgG1 and IgG2c) were evaluated on day 28 in WT (n=13) or NALP3⁻/⁻ (n=5) mice with serum dilutions of 1:900. (J) pMφ were treated with TNF-α alone or in combination with ISCOMATRIX (IMX). ASC speck formation was evaluated by confocal microscopy. (K) CD69 up-regulation on NK cells isolated from the draining lymph nodes of WT (n=5) or TNFR1/2⁻/⁻ (n=4-5) mice 16 h after ISCOMATRIX administration.
Phagocyte

IMX

Inflammasome independent

DC activation
CD8+ T cell priming
B cell priming

Lysosomal destabilization
Metabolic stress
ROS
Ag translocation
Caspase 3/7 activation
Phagocytic cell death

Neutrophil

DC

MØ

Phagocyte

IMX

Lysosomal destabilization
Metabolic stress
ROS
Ag translocation
Caspase 3/7 activation
Phagocytic cell death

Neutrophil

Inflammasome independent

Neutrophil proteases? Caspase-8?

Inflammasome dependent

IL18

CD8+ T cell

CD4+ T_{h,1} cell

CD8+ T cell

MHC-I

TCR

NK cell

IFN-γ

NK cell activation

B cell

IgG2c

DC

DC activation
CD8+ T cell priming
B cell priming

IL18
Supplemental Figure 4: IL-18R pathway links innate and adaptive immunity to ISCOMATRIX in vivo

Proposed model: At the local injection site ISCOMATRIX (IMX) is captured by APCs, which results in lysosomal maturation and destabilization, leading to the release of lysosomal contents into the cytosol. This lysosomal stress pathway initiates reactive oxygen species production (ROS), caspase-3/7 activation, ATP depletion, cumulatively leading to a rapid form of sterile cell death. Neutrophils and monocytes respond to dying APCs by rapidly infiltrating into the injection site (38). Neutrophils may contribute extracellular traps, containing enzymes that can cleave pro-IL18 released by themselves and/or from dying APCs. This process appears to be independent of caspase-1, and may involve caspase-8. Early inflammatory events resulting from APC cell death lead to TNFα production in the lymph nodes draining from the injection site. This may enhance innate and adaptive immune cell activation, including up-regulation of the high affinity version of the IL18R. Moreover, TNFα may act as a physiological priming signal for inflammasome-dependent IL18 (and IL1β) production. Bioactive IL18 acts as the key cytokine for NK cell activation, and is required for the innate and Th1-like adaptive immune responses to our model ISCOMATRIX vaccine in mice. M0 – macrophages; DC- dendritic cells.