

Cutting Edge: Inflammasome Activation by Alum and Alum's Adjuvant Effect Are Mediated by NLRP3¹

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Alum is the only adjuvant approved for routine use in humans, although the basis for its adjuvanticity remains poorly understood. We have recently shown that alum activates caspase-1 and induces secretion of mature IL-1 β and IL-18. In this study we show that, in human and mouse macrophages, alum-induced secretion of IL-1 β , IL-18, and IL-33 is mediated by the NLR (nucleotide-binding domain leucine-rich repeat-containing) protein NLRP3 and its adaptor ASC, but not by NLRC4. Other particulate adjuvants, such as QuilA and chitosan, induce inflammasome activation in a NLRP3-dependent fashion, suggesting that activation of the NLRP3-inflammasome may be a common mechanism of action of particulate adjuvants. Importantly, we demonstrate that Ag-specific Ab production elicited by vaccines that contain alum is significantly impaired in NLRP3-deficient mice. Our results demonstrate for the first time a role for the NLRP3-inflammasome during development of the immune response elicited by alum-enhanced vaccination and suggest that therapeutic intervention aimed at NLRP3 may improve adjuvant efficacy. *The Journal of Immunology*, 2008, 181: 17–21.

Aluminum hydroxide and aluminum phosphate adjuvants (herein referred to as alum) have been used for decades and are the only adjuvants licensed for routine human vaccination. Alum has proven its efficacy in several vaccine formulations (reviewed in Ref. 1), although its mode of action remains unclear. Alum is unable to act as a TLR agonist and does not promote dendritic cell (DC)³ maturation (2); hence, whether its activity is mediated by pattern recognition receptors is unknown. It is generally accepted that adsorption of the Ag on the alum particle forms an Ag depot at the injection site. This is believed to maximize the interaction time between the Ag and the APC and would ensure that Ag and adjuvant are

delivered to the same population of APC. This notion however has been challenged by several reports. It is likely that multiple mechanisms (reviewed in Ref. 3) are responsible for the adjuvant effect of alum and other particulate adjuvants such as chitosan and QuilA/saponin. Alum has been shown to fix complement (4), cause granulomas containing Ab-producing plasma cells and neutrophils (5), and induce the appearance in the spleen of Gr1⁺, IL-4-secreting eosinophils that prime B cells (6, 7). How alum achieves these effects and their role in alum adjuvanticity remains unknown.

We have recently shown that alum is capable of activating caspase-1 and inducing the release of IL-1 β and IL-18 (8) in a MyD88-independent fashion, thus suggesting a possible mechanism for alum's adjuvant effect. IL-1 β and IL-18 are prototypical proinflammatory cytokines with pleiotropic functions (reviewed in Ref. 9), including adjuvant capacity (10, 11). Their biosynthesis is regulated at several levels, with two key steps playing a prominent role. One is the transcription and translation of their mRNA into the immature proteins pro-IL-1 β and pro-IL-18. This step is regulated by classical proinflammatory stimuli, most notably TLR agonists. The second step is required for their secretion and involves the proteolytic processing of pro-IL-1 β and pro-IL-18, which is conducted by activated caspase-1. Caspase-1 activity is also required for secretion of IL-33, a recently identified member of the IL-1 family (12). Caspase-1 activation is not triggered by TLR signaling and occurs in the context of a multiprotein complex termed the inflammasome. Key components of the inflammasome are cytoplasmic sensors of pathogen-associated molecules that belong to a subgroup of the nucleotide-binding domain leucine-rich repeat-containing (NLR) family (reviewed in Ref. 13). Two of the best characterized NLR molecules are NLRP3 (also known as Cryopyrin, NALP3, CIAS1, and Pypaf1) and NLRC4 (also known as IPAF and CARD12). Both molecules have been shown to mediate caspase-1 activation in response to a growing number of intracellular bacteria, toxins, and danger signals. Inflammasome activation by both

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Received for publication March 27, 2008. Accepted for publication April 28, 2008.

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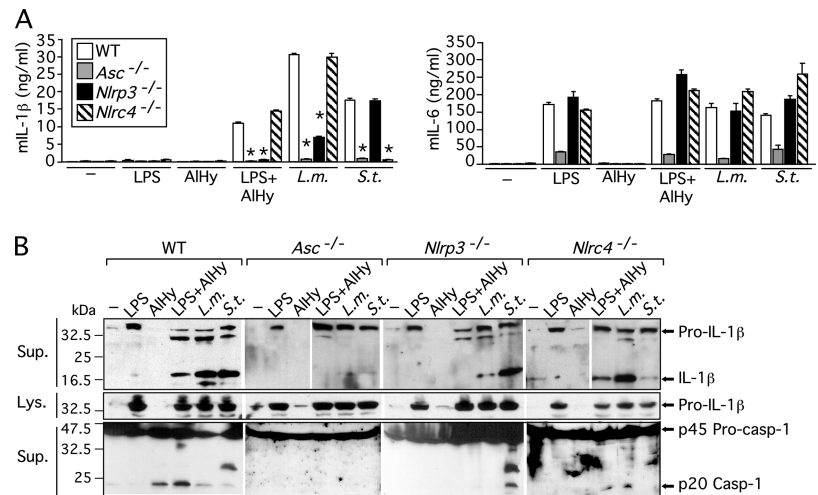
¹ This work was supported by National Institutes of Health Grants R21AI076835-01 (to F.R.) and R01AI057157, R01AI063031, R01DE16326, and SERCEB A1-02-031 (to J.P.-Y.T.).

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³ Abbreviations used in this paper: DC, dendritic cell; BMM, bone marrow-derived mononuclear cell; DT, diphtheria toxoid; LF, flocculation unit; NLR, nucleotide-binding domain leucine-rich repeat-containing (protein/family); shRNA, short hairpin RNA; TT, tetanus toxoid; Z-VVAD-FMK, N-benzyloxycarbonyl-Tyr-Val-Ala-Asp-fluoromethyl ketone.

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FIGURE 1. Alum activates the NLRP3-inflammasome. Wild-type (WT), ASC^{-/-}, NLRP3^{-/-}, or NLRC4^{-/-} BMM were stimulated for 9 h with LPS (5 ng/ml) in the presence or absence of aluminum hydroxide (AlHy; 130 μg/ml) or for 4 h with *L. monocytogenes* (*L.m.*) or *S. typhimurium* (*S.t.*) (multiplicity of infection = 10). **A**, IL-1β or IL-6 was measured in conditioned supernatants. **B**, The presence of the immature and mature forms of IL-1β and caspase-1 (Casp-1) in cell culture supernatants (Sup.) or cell lysates (Lys.) were analyzed by immunoblotting. One experiment representative of five (WT and NLRP3^{-/-}) or two (ASC^{-/-} and NLRC4^{-/-}) is shown. Values are mean ± SEM. Asterisks denote significant difference vs WT ($p < 0.001$).



proteins depends on the adaptor molecule ASC (14, 15), although the details of the interaction between NLRC4 and ASC are not clear.

Our discovery that alum activates caspase-1 and triggers IL-1β and IL-18 secretion suggests that the activity of alum may be mediated by NLR molecules. In this report we show that inflammasome activation by alum and alum's adjuvanticity are mediated by NLRP3 and ASC but not NLRC4.

Materials and Methods

Mice

NLRP3^{-/-} mice were provided by Millenium Pharmaceuticals and backcrossed for nine generations to C57BL/6 mice. ASC^{-/-} and NLRC4^{-/-} mice have been backcrossed for over nine generations to C57BL/6 mice and were provided by Dr. V. Dixit of Genentech. Age- and sex-matched C57BL/6 mice were purchased from the National Cancer Institute (Bethesda, MD). All experiments using mice were approved by the University of Tennessee Health Science Center (Memphis, TN) and the University of North Carolina Institutional Animal Care and Use Committee (Chapel Hill, NC).

Reagents

Alhydrogel is an aluminum hydroxide gel (Sigma-Aldrich). Imject alum and CFA were from Pierce. Tetanus toxoid (TT) and diphtheria toxoid (DT) adsorbed for pediatric use were from Sanofi-Pasteur. Endotoxin-free chicken OVA was from Profos. Chitosan was from Sigma-Aldrich. QuilA was from Brenntag. Caspase-1 inhibitor *N*-benzyloxycarbonyl-Tyr-Val-Ala-Asp-fluoromethyl ketone (Z-VYAD-FMK) was from Alexis Biochemicals and was used at 10 μM. The following Abs were used: rabbit anti-caspase-1 (Upstate Biotechnologies); goat anti-mouse IL-1β (R&D Systems); anti-human IL-1β 3ZD mAb (National Cancer Institute's Biometrics Research Branch Preclinical Repository); and rabbit anti-IL-33 (Alexis Biochemicals).

PBMC and bone marrow-derived mononuclear cells (BMM) isolation

Human PBMC were isolated from Leukopacks by Ficoll-Hystopaque density gradient centrifugation. Mouse mononuclear cells were generated by incubating bone marrow in RPMI 1640 plus 10% FCS supplemented with recombinant mouse GM-CSF (20 ng/ml) (R&D Systems) for 8 days. This procedure routinely results in 60–80% CD11c⁺ cells.

Lentiviral short hairpin RNA (shRNA) constructs

Expression of ASC and NLRP3 was silenced using a derivative of the lentiviral vector pLL3.7 (Addgene), where enhanced GFP was replaced with the puromycin resistance gene. The following shRNA target sequences were selected: mASC-45, 5'-GTCAGGGGATGAACCTCAA-3'; human ASC-327, 5'-GC CAGGCTGCACCTTTATA-3'; and human NLRP3-52, 5'-GTGGACTT GAAGAAATTTA-3'. To construct the shRNA-resistant form of human ASC, the following silent mutations (in bold type, compared with the shRNA target sequence shown above) were introduced: GCCGGG**TTT**ACATTTTCATA. The resulting mutated ASC was cloned into the retroviral construct pLXIZ (a

derivative of pLXIN from Clontech, where the neomycin-resistant marker is substituted with the zeocin marker). The THP-1 macrophage cell line was infected with pLL3.7 lentiviral stocks and selected with puromycin. The efficiency of silencing was confirmed by an RNase protection assay and by immunoblotting. To rescue ASC expression, the shRNA-expressing ASC-THP-1 cell line was infected with the pLXIZ-mutASC stocks and selected with zeocin.

Cytokines measurements

Cytokines levels in conditioned supernatants were measured by ELISA using the following paired Abs kits: human IL-1β and human IL-18 (R&D Systems); mouse IL-1β and mouse IL-6 (eBioscience); and mouse IL-18 (MBL Nagoya).

Mice vaccination

Mice were vaccinated i.p. with 50 μg of OVA adsorbed to alum (50 μl) or CFA (100 μl) or one-tenth of a human dose of the DT/TT vaccine (50 μl; 0.67 flocculation units (Lf) of DT, 0.5 Lf of TT) in a total volume of 200 μl of saline. Boost injections had the same composition (except that IFA was used in place of CFA) and were administered i.p. 2 wk after the first immunization. On days 14, 28, and 56 blood samples were collected by retroorbital puncture.

Determination of Ab titers

OVA- or DT-specific Ig levels in sera were measured by ELISA. Serial dilutions of sera were plated in 96-well plates coated with OVA (10 μg/ml) or DT (2.5 Lf/ml). HRP-conjugated goat anti-mouse IgG1 (Southern Biotech Associates) was added followed by a tetramethylbenzidine substrate. Ag-specific serum IgG1 titers were expressed as the average ± SEM of the reciprocal of the dilution at which absorbance was 0.15 U higher than the strongest negative control reading (preimmune sera). Total IgE levels were measured using a commercial ELISA kit (Bethyl Laboratories).

Statistical analysis

All data were expressed as mean ± SEM. Student's *t* test was used for statistical evaluation of the results. Significance was set at $p < 0.05$.

Results and Discussion

Alum activates the NLRP3-inflammasome

To determine the mechanism through which alum induces caspase-1 activation and secretion of mature IL-1β, BMM from wild-type mice or mice deficient in the NLR molecules NLRP3 and NLRC4 or the adaptor molecule ASC were stimulated with LPS in the presence or absence of alum. Cells were also infected with *Listeria monocytogenes* or *Salmonella typhimurium*, which activate the inflammasome through NLRP3 and NLRC4, respectively (15–18). In agreement with our previous results (8), cells stimulated with LPS alone or alum alone secreted negligible amounts of IL-1β (Fig. 1A), whereas stimulation of wild-type cells with LPS in the presence of alum or infection with *L. monocytogenes* or *S. typhimurium* resulted in the secretion of a

large amount of mature IL-1 β . In contrast, the secretion of IL-1 β in response to LPS plus alum was abolished in NLRP3 $^{-/-}$ and ASC $^{-/-}$ cells but not in NLRC4 $^{-/-}$ cells. IL-1 β secretion was induced to the same extent in wild-type and Nod2 $^{-/-}$ BMM stimulated with LPS plus alum (data not shown). IL-6, whose production does not depend on caspase-1 activation, was secreted at comparable levels by cells stimulated with LPS alone or LPS plus alum and by wild-type, NLRP3 $^{-/-}$ or NLRC4 $^{-/-}$ cells (Fig. 1A), confirming that the observed defects are not due to the potentiation of LPS signaling by alum or to the generalized hyporesponsiveness of NLRP3 $^{-/-}$ cells and are specific for inflammasome-dependent cytokines. Interestingly, IL-6 secretion was compromised in ASC-deficient cells, a phenomenon we previously observed in a human macrophage cell line with knocked-down ASC expression (19), suggesting that ASC may be involved in signaling pathways other than inflammasome activation. Immunoblotting experiments (Fig. 1B) confirmed that the mature form of IL-1 β could be detected in the supernatant of wild-type and NLRC4 $^{-/-}$ cells treated with LPS plus alum but not in supernatants of NLRP3 $^{-/-}$ or ASC $^{-/-}$ cells. Importantly, LPS-stimulated BMM of all four genotypes expressed pro-IL-1 β at comparable levels (Fig. 1B, middle row), indicating that the absence of secreted mature IL-1 β is not due to the hyporesponsiveness of selected BMM but is specific for the inflammasome-dependent maturation step. Caspase-1 activation depends on an autocatalytic processing step of the 45-kDa caspase-1 that generates the p20 and p10 active subunits. As shown in Fig. 1B (lower row), the p20 subunit of caspase-1 could be detected in the supernatant of wild-type or NLRC4 $^{-/-}$ cells treated with alum but not in the supernatant of NLRP3 $^{-/-}$ or ASC $^{-/-}$ cells. Importantly, while IL-1 β secretion was dependent on a concomitant LPS plus alum stimulation, caspase-1 activation was detected in response to alum alone, as we previously showed (8). Thus, NLRP3-mediated caspase-1 activation in response to alum does not require the presence of LPS, whereas pro-IL-1 β expression is dependent on LPS. These results demonstrate that caspase-1 activation and processing and secretion of IL-1 β in response to alum is mediated by the NLRP3-inflammasome and not the NLRC4-inflammasome.

ASC- and NLRP3-knockdown shRNA prevents inflammasome activation by alum in THP-1 macrophage cell lines

The involvement of NLRP3 and ASC in the inflammasome activation by alum was confirmed in the human macrophage cell line THP-1 through the use of shRNA. THP-1 cell lines with silenced or severely diminished ASC and NLRP3 expression were generated (Fig. 2A). A THP-1 cell line that expresses a shRNA specific for mouse ASC sequence not present in the human genome was used as control reference. As shown in Fig. 2B, IL-1 β secretion in response to alum (or ATP, *L. monocytogenes*, or *S. typhimurium*) was abolished in the ASC-shRNA cell line but not the control shRNA cell line. Importantly, expression of a shRNA-resistant form of ASC in the ASC-shRNA cell line rescued the IL-1 β secretion in response to alum (or the other agonists). Alum-induced IL-1 β secretion was also drastically decreased in a THP-1 cell line in which NLRP3 expression was knocked-down through shRNA, confirming NLRP3 involvement in alum effect. Secretion of IL-1 β in response to *L. monocytogenes* or ATP was also abolished in this cell line while the response to *S. typhimurium* was minimally affected, showing

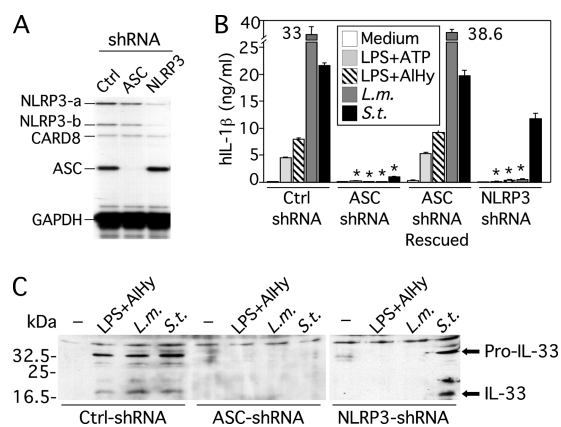


FIGURE 2. ASC- and NLRP3-knockdown shRNA prevents inflammasome activation by alum in THP-1 cell lines. *A*, Expression of ASC or NLRP3 was measured in different THP-1 cell lines by RNase protection assay. NLRP3-a and NLRP3-b are two probes of different sizes, both detecting NLRP3. Ctrl, Control. *B*, Ctrl-, ASC-, and NLRP3-knockdown THP-1 cell lines were stimulated with LPS (10 ng/ml) in the presence of 5 mM ATP, aluminum hydroxide (AlHy; 130 μ g/ml), *L. monocytogenes* (*L.m.*), or *S. typhimurium* (*S.t.*) for 10 h. IL-1 β was measured in conditioned supernatants. One experiment representative of five is shown. Values are mean $\pm$ SEM. Asterisks denotes significant difference vs control shRNA cell line; $p < 0.001$. *C*, THP-1 knockdown cell lines were stimulated as indicated and IL-33 expression was analyzed by immunoblotting in cell supernatants.

the NLRP3 specificity of the shRNA. The inhibition of IL-1 β secretion in the knocked-down THP-1 cell lines occurred at the level of inflammasome activation and was not due to reduced responsiveness of the different cell lines to LPS as demonstrated by the fact that similar levels of pro-IL-1 β were detected in the cytoplasmic extracts of all cell lines stimulated with LPS plus alum (data not shown). Remarkably, secretion of mature IL-33 was also triggered by alum treatment in THP-1 cells and depended on ASC and NLRP3 (Fig. 2C). Thus, inflammasome activation by alum is mediated by NLRP3 in both mouse and human cells.

Inflammasome activation by particulate adjuvants is mediated by NLRP3

We next tested whether inflammasome activation is triggered by other particulate adjuvants (reviewed in Ref. 20). QuilA, a saponin extracted from the bark of the *Quillaja saponaria* tree, is an adjuvant that is incorporated into liposome particles to form the immunostimulating complex "ISCOM". Chitosan, a biodegradable polysaccharide derived from chitin, is another particulate material that is known to act as an adjuvant. The mechanism responsible for QuilA and chitosan adjuvant effects is unknown. As shown in Fig. 3A, QuilA and chitosan particles were able to induce IL-1 β and IL-18 secretion in human PBMC. Secretion of both cytokines in response to alum, QuilA, or chitosan reflected caspase-1 activation, because it was inhibited by the caspase-1 inhibitor Z-YVAD-FMK. In mouse BMM, inflammasome activation by QuilA or chitosan appeared to occur with modalities similar to alum's, because IL-1 β and IL-18 secretion in response to both particles was abolished in NLRP3 $^{-/-}$ cells (Fig. 3B). This result suggests that NLRP3-mediated inflammasome activation and IL-1 β and IL-18 secretion may be a common mechanism of action of particulate adjuvants. Interestingly, particulate adjuvants stimulated the release of low amount of IL-18 independently of LPS,

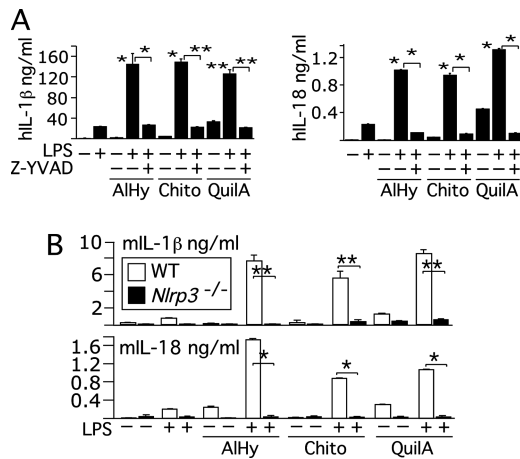


FIGURE 3. Inflammasome activation by particulate adjuvants is mediated by NLRP3. Human PBMC (A) or wild-type (WT) and NLRP3^{-/-} BMM (B) were stimulated for 9 h with LPS (5 ng/ml) in the presence or absence of aluminum hydroxide (AlHy; 130 μg/ml), QuilA (5 μg/ml), or chitosan (Chito) (200 μg/ml). IL-1β or IL-18 was measured in conditioned supernatants (hIL, human IL; mIL, mouse IL). Z-VYAD-FMK (Z-VYAD) is a caspase-1 inhibitor. One experiment representative of two is shown. Values are mean ± SEM. Asterisks denote significant difference vs treatment with LPS alone or treatment in presence of Z-VYAD, as indicated. *, $p < 0.001$; **, $p < 0.005$.

suggesting that IL-18, which is constitutively expressed by several cell types and can stimulate IL-1β transcription, may initiate a proinflammatory cascade during alum vaccination. In agreement with this notion, the level of IL-18 present in the peritoneal lavage (400 μl) of mice injected with alum ($n = 5$) for 24 h was significantly higher ($p < 0.05$) in wild-type mice (168 ± 81 pg/ml) than in NLRP3^{-/-} mice (83 ± 36 pg/ml). IL-1β was not detected. It was recently demonstrated (21) that in vivo some of alum's effects are mediated by uric acid, a NLRP3 activator (22). In our in vitro experiments, secretion of IL-1β by mouse or human cells stimulated with alum, QuilA, or chitosan was not affected by uricase treatment (data not shown), suggesting that in vitro NLRP3-inflammasome activation by particulate adjuvants is not mediated by uric acid. Interestingly, uric acid induces in vitro DC maturation while alum does not (23), although both activate NLRP3. However, in vivo alum does induce DC maturation (21), suggesting that uric acid may mediate this effect by stimulating additional pathways. An additional difference between uric acid and alum is that the former promotes Th1 responses (23) while the latter does not.

Alum adjuvant effect is mediated by NLRP3

We next tested whether the adjuvant effect of alum depends on NLRP3. Wild-type and NLRP3-deficient mice were vaccinated using a pediatric DT/TT vaccine that contains alum as adjuvant. Mice were also vaccinated with chicken OVA mixed to CFA or adsorbed to alum. As shown in Fig. 4A, wild-type mice vaccinated with the DT/TT vaccine developed a potent Ab response characterized by high IgE and DT-specific IgG1 titers (the characteristic Ig profile elicited by alum). In contrast, the titer of total IgE and DT-specific IgG1 was significantly reduced in NLRP3-deficient mice. A similar reduction in total IgE and OVA-specific IgG1 titers was also observed between the wild-type and NLRP3-deficient mice vaccinated with OVA-alum (Fig. 4B). OVA-specific IgG1 titers were not significantly different among wild-type and NLRP3-deficient

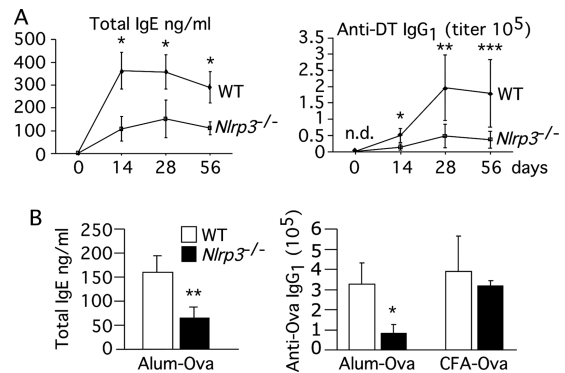


FIGURE 4. Alum adjuvant effect is mediated by NLRP3. Wild-type (WT) ($n = 5$) and NLRP3-deficient mice ($n = 5$) were vaccinated with alum-adsorbed DT (A) or with OVA adsorbed to alum or CFA (B). Boost injections were administered on day 14. Total IgE or DT- and OVA-specific IgG1 were measured in sera obtained at the indicated time points (A) or at 14 days (B). Values are mean ± SEM. *, $p < 0.005$; **, $p < 0.01$; ***, $p < 0.05$; n.d., not detectable.

mice vaccinated with OVA-CFA. As expected, because alum is known to bias the response toward Th2, the OVA- or DT-specific IgG2a titers were modest and were not different between the wild-type and NLRP3-deficient mice groups (not shown).

In conclusion, our results demonstrate that the ability of alum and other particulate adjuvants to induce caspase-1 activation and trigger IL-1β, IL-18, and IL-33 secretion is mediated by the NLRP3-inflammasome. More importantly, we show that alum's adjuvant effect is in good part dependent on NLRP3. The fact that the Ab response of NLRP3-deficient mice is not completely compromised suggests that other pathways activated by alum may contribute to its adjuvant effect. Although our results did not reveal a role for the NLRP3-inflammasome in the CFA adjuvant effect, this possibility could not be ruled out. The powerful stimulation of TLR-mediated signaling by the mycobacterial products found in CFA may in fact be sufficient to elicit the immune response in the absence of NLRP3. The analysis of CFA adjuvant effect in mice deficient in both TLR signaling and inflammasome activation may shed light on the contribution of each pathway to the immune response during vaccination and may provide an explanation for the contrasting results that have been reported regarding the role of TLR signaling in the CFA adjuvant effect (24, 25). One important implication suggested by our results is that cytokines belonging to the IL-1 family may play an important role during alum-assisted vaccination. IL-1β is known to possess adjuvant capacity (10, 11) and to preferentially activate Th2 cells and enhance Ab production (26). IL-33 is particularly active on Th2 cells and mast cells and strongly promotes production of the Th2-associated cytokines IL-4, IL-5, and IL-13 (12), a pattern consistent with the type of immune response elicited by alum. Studies in mice have indicated a role, modest but not negligible, for IL-1β or IL-18 in alum assisted vaccination (27, 28). Because the IL-1-family cytokines share common signaling pathways and overlapping activities, more conclusive results may be obtained by the analysis of compounded knockout mice.

Our results identified for the first time NLRP3 as an important player in alum's adjuvant effect and indicate an important role for the inflammasome in the development of adaptive immunity. The ability to activate the inflammasome must be one

of the properties to be considered during the development of new generation vaccines (3).

Acknowledgments

We are grateful to Vishva Dixit, Genentech, for ASC^{-/-}, and NLRC4^{-/-} mice, to Millenium Pharmaceutical for NLRP3^{-/-} mice, to Peter Murray, St. Jude Children's Hospital, for Nod2^{-/-} bone marrow, to Bernard Metz, The Netherlands Vaccine Institute, for diphtheria toxoid, and Coy Allen for help with animals.

Disclosures

The authors have no financial conflict of interest.

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