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Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants

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Abstract

Aluminium adjuvants, typically referred to as ‘alum’, are the most commonly used adjuvants in human and animal vaccines worldwide, yet the mechanism underlying the stimulation of the immune system by alum remains unknown. Toll-like receptors are critical in sensing infections and are therefore common targets of various adjuvants used in immunological studies. Although alum is known to induce the production of proinflammatory cytokines *in vitro*, it has been repeatedly demonstrated that alum does not require intact Toll-like receptor signalling to activate the immune system^{1,2}. Here we show that aluminium adjuvants activate an intracellular innate immune response system called the Nalp3 (also known as cryopyrin, CIAS1 or NLRP3) inflammasome. Production of the pro-inflammatory cytokines interleukin-1 β and interleukin-18 by macrophages in response to alum *in vitro* required intact inflammasome signalling. Furthermore, *in vivo*, mice deficient in Nalp3, ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain) or caspase-1 failed to mount a significant antibody response to an antigen administered with aluminium adjuvants, whereas the response to complete Freund’s adjuvant remained intact. We identify the Nalp3 inflammasome as a crucial element in the adjuvant effect of aluminium adjuvants; in addition, we show that the innate inflammasome pathway can direct a humoral adaptive immune response. This is likely to affect how we design effective, but safe, adjuvants in the future.

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Shortly after the discovery that alum could be used as an adjuvant in the 1920s (ref. 3), a hypothesis was put forth that alum stimulated an immune response by acting as a 'depot'; antigens were proposed to be slowly released in a particulate form that was favourable for uptake by antigen-presenting cells (APCs), thereby enhancing the immune response to the antigen (reviewed in ref. 4). Since then, many of the signals used by APCs to initiate T-cell responses have been identified along with the immune stimuli (for example, Toll-like receptor (TLR) ligands) required to enhance interactions between APCs and T cells. However, the cellular signalling pathways triggered by alum that induce effective immunity against antigens have remained elusive.

The initiation of adaptive immune responses is controlled by innate immune signals. Regulation of these immune signals relies on a large group of intracellular and extracellular receptors called pattern recognition receptors⁵. The best-described class of these receptors is the TLRs, which sense conserved molecular patterns from a wide range of microbes. Whereas TLRs sense non-self motifs of infectious organisms, another class of intracellular pattern recognition receptors, the NOD-like receptors (NLRs), can sense stimuli of microbial origin as well as endogenous markers of cellular damage (for example ATP or uric acid crystals)^{6,7}. Nalp3, a member of the NLR family, along with ASC (also known as Pycard) and caspase-1, forms a molecular platform called the inflammasome, which regulates the cleavage and release of the potent pro-inflammatory cytokines interleukin (IL)-1 β , IL-18 and IL-33 (ref. 8). One recently described endogenous molecule that activates the Nalp3 inflammasome is crystalline (but not soluble) uric acid (monosodium urate; MSU)^{9–11}.

Aluminium particles of various aluminium adjuvants form insoluble particles that can aggregate, are readily phagocytosed by macrophages and have been shown to stimulate IL-1 β and IL-18 production *in vitro*^{12–15}. We formed the hypothesis that the particulate nature of alum might be recognized by NLRs, much like crystalline MSU. To test whether alum activates the Nalp3 inflammasome, we used primary peritoneal macrophages from mice deficient in critical signalling components of the Nalp3 inflammasome. Because inflammasome activation requires two signals for the production of mature IL-1 β , we first primed macrophages with lipopolysaccharide (LPS) and then exposed them to aluminium adjuvants. Consistent with previous reports^{13–15}, aluminium adjuvants induced the production of IL-1 β and IL-18 from wild-type (C57BL/6; WT) primary murine macrophages (Fig. 1a, d), bone-marrow-derived macrophages (Supplementary Fig. 1a) and bone-marrow-derived dendritic cells (Supplementary Fig. 1b) *in vitro*. IL-1 β secretion was dependent on the dose of alum (Fig. 1b) and peaked between 8 and 10 h of stimulation with alum in WT macrophages, but continued out to 48 h (Fig. 2c and data not shown).

In contrast, macrophages from animals deficient in Nalp3, ASC or caspase-1 failed to produce IL-1 β or IL-18 on stimulation with multiple types of aluminium adjuvant (Fig. 1c, d, Supplementary Fig. 1b and data not shown). Another member of the NLR family, Ipaf (also known as NLRC4), also forms an inflammasome, which is capable of activating caspase-1 in response to several different Gram-negative bacteria^{16,17}. Ipaf-deficient macrophages were fully capable of secreting IL-1 β in response to LPS and alum (Fig. 1c), suggesting that alum-induced IL-1 β secretion is specifically dependent on the Nalp3 inflammasome. Consistent with NLR, but not TLR activation, alum did not induce the

production of IL-6 or tumour necrosis factor (TNF)- α by primary macrophages *in vitro* (Supplementary Fig. 2a, b). Neither of two other common adjuvants, complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA), induced IL-1 β production by macrophages (Fig. 1e).

Caspase-1 activation involves autocatalytic processing of the 45-kDa pro-caspase-1 to generate two subunits, p20 and p10. Caspase-1 activation in LPS-primed WT macrophages stimulated with alum was detected by western blotting by the appearance of the p10 cleavage product 4 h after the addition of alum (Fig. 2a). Consistent with the lack of IL-1 β production, caspase-1 activation was absent in macrophages deficient in Nalp3 and ASC that were exposed to LPS and alum (Fig. 2a, b). Nalp3 knockout macrophages did not show caspase-1 activation or IL-1 β production even at later time points, arguing against delayed caspase-1 activation by alum in the absence of Nalp3 (Fig. 2a, c). These data demonstrate that alum activates macrophages *in vitro* to secrete mature IL-1 β in a manner dependent on the Nalp3 inflammasome.

To understand how alum might stimulate the inflammasome pathway, we first tested whether the endocytic ability of macrophages was required for the alum-stimulated production of IL-1 β . Inhibiting actin or tubulin polymerization with either cytochalasin B or colchicine, respectively, inhibited IL-1 β production by LPS and alum (Fig. 3a) but did not affect secretion of the inflammasome-independent cytokines TNF- α or IL-6 (Supplementary Fig. 2a, b). Neither cytochalasin B nor colchicine decreased IL-1 β production in response to stimulation with ATP, which uses the P2X7 receptor (P2X7R) to activate the Nalp3 inflammasome^{18,19}, confirming that macrophages were still viable and capable of secreting inflammasome-dependent IL-1 β (Fig. 3a).

ATP and MSU released from dying and injured cells into the extracellular milieu may activate the Nalp3 inflammasome^{8,9,19}. *In vitro*, alum induced cell death at very high doses (that did not induce significant IL-1 β) in WT macrophages and in macrophages deficient in Nalp3 and Caspase-1 (Fig. 3b); however, the induction of IL-1 β by alum did not depend on the presence of MSU because the addition of uricase, which degrades MSU crystals and prevents the induction of IL-1 β (ref. 10), had no effect on IL-1 β production in response to LPS and alum (Fig. 3c). To exclude the possibility that Nalp3 inflammasome activation was in response to ATP release caused by alum-induced cellular damage, we used macrophages from P2X7R knockout mice. P2X7R-deficient macrophages showed no defect in IL-1 β production after stimulation with LPS and alum (Fig. 3d). Taken together, these data support a model of active endocytosis of alum by viable macrophages leading to Nalp3 inflammasome activation with the resultant secretion of pro-inflammatory cytokines.

Although several diverse stimuli activate the Nalp3 inflammasome, the efflux of cellular potassium seems to be a common step shared by these stimuli and is required for Nalp3-dependent caspase-1 activation; it has therefore been suggested that the inflammasome acts as a sensor of cellular membrane disruption⁷. Preventing this potassium efflux by increasing extracellular potassium inhibits inflammasome activation with a variety of Nalp3 triggers²⁰. Indeed, increased extracellular potassium significantly inhibited alum-induced IL-1 β production from macrophages (Fig. 3e) but not the LPS-dependent production of TNF- α or

IL-6 (data not shown). Pannexin pores are thought to have a function in inflammasome activation induced by ATP, nigericin or maitotoxin, possibly by facilitating potassium efflux²¹. We did not detect a significant difference in IL-1 β secretion between macrophages exposed to a pannexin-pore-blocking peptide and those exposed to a scrambled peptide. Therefore we do not currently have evidence that pannexin pores mediate alum-induced inflammasome activation. Alum is therefore a new Nalp3 trigger and, like other triggers, may induce inflammasome activation through membrane disruption with resultant potassium efflux.

Aluminium adjuvants are used in human vaccines to induce a potent humoral response; alum is also used as a potent adjuvant to induce T helper type 2 (T_H2)-mediated inflammation in murine allergy/asthma models. Given the Nalp3-dependent activation of macrophages that we observed *in vitro*, we tested whether immunity in mice against a model protein antigen, ovalbumin, required a functional Nalp3 inflammasome. Ovalbumin-specific IgG1 antibody induction was significantly decreased in Nalp3-deficient, ASC-deficient and caspase-1-deficient mice primed intraperitoneally with ovalbumin and alum (Fig. 4a) or subcutaneously with another protein antigen, human serum albumin (HSA) in alum (Supplementary Fig. 3), but was not affected in MyD88 knockout mice (Supplementary Fig. 4; ref. 2). We tested whether Nalp3 and ASC knockout mice have a general antibody-production defect by immunizing them with the adjuvant CFA. Ovalbumin-specific IgG2c (Fig. 4b) and IgG1 (not shown) in Nalp3 and ASC knockout mice were equivalent to levels in WT mice but, as expected, CFA-induced ovalbumin-specific IgG2c was completely dependent on MyD88 (Fig. 4b).

T_H2 cell priming was also impaired in Nalp3, ASC and caspase-1 knockout mice as demonstrated by decreased airway eosinophilia and hilar lymph-node IL-5 production in an alum-dependent model of asthma (Fig. 4c, d). The overall inflammation was decreased in these knockout mice without evidence of a switch to a T_H1 response (typically characterized by airway neutrophilia and IgG2c induction). Consistent with previous reports, alum-induced T_H2 responses are not affected in mice lacking MyD88 (ref. 2) or lacking both MyD88 and TRIF (ref. 1; Fig. 4c and Supplementary Fig. 4). Previous studies have suggested that antigen must be physically associated with (although not necessarily adsorbed on) alum for it to have an adjuvant effect²². Indeed, we saw a significantly impaired antibody response (Supplementary Fig. 5a) and an absence of T_H2 inflammation in the airways when alum and ovalbumin were injected separately into the peritoneum (Supplementary Fig. 5b). In mouse cells, but not in human cells, there is a clear requirement *in vitro* for two signals to activate the inflammasome and to produce pro-IL-1 β (LPS and alum), yet it is not clear what is providing the first signal for alum *in vivo* (or other Nalp3 stimuli including MSU). We have preliminary evidence from *in vitro* studies that IL-1 β itself can prime macrophages for alum-induced inflammasome activation (data not shown); these results are consistent with previous reports that IL-1 β can act in an autocrine manner to induce its own gene expression²³. Other groups have similarly seen macrophage priming with cytokines (for example TNF- α) instead of LPS¹³. Combining the above information with the fact that alum must be encountered simultaneously with antigen *in vivo* for efficient priming suggests that the antigen might provide the first signal either directly, or indirectly

by inciting the production of local pro-inflammatory cytokines from resident monocytes or specialized cells recruited by alum²⁴. Once the first signal has primed the cell, alum provides the second signal for activation of the Nalp3 inflammasome. These two stimuli must be sensed by the same cell for effective immune activation, thereby increasing the specificity of an immune response and perhaps explaining why alum (which readily adsorbs antigens) is such an effective adjuvant.

Thus, by eliminating signalling through the Nalp3 inflammasome, we have eliminated one critical pathway used by alum to initiate humoral and cellular immunity. In doing so, aluminium hydroxide adjuvants ‘hijack’ an innate immune pathway that is exquisitely sensitive to cellular damage, perhaps as a result of the similarity to MSU in its physical structure. Although intraperitoneal MSU induces peritonitis⁹ and subcutaneous MSU in concert with antigen injection has been used *in vivo* to initiate CD8 T-cell responses¹⁰, we predicted, on the basis of our findings, that this Nalp3 stimulant would also induce a significant antibody response to a protein antigen. Indeed, MSU injected intraperitoneally with antigen induces an IgG1-type antibody response similar in nature to that induced by alum in WT mice but not in Nalp3-deficient mice (Supplementary Fig. 6). These mice did not develop a significant T_H1-type antibody response (IgG2c) under these immunization conditions (data not shown), suggesting that MSU and alum induce a similar pattern of inflammation when injected at similar doses in the same location.

A critical question regarding the mechanism by which alum influences immunity is how alum initiates lymphocyte activation and how it favours T_H2 differentiation over T_H1 differentiation. Inflammasome-dependent cytokines have been implicated in various aspects of T_H2 responses: IL-1 has classically been thought to promote T_H2 cell proliferation^{25,26}, IL-33 is a potent pro-T_H2 stimulus²⁶ and IL-18 has been shown to augment IgE antibody production (although it primarily potentiates T_H1 responses)²⁷. On the basis of our *in vitro* findings, we would predict that local production of IL-1 β , IL-18 and/or IL-33 could induce the requisite signals for activation of the adaptive immune system. Indeed, we found a lower expression of *Il1b* mRNA from peritoneal cells of Nalp3-deficient mice immunized with ovalbumin and alum than in WT mice (Supplementary Fig. 7). In further support of an IL-1-dependent model, the antibody response in another immunization model has been shown to be defective in IL-1 α /IL-1 β knockout mice as the result of a defect in the induction of CD40L on T cells by activated APCs²⁸. However, there is no antibody production or T_H2 defect after alum priming in MyD88 knockout mice. MyD88 is critical in the IL-1 receptor signalling cascade²⁹, although one recent study has identified a MyD88-independent IL-1 pathway³⁰. It will therefore be of interest to study the relative roles of IL-1 family members in alum-dependent priming in future work. In addition, as new functions of caspase-1 and the inflammasome are uncovered, we will further understand how stimulation of this potent pro-inflammatory machinery results in activation of the adaptive immune response.

METHODS SUMMARY

Mice

The generation of mice deficient in Nalp3, ASC, Ipaf, caspase-1 and P2X7R has been reported previously^{8,16,18}. Nalp3-deficient, Caspase-1-deficient and ASC-deficient mice

were backcrossed nine generations, and Ipaf-deficient mice were backcrossed six generations onto a C57BL/6 background. Age-matched and sex-matched C57BL/6 mice from the National Cancer Institute were used as all WT controls. All protocols used in this study were approved by the Yale Institutional Animal Care and Use Committee.

Macrophages

The generation of thioglycollate-elicited peritoneal and bone-marrow-derived macrophages and bone-marrow-derived dendritic cells has been described previously^{2,8}. Unless indicated, macrophages were primed by stimulating with 50 ng ml⁻¹ LPS from *Escherichia coli* serotype 0111:B4 (Invivogen) for 16–18 h before stimulation with Imject alum (unless otherwise indicated), MSU or ATP. For ATP-stimulated cells, the medium was changed at 20 min and all stimulants were replaced. Macrophage cell death was measured by the release of lactate dehydrogenase with a cytotoxicity detection kit (Promega).

Sensitizations

For intraperitoneal sensitization, 6–8-week-old mice were injected intraperitoneally on day 0 with 50 µg of ovalbumin (Grade V; Sigma) adsorbed on 4 mg of Imject alum and again on day 10 with 25 µg of ovalbumin adsorbed on 4 mg of Imject alum. Mice were challenged intranasally with 25 µg of ovalbumin in PBS on days 21, 22 and 23. Mice were killed for analysis on day 25. For subcutaneous sensitization, mice were injected subcutaneously on day 0 with 50 µg of ovalbumin in 400 µg (180 µl) of CFA and again on day 10 with 25 µg of ovalbumin in 180 µl of IFA.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Aluminium-containing adjuvants stimulate macrophages to produce the pro-inflammatory cytokines IL-1 β and IL-18 in a Nalp3 inflammasome-dependent manner
a, Macrophages were stimulated with 50 ng ml $^{-1}$ LPS for 18 h and then 500 μ g ml $^{-1}$ Inject alum ('Alum1') or aluminium hydroxide gel ('Alum2') for 8 h. IL-1 β released into culture supernatants was measured by ELISA with a minimum detection level of 200 pg ml $^{-1}$. **b**, LPS-primed macrophages were stimulated with the indicated amount of Inject alum for 8 h and analysed as in **a**. **c**, Unprimed or LPS-primed WT, ASC-deficient, Nalp3-deficient, caspase-1-deficient (Casp1) and Ipaf-deficient macrophages were stimulated with Inject

alum ($500 \mu\text{g ml}^{-1}$) for 8 h, and the IL-1 β released into the culture supernatants was measured by ELISA. **d**, WT or Nalp3-deficient macrophages were stimulated as in **c**, and the IL-18 released was measured by ELISA. **e**, WT or Nalp3-deficient LPS-primed macrophages were stimulated with either Imject alum, CFA ($120 \mu\text{g ml}^{-1}$) or IFA ($30 \mu\text{l ml}^{-1}$) for 8 h and analysed as in **a**. Determinations were performed in triplicate and are expressed as means and s.d.; data are from one of at least three independent experiments.

Figure 2. Caspase-1 activation by aluminium adjuvants requires Nalp3 and ASC

a, b, LPS-primed or unprimed macrophages from WT mice (**a, b**), Nalp3-deficient mice and caspase-1-deficient (Casp1) mice (**a**) and from ASC-deficient mice (**b**) were stimulated with Inject alum (500 $\mu\text{g ml}^{-1}$) for the indicated durations, and cell lysates were immunoblotted with antibodies against the p10 subunit of caspase-1. Macrophages from caspase-1 knockout mice were stimulated as indicated to provide a reference for some of the non-specific bands seen with this antibody. Indeed, the band at 15 kDa in the Nalp3 knockout samples in **a** and in all samples in **b** is distinct from the p10 band representing active caspase-1. **c**, LPS-primed macrophages from WT or Nalp3-deficient mice were stimulated with Inject alum (500 $\mu\text{g ml}^{-1}$) for the indicated durations, and the IL-1 β released into culture supernatants was measured by ELISA. Results are shown as means $\pm$ s.d. from one of three independent experiments.

Figure 3. Alum requires intact endocytic macrophage machinery and causes potassium-gradient-dependent IL-1 β secretion without causing significant cell death

a, LPS-primed peritoneal macrophages were treated with either colchicine (28 μ g ml $^{-1}$) or cytochalasin B (10 μ M) for 1 h before the addition of Imject alum (500 μ g ml $^{-1}$), ATP (5 mM) or MSU (200 μ g ml $^{-1}$). **b**, Lactate dehydrogenase (LDH) release was measured from LPS-primed WT, Nalp3-deficient and caspase-1-deficient (Casp1) macrophage culture supernatants stimulated with the indicated amounts of Imject alum. **c**, Unprimed or LPS-primed WT macrophages were stimulated for 8 h with either Imject alum (500 μ g ml $^{-1}$) or

MSU ($200 \mu\text{g ml}^{-1}$) in the presence or absence of 2 U ml^{-1} uricase. **d**, LPS-primed macrophages from WT or P2X7R-deficient (P2X7) mice were stimulated with Imject alum ($500 \mu\text{g ml}^{-1}$) or ATP (5 mM) and samples were analysed as in **a**. **e**, Unprimed or LPS-primed WT or Nalp3-deficient macrophages were stimulated with Imject alum in serum-free buffer with either 150 mM NaCl or 150 mM KCl and analysed as in **a**. Determinations were performed in triplicate and are expressed as means and s.d.; data are from one of at least three independent experiments.

Figure 4. Antibody production and T_H2-dependent inflammation induced by aluminium adjuvants are decreased in the absence of Nalp3, ASC and caspase-1

a, WT, Nalp3-deficient, ASC-deficient or caspase-1-deficient (Casp1) mice (three to five mice per group) 6–8 weeks old were injected intraperitoneally with ovalbumin adsorbed on Imject alum on day 0 and again on day 10. Mice were challenged intranasally with ovalbumin on days 21, 22 and 23. Sera were collected from mice on day 25 and analysed for ovalbumin-specific IgG1 by ELISA as described previously². Asterisk, $P < 0.03$; nonparametric Mann–Whitney U -test. **b**, WT, Nalp3-deficient, ASC-deficient or MyD88-deficient mice (three to five mice per group) were primed subcutaneously with ovalbumin in CFA on day 0 and on day 10 in IFA. Sera were collected on day 21 and analysed for ovalbumin-specific IgG2c by ELISA. **c**, Three to five mice per group were primed and challenged as in **a**; bronchoalveolar lavage was collected on day 25 and analysed as

described previously² (see Methods) (total cell number; means and s.d. are shown). Asterisk, $P < 0.03$; nonparametric Mann–Whitney U -test. **d**, Lung draining (hilar) lymph nodes were collected from WT and Nalp3-deficient mice primed and challenged as in **a** and pooled within each group for restimulation; cells were restimulated *in vitro* with (+) or without (–) $200 \mu\text{g ml}^{-1}$ ovalbumin and mitomycin-C-treated splenocytes for 48 h. Supernatants were analysed for IL-5 (filled bars) or IFN- γ (open bars).