Frontline

The Nlrp3 inflammasome is critical for aluminium hydroxide-mediated IL-1β secretion but dispensable for adjuvant activity

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Aluminum hydroxide (alum) is the most widely used adjuvant in human vaccines, but the immune mechanisms that are activated by alum remain poorly understood. Alum has recently been shown to promote caspase-1 activation and IL-1β secretion, but the cellular pathways involved remain elusive. Here we report that the release of IL-1β triggered by alum is abrogated in macrophages deficient in the NLR family, pyrin domain containing 3 (Nlrp3) protein and the apoptosis-associated speck-like protein containing a caspase recruitment domain (Asc) but not the NLR family, CARD domain containing 4 (Nlrc4) protein. The requirement of the Nlrp3 inflammasome was specific for IL-1β in that secretion of TNF-α was independent of Nlrp3 or Asc. Consistently, processing of pro-caspase-1 induced by alum was abolished in macrophages lacking Nlrp3 or Asc. Unlike caspase-1 processing and IL-1β secretion triggered by LPS, alum-mediated activation of the inflammasome did not require exogenous ATP. Importantly, induction of IgG production against human serum albumin by alum was unimpaired in mice deficient in Nlrp3. These results indicate that alum induces IL-1β *via* the Nlrp3 inflammasome but this activity is dispensable for alum-mediated adjuvant activity.

Key words: Adjuvant · Aluminum hydroxide · Caspase-1 · NOD-like receptor

See accompanying article by De Gregorio *et al.*

Introduction

Aluminum hydroxide, typically referred as "alum", and other aluminum salts are the most widely used adjuvants in human and animal vaccines [1]. Despite the routine use of alum, the immune responses that it induces and the mechanisms that mediate its ability to boost antigen-specific antibody production have remained poorly understood. Several investigators have proposed that alum activates immune responses by acting as a "depot" that promotes the slow release of antigen as a particulate form and its uptake by antigen-presenting cells [2]. In addition, alum is known to induce inflammatory responses which may serve to recruit and activate antigen-presenting cells [3, 4]. Finally, recent studies have suggested that alum promotes the recruitment of monocytes to draining lymph nodes through the production of uric acid [5]. However, the cellular signaling pathways by which alum induces immune responses have remained elusive.

The activation of adaptive immunity including humoral responses to antigens is controlled by innate immunity signaling pathways. Agonists to several Toll-like receptors (TLR) are known to act as adjuvants for antigen-specific antibody responses [6]. TLR recognize multiple microbial moieties and activate innate immune responses *via* NF-κB, MAPK and type-1 interferons [7, 8]. There is evidence that TLR signaling pathways are important for adjuvant activity induced by TLR agonists including LPS [9]. However, the role of TLR signaling in mediating boosting antibody responses has

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recently been challenged [10]. An alternative cellular system involved in the recognition of microbial molecules is that mediated *via* NOD-like receptors (NLR) [11, 12]. Unlike TLR, the NLR protein family senses microbial molecules in the cytosol [13–15]. NLR family, pyrin domain containing 3 (Nlrp3, also called Nalp3 or cryopyrin) is an NLR protein that is critical for caspase-1 activation and secretion of IL-1β and IL-18 in response to multiple microbial molecules and endogenous urate crystals [16–18]. We have therefore investigated a role for Nlrp3 in the activation of caspase-1 and adjuvant activity induced by alum.

Results and discussion

Alum induces IL-1β secretion through the Nlrp3 inflammasome

Recent studies have shown that alum promotes IL-1β secretion in dendritic cells, but the mechanism involved is unknown [3, 4]. To determine the mechanism, we assessed the role of the Nlrp3 inflammasome in cytokine responses induced by alum in wild-type and Nlrp3-deficient macrophages. In the absence of exogenous ATP, stimulation of wild-type macrophages with LPS did not induce the secretion of IL-1β, which is consistent with previous results [17, 19]. Notably, alum elicited release of IL-1β in the absence of ATP stimulation in macrophages primed with LPS (Fig. 1A). Secretion of IL-1β by alum was inhibited more than 90% in macrophages lacking Nlrp3 or the apoptosis-associated speck-like protein containing a caspase recruitment domain (Asc), an

adaptor required for caspase-1 activation *via* Nlrp3 (Fig. 1A). The role of the Nlrp3 inflammasome in the alum-mediated IL-1β secretion was specific in that the production of TNF-α was unimpaired (Fig. 1B). These results indicate that the Nlrp3 inflammasome mediates IL-1β secretion in response to alum independently of ATP stimulation.

The Nlrp3 inflammasome is required for caspase-1 activation induced by alum

We next tested the role of the Nlrp3 inflammasome in caspase-1 activation, a step that is required for IL-1β release [20, 21]. To assess caspase-1 activation, extracts were prepared from macrophages stimulated with alum and immunoblotted with an antibody that recognizes the p20 subunit of caspase-1. Stimulation of macrophages with LPS or alum alone did not induce the processing of pro-caspase-1 into the mature p20 subunit (Fig. 2). However, co-stimulation of macrophages with LPS and alum, which elicited IL-1β release, triggered caspase-1 activation as did co-stimulation with LPS and ATP, a potent stimulus of pro-caspase-1 processing (Fig. 2). Importantly, activation of caspase-1 induced by alum was abrogated in LPS-primed macrophages lacking Nlrp3 or Asc, but not the NLR family, CARD domain containing 4 (Nlrc4) protein (Fig. 2). These results indicate that alum promotes caspase-1 activation *via* the Nlrp3 inflammasome.

Nlrp3 is dispensable for adjuvant activity *in vivo*

To determine the role of Nlrp3 in adjuvant activity, we tested the ability of alum to serve as an adjuvant for the production of IgG against human serum albumin (HSA), a T cell-dependent antigen. WT and Nlrp3$^{-/-}$ mice were immunized with HSA i.p. in the presence of alum, and boosted with HSA after 2 wk. High titers of HSA-specific IgG1, IgG2a, IgG2b and IgG3 were detected in the serum of wild-type mice but not in unimmunized mice (Fig. 3). Significantly, comparable enhancement of the anti-HSA IgG responses was observed in Nlrp3$^{-/-}$ animals (Fig. 3). These results indicate that Nlrp3 is dispensable for alum-mediated IgG adjuvant activity.

Our results indicate that alum triggers activation of the Nlrp3 inflammasome, leading to release of IL-1β, which is consistent with reports showing that alum induces IL-1β secretion from dendritic cells [3, 4]. Alum alone was insufficient to trigger caspase-1 activation in that it requires priming or co-stimulation with LPS. Unlike TLR or Nod2 agonists, alum induced caspase-1 activation *via* Nlrp3 in the absence of ATP stimulation. Thus, the results suggest that alum acts in a similar manner as ATP to promote caspase-1 activation *via* the Nlrp3 inflammasome. In the case of muramyl dipeptide, ATP mediates its release from acidified vesicles to the cytosol *via* the P2X7 receptor and the pannexin-1 pore to trigger Nlrp3-dependent caspase-1 activation [14]. Similarly, we suggest that alum is internalized in acidified vesicles and mediates the release of microbial molecules or endogenous
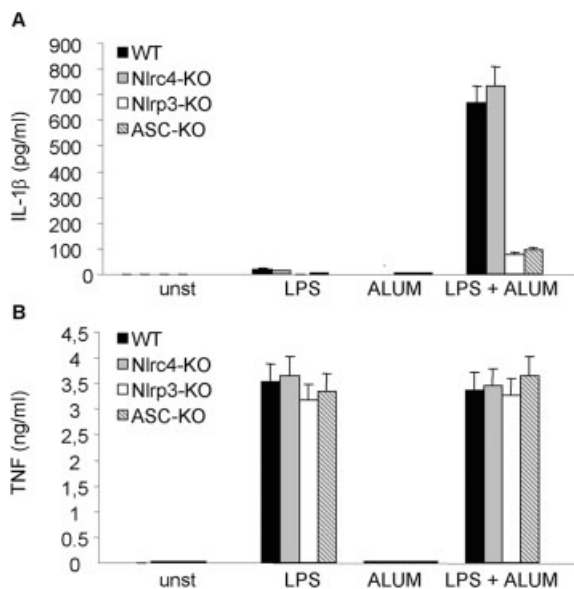

Figure 1. Alum-induced IL-1β production is Nlpr3-dependent. Bone-marrow derived macrophages form WT, Nlrp3-KO, Nlrc4-KO and Asc-KO mice were stimulated with LPS (1 μg/mL), alum (250 μg/mL) or LPS + alum for 6 h. Cell-free supernatants were analyzed for production of IL-1β (A) and TNF-α (B) by ELISA. Results are representative of three separate experiments.
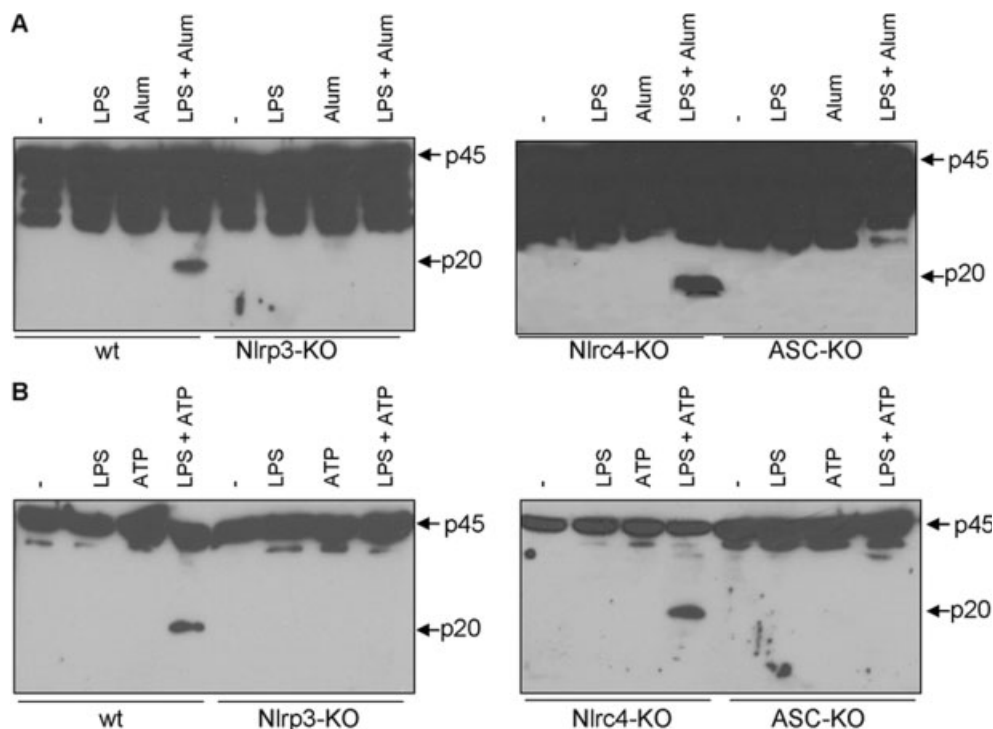
Figure 2. Alum-induced caspase-1 activation is Nlpr3- and Asc-dependent. Bone-marrow derived macrophages from WT, Nlrp3-KO, Nlrc4-KO and Asc-KO mice were stimulated for 6 h with alum (250 µg/mL), LPS (1 µg/mL) or LPS + alum (A); or stimulated for 6 h with LPS (1 µg/mL) $\pm$ ATP (5 mM) for the last 30 min (B). Extracts were prepared from cell and culture supernatants and immunoblotted with caspase-1 antibody. Arrows denote pro-caspase-1 (p45) and its processed p20 subunit. Results are representative of three separate experiments.

molecules leading to inflammasome activation. Because uricase treatment has been shown to abolish the ability of alum to enhance monocyte-driven stimulatory responses [5], it is possible that alum promotes inflammasome activation by delivering uric acid to the cytosol. Alternatively, alum may disrupt acidified vesicles which may trigger induction of reactive oxygen species or other cellular signals to activate the Nlrp3 inflammasome [22].

Consistent with our results, Eisenbart *et al.* [23] recently reported that alum promotes caspase-1 activation and IL-1β secretion through the Nlrp3 inflammasome. In contrast to our results, however, these authors found a critical role for the Nlrp3 inflammasome in the induction of IgG1 adjuvant activity by alum [23]. We do not have a clear explanation for the difference in results, although it may be due to the use of different immunization protocols. We immunized mice with antigen in alum i.p., boosted the animals with antigen i.p. and serum was collected at day 35. In contrast, Eisenbart *et al.* immunized with antigen in alum twice i.p., challenged the animals with antigen intranasally three times (on days 21, 22 and 23) and serum was collected at day 25. Further studies are needed to clarify the role of the Nlrp3 inflammasome in cellular responses induced by alum and the mechanisms by which it promotes boosting of humoral immunity.

Concluding remarks

The Nlrp3 inflammasome was found to mediate caspase-1 activation and IL-1β release induced by alum in LPS-primed macrophages. However, Nlrp3 was not required for alum-mediated IgG adjuvant activity against HSA, a T cell-dependent antigen.

Material and methods

Mice and cells

Mice deficient in Nlrp3, Nlrc4 and Asc have been described previously [24–26]. Mice were housed in a pathogen-free facility. Bone marrow-derived macrophages were isolated as previously described [27]. The animal studies were conducted under approved protocols by the University of Michigan Committee on Use and Care of Animals.

Immunoblotting

Cells were lysed together with the cell supernatant by the addition of 1% NP-40, complete protease inhibitor cocktail (Roche, Mannheim, Germany) and 2 mM dithiothreitol. Clarified lysates
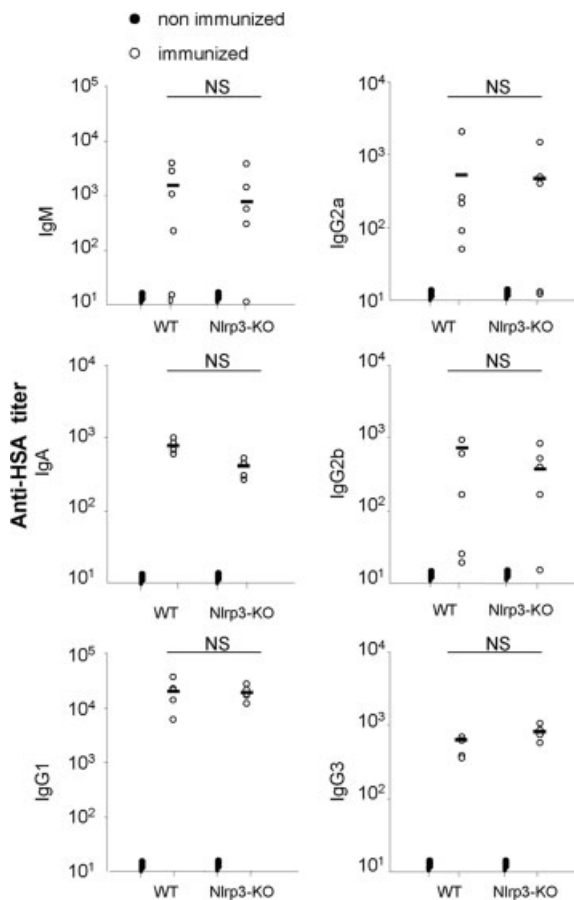
Figure 3. Alum-induced Ig antibody production is Nlpr3-independent. Twelve-week-old mice were immunized with a mixture of HSA (100 μg in PBS) and alum by i.p. injection on day 0 and boosted with antigen on day 21. Blood samples were obtained on day 35 and antibody titers measured in serum. Each point represents the serum antibody titer to HSA for one individual mouse. Results are representative of three independent experiments; NS, not significant.

were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes by electro-blotting. The rabbit anti-mouse caspase-1 antibody was a kind gift from Dr. Vandanabeele (Ghent University, Ghent, Belgium). LPS from *Escherichia coli* (O111B) and alum were from Sigma.

Immunization of mice and serum Ig analysis

Groups of mice were immunized with a mixture of alum (100 μL) and HSA (clinical grade, 100 μg in PBS) by i.p. injection. Mice were boosted with HSA (100 μg) after 21 days. Serum samples were obtained 2 wk after the boost. Diluted sera from immunized animals were incubated on HSA-coated ELISA plates at 100 μg/mL, and bound antibody detected using biotinylated isotype-specific antibodies (Southern Biotechnology) followed by p-nitrophenyl phosphate substrate. Antibody titers were calculated by plotting the dilution sera that gave half-maximal signal. When no

signal was detected, a dilution of 10 (IgM), 100 (IgG1), 10 (IgG2a), 10 (IgG2b), 10 (IgG3) and 10 (IgGA) was assigned.

Measurements of cytokines

Mouse cytokines were measured in culture supernatants with ELISA kits (R&D Systems, Minneapolis, MN). Assays were performed in triplicate for each independent experiment.

Statistical analysis

For all experiments, the difference between groups was calculated using the Mann–Whitney U-test for unpaired data. Difference were considered significant when $p < 0.05$.

References

1 **Lindblad, E. B.,** Aluminium compounds for use in vaccines. *Immunol. Cell Biol.* 2004. **82:** 497–505.

2 **Mannhalter, J. W., Neychev, H. O., Zlabinger, G. J., Ahmad, R. and Eibl, M. M.,** Modulation of the human immune response by the non-toxic and non-pyrogenic adjuvant aluminium hydroxide: Effect on antigen uptake and antigen presentation. *Clin. Exp. Immunol.* 1985. **61:** 143–151.

3 **Sokolovska, A., Hem, S. L. and HogenEsch, H.,** Activation of dendritic cells and induction of CD4(+) T cell differentiation by aluminum-containing adjuvants. *Vaccine* 2007. **25:** 4575–4585.

4 **Li, H., Nookala, S. and Re, F.,** Aluminum hydroxide adjuvants activate caspase-1 and induce IL-1beta and IL-18 release. *J. Immunol.* 2007. **178:** 5271–5276.

5 **Kool, M., Soullie, T., van Nimwegen, M., Willart, M. A., Muskens, F., Jung, S., Hoogsteden, H. C. et al.,** Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. *J. Exp. Med.* 2008. **205:** 869–882.

6 **Ishii, K. J. and Akira, S.,** Toll or toll-free adjuvant path toward the optimal vaccine development. *J. Clin. Immunol.* 2007. **27:** 363–371.

7 **Akira, S., Uematsu, S. and Takeuchi, O.,** Pathogen recognition and innate immunity. *Cell* 2006. **124:** 783–801.

8 **Uematsu, S. and Akira, S.,** Toll-like receptors and type I interferons. *J. Biol. Chem.* 2007. **282:** 15319–15323.

9 **Pasare, C. and Medzhitov, R.,** Control of B-cell responses by Toll-like receptors. *Nature* 2005. **438:** 364–368.

10 **Gavin, A. L., Hoebe, K., Duong, B., Ota, T., Martin, C., Beutler, B. and Nemazee, D.,** Adjuvant-enhanced antibody responses in the absence of Toll-like receptor signaling. *Science* 2006. **314:** 1936–1938.

11 **Franchi, L., Park, J. H., Shaw, M. H., Marina-Garcia, N., Chen, G., Kim, Y. G. and Nunez, G.,** Intracellular NOD-like receptors in innate immunity, infection and disease. *Cell. Microbiol.* 2008. **10:** 1–8.

12 **Franchi, L., McDonald, C., Kanneganti, T. D., Amer, A. and Nunez, G.,** Nucleotide-binding oligomerization domain-like receptors: Intracellular pattern recognition molecules for pathogen detection and host defense. *J. Immunol.* 2006. **177:** 3507–3513.

13 **Kim, Y. G., Park, J. H., Shaw, M. H., Franchi, L., Inohara, N. and Nunez, G.,** The cytosolic sensors Nod1 and Nod2 are critical for bacterial recognition and host defense after exposure to Toll-like receptor ligands. *Immunity* 2008. **28:** 246–257.

14 **Marina-Garcia, N., Franchi, L., Kim, Y. G., Miller, D., McDonald, C., Boons, G. J. and Nunez, G.,** Pannexin-1-mediated intracellular delivery of muramyl dipeptide induces caspase-1 activation *via* cryopyrin/NLRP3 independently of Nod2. *J. Immunol.* 2008. **180:** 4050–4057.

15 **Kanneganti, T. D., Lamkanfi, M., Kim, Y. G., Chen, G., Park, J. H., Franchi, L., Vandenabeele, P. and Nunez, G.,** Pannexin-1-mediated recognition of bacterial molecules activates the cryopyrin inflammasome independent of Toll-like receptor signaling. *Immunity* 2007. **26:** 433–443.

16 **Martinon, F., Petrilli, V., Mayor, A., Tardivel, A. and Tschopp, J.,** Gout-associated uric acid crystals activate the Nalp3 inflammasome. *Nature* 2006. **440:** 237–241.

17 **Mariathasan, S., Weiss, D. S., Newton, K., McBride, J., O'Rourke, K., Roose-Girma, M., Lee, W. P. et al.,** Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* 2006. **440:** 228–232.

18 **Kanneganti, T. D., Ozoren, N., Body-Malapel, M., Amer, A., Park, J. H., Franchi, L., Whitfield, J. et al.,** Bacterial RNA and small antiviral compounds activate caspase-1 through cryopyrin/Nalp3. *Nature* 2006. **440:** 233–236.

19 **Sutterwala, F. S., Ogura, Y., Szczepanik, M., Lara-Tejero, M., Lichtenberger, G. S., Grant, E. P., Bertin, J. et al.,** Critical role for Nalp3/CIAS1/cryopyrin in innate and adaptive immunity through its regulation of caspase-1. *Immunity* 2006. **24:** 317–327.

20 **Qu, Y., Franchi, L., Nunez, G. and Dubyak, G. R.,** Nonclassical IL-1 beta secretion stimulated by P2X7 receptors is dependent on inflammasome activation and correlated with exosome release in murine macrophages. *J. Immunol.* 2007. **179:** 1913–1925.

21 **Brough, D. and Rothwell, N. J.,** Caspase-1-dependent processing of pro-interleukin-1beta is cytosolic and precedes cell death. *J. Cell Sci.* 2007. **120:** 772–781.

22 **Dostert, C., Petrilli, V., Van Bruggen, R., Steele, C., Mossman, B. T. and Tschopp, J.,** Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science* 2008. **320:** 674–677.

23 **Eisenbarth, S. C., Colegio, O. R., O'Connor, W., Sutterwala, F. S. and Flavell, R. A.,** Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. *Nature* 2008. DOI 10.1038/nature06939.

24 **Kanneganti, T. D., Body-Malapel, M., Amer, A., Park, J. H., Whitfield, J., Franchi, L., Taraporewala, Z. F. et al.,** Critical role for cryopyrin/Nalp3 in activation of caspase-1 in response to viral infection and double-stranded RNA. *J. Biol. Chem.* 2006. **281:** 36560–36568.

25 **Franchi, L., Amer, A., Body-Malapel, M., Kanneganti, T. D., Ozoren, N., Jagirdar, R., Inohara, N. et al.,** Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1beta in *Salmonella*-infected macrophages. *Nat. Immunol.* 2006. **7:** 576–582.

26 **Ozoren, N., Masumoto, J., Franchi, L., Kanneganti, T. D., Body-Malapel, M., Erturk, I., Jagirdar, R. et al.,** Distinct roles of TLR2 and the adaptor ASC in IL-1beta/IL-18 secretion in response to *Listeria monocytogenes*. *J. Immunol.* 2006. **176:** 4337–4342.

27 **Amer, A., Franchi, L., Kanneganti, T. D., Body-Malapel, M., Ozoren, N., Brady, G., Meshinchi, S. et al.,** Regulation of *Legionella* phagosome maturation and infection through flagellin and host Ipaf. *J. Biol. Chem.* 2006. **281:** 35217–35223.

Abbreviations: **Asc:** apoptosis-associated speck-like protein containing a caspase recruitment domain · **Alum:** aluminum hydroxide · **HSA:** human serum albumin · **NLR:** NOD-like receptor · **Nlrc4:** NLR family, CARD containing 4 · **Nlrp3:** NLR family, pyrin domain containing 3

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