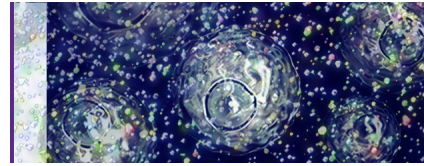


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## Aluminum Hydroxide Adjuvants Activate Caspase-1 and Induce IL-1 $\beta$ and IL-18 Release

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Hanfen Li, Suba Nookala and Fabio Re

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# Aluminum Hydroxide Adjuvants Activate Caspase-1 and Induce IL-1 $\beta$ and IL-18 Release<sup>1</sup>

Hanfen Li, Suba Nookala, and Fabio Re<sup>2</sup>

**Aluminum hydroxide (Alum) is the only adjuvant approved for routine use in humans, although the basis for its adjuvanticity remains poorly understood. In this study, we show that Alum activates caspase-1 and induce secretion of mature IL-1 $\beta$  and IL-18. Human PBMC or dendritic cells stimulated with pure TLR4 and TLR2 agonists released only traces of IL-1 $\beta$  or IL-18, despite the fact that the IL-1 $\beta$  mRNA was readily induced by both TLR agonists. In contrast, cells costimulated with TLR agonists plus Alum released large amount of IL-1 $\beta$  and IL-18. Alum-induced IL-1 $\beta$  and IL-18 production was not due to enhancement of TLR signaling but rather reflected caspase-1 activation and in mouse dendritic cells occurred in a MyD88-independent fashion. Secretion of other proinflammatory cytokines such as IL-8 was not affected by Alum treatments. However, TLR-induced production of IL-10 was increased and that of IFN- $\gamma$ -inducible protein decreased by Alum cotreatment. Considering the immunostimulatory activities of these cytokines and the ability of IL-1 $\beta$  to act as adjuvant, our results suggest a mechanism for the adjuvanticity of Alum. *The Journal of Immunology*, 2007, 178: 5271–5276.**

**T**he achievement of protective immunity through vaccination depends in great part on the activation of the appropriate Ag-specific T cells. This process is regulated primarily by signaling events emanating from the TCR (signal 1) and costimulatory molecules (signal 2). A third signal has been proposed to exist in the form of proinflammatory cytokines, such as IL-1 $\beta$  and IL-12, produced by activated APCs and acting directly on T cells (signal 3) (1).

During the early phase of a natural infection, recognition of microbial products by pattern recognition receptors (PRR)<sup>3</sup> such as TLR expressed on APC is responsible for the generation of signal 2 and signal 3 (reviewed in Ref. 2). In contrast, in the artificial setting of vaccination, triggering of signal 2 and signal 3 can be achieved through the use of adjuvants (reviewed in Ref. 3), a variegated group of compounds that includes oil emulsions, surfactants, synthetic or natural bacterial products, and mineral compounds such as aluminum hydroxide (Alum) gels. Unfortunately, the most potent adjuvants also possess high toxicity, which prevents their use for human vaccination. For several decades, aluminum phosphate and Alum have been widely used as adjuvants in humans, representing an acceptable compromise between toxicity and adjuvanticity (reviewed in Ref. 4). Alternative adjuvants, such as derivatives of muramyl dipeptide (MDP), monophosphoryl lipid

A, liposomes, QS21, MF-59, and immunostimulating complexes have been developed and are being evaluated for use in humans (reviewed in Ref. 5).

Despite the routine use of Alum, its mechanism of action remains somewhat elusive. It is believed that adsorption of the Ag onto the Alum-insoluble particles leads to the formation of a depot of Ag that is released slowly, prolonging the time of interaction between Ag and APC. This notion however has been challenged by several reports that demonstrated that the adsorbed Ag is released from Alum particles within hours of the injection (6, 7). It is believed that adsorption of the Ag on Alum allows it to be presented in particulate form, which appears to be more antigenic and can be efficiently phagocytosed. Although these features are likely determinants of Alum adjuvanticity, other mechanisms may be involved, possibly some that directly lead to the generation of signal 2 and signal 3.

In fact, it has been demonstrated that Alum is capable of activating the complement cascade (8), and injection of Alum leads to formation of an inflammatory focus that attracts immunocompetent cells, thus resulting in granulomas that contain Abs producing plasma cells (9). Interestingly, the extent of the Ab response has been shown to be dependent and proportional to the local inflammatory reaction (10). Still, it is unclear how this inflammatory response is instigated by Alum. Inability of Alum to induce dendritic cell (DC) activation and production of cytokines (11) suggests that Alum is incapable of directly triggering the generation of signal 2 or signal 3 through stimulation of PRR such as TLR. It has been proposed (12), though not proved, that somehow Alum may damage cells through a cytotoxic mechanism leading to leakage of cytosolic “danger signals” that eventually instigate inflammation through TLR. Whether this scenario exists in vivo is unknown, but it clearly does not occur in vitro (11).

IL-1 $\beta$ , a prototypic proinflammatory cytokine, displays pleiotropic biological effects (reviewed in Ref. 13) and has been shown to possess adjuvant activity (14, 15) and proposed to represent a third signal for activation of naive T cells (1). Production of bioactive IL-1 $\beta$  is regulated at many levels, including the transcription and stability of the mRNA, the translation of the precursor immature pro-IL-1 $\beta$ , maturation, and secretion. Maturation of pro-IL-1 $\beta$  requires a proteolytic cleavage that is conducted by caspase-1 and is

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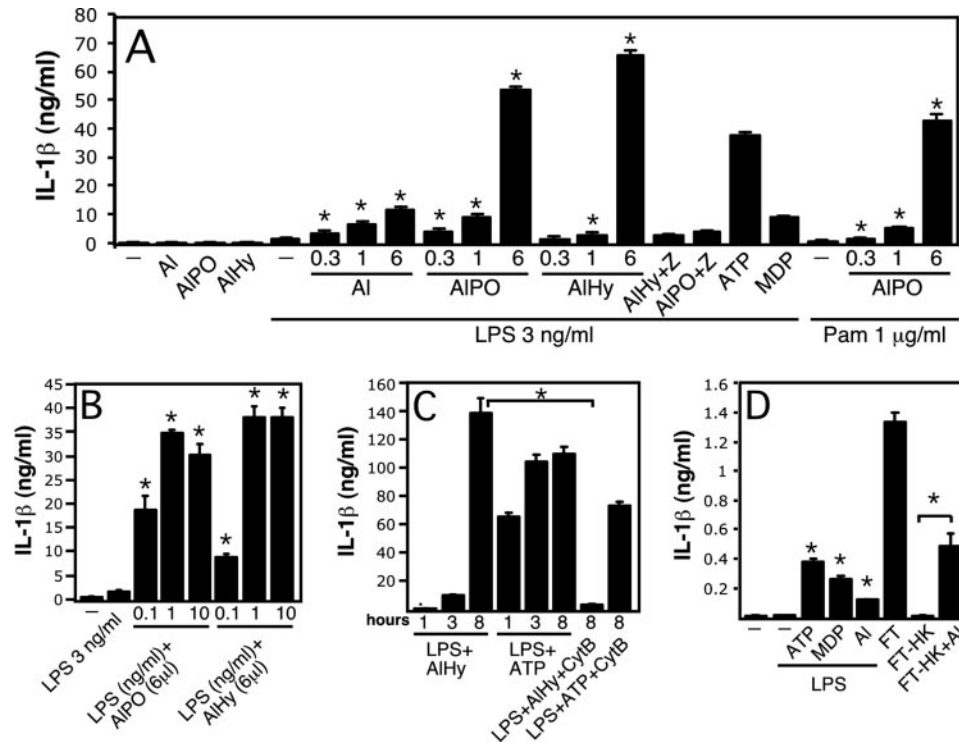
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<sup>3</sup> Abbreviations used in this paper: PRR, pattern recognition receptor; MSU, monosodium urate; Alum, aluminum hydroxide; MDP, muramyl dipeptide; DC, dendritic cell; IP-10, IFN- $\gamma$ -inducible protein.

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**FIGURE 1.** Alum adjuvants induce IL-1 $\beta$  release. Human PBMC (A–C) or DC (D) were stimulated with pure TLR4 or TLR2 agonists (LPS at 3 ng/ml or 0.1, 1, and 10 ng/ml as indicated, and synthetic Pam<sub>3</sub>Cys, 1  $\mu$ g/ml) in the presence or absence of Alum adjuvants (in the amount of microliters per 0.5 ml of culture). Mature IL-1 $\beta$  was measured in conditioned supernatants 24 h later (A and B). C, Human PBMC were stimulated for 18 h with LPS (3 ng/ml), resuspended in fresh medium and stimulated with Alhydrogel (AlHy, 6  $\mu$ l/0.5 ml culture) or ATP (5 mM). Aliquots of medium were removed at the indicated time for IL-1 $\beta$  measurement. D, Human DC were stimulated with LPS (3 ng/ml), or live or heat-killed *F. tularensis* (FT-HK) in the presence or absence of Alum (2  $\mu$ l). Mature IL-1 $\beta$  was measured in conditioned supernatants 24 h later. Data are triplicate  $\pm$  SEM. \*,  $p < 0.05$  vs LPS, Pam<sub>3</sub>Cys, or heat-killed *F. tularensis* treatments alone (A, B, and D) or as indicated in C. For all stimulation, ATP concentration was 5 mM and MDP 10  $\mu$ g/ml. Al, Alum Imject; AIPO, aluminum phosphate; CytB, cytochalasin B; Z, Z-YVAD.

necessary for optimal activity and secretion. Caspase-1 is part of a multiprotein complex known as inflammasome (reviewed in Refs. 16, 17) that is also involved in the processing of IL-18, a cytokine with structural similarities to IL-1 $\beta$ .

Monosodium urate (MSU) crystals were recently found to represent an endogenous danger signal (18) capable of activating the Nalp3 inflammasome leading to activation of caspase-1 and release of mature IL-1 $\beta$  and IL-18 (19). The crystalline nature of Alum prompted us to test its ability to act in a manner similar to urate crystals and stimulate IL-1 $\beta$  and IL-18 production.

The present study demonstrates for the first time that Alum can activate caspase-1 inducing the release of mature IL-1 $\beta$  and IL-18 in cells activated by TLR agonists. Considering the immunostimulatory activities of these cytokines and the ability of IL-1 $\beta$  to act as adjuvant our results suggest a mechanism for the adjuvanticity of Alum.

## Materials and Methods

### Reagents

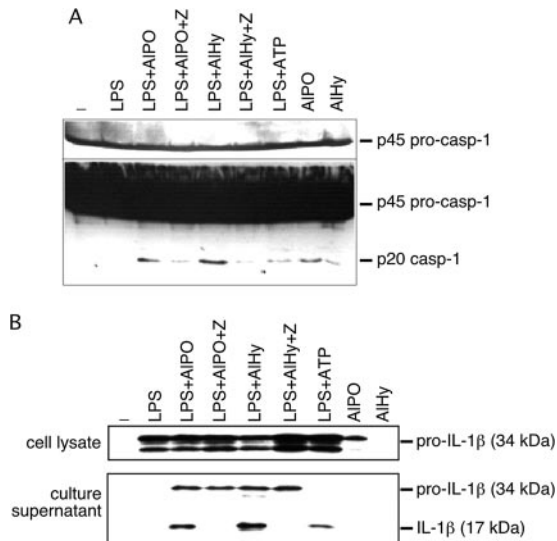
Three formulations of Alum were tested. Imject Alum (Pierce) is a mixture of Alum and magnesium hydroxide (40 mg/ml). Alhydrogel (Sigma-Aldrich) is an Alum gel (13 mg/ml). Adju-Phos (Brenntag) is an aluminum phosphate gel (2 mg/ml). LPS (*Escherichia coli* K12 LCD25) was from List Biological Laboratories. It was purified from contaminant lipoproteins normally found in commercially available LPS preparations by double phenol extraction. Triacylated synthetic lipopeptide Pam<sub>3</sub>-Cys-Ala-Gly was purchased from Bachem. Caspase-1 inhibitor Z-YVAD-FMK was from Alexis Biochemicals and was used at 10  $\mu$ M. Cytochalasin B was from Sigma-Aldrich and was used at 10  $\mu$ M. Rabbit polyclonal anti-caspase-1 antiserum (06-503) was from Upstate Biotechnology. Anti-IL-1 $\beta$  mAb

3ZD was from the National Cancer Institute Biological Resources Branch Preclinical Repository (Frederick, MD).

### PBMC, monocytes, and DCs isolation and stimulation

Human PBMC were isolated from Leukopacks by Ficoll-Histopaque density gradient centrifugation. Monocytes were purified from human PBMC using MACS CD14 microbeads (Miltenyi Biotec). Purity was checked by staining with a FITC conjugated anti-CD14 Ab (Sigma-Aldrich) and FACScan analysis, and routinely found to be higher than 94%. Immature DC were obtained by incubating monocytes at  $3 \times 10^6$ /ml in RPMI 1640/10% FCS supplemented with recombinant human GM-CSF (10 ng/ml) and recombinant human IL-4 (10 ng/ml) (both from R&D Systems) for 8 days. Fresh complete medium was replaced on day 4. For stimulations,  $5 \times 10^6$  PBMC, or  $1 \times 10^6$  DC, were plated in 24-well plates in 0.5 ml of RPMI 1640/10% FCS and stimulated with the various agonists as detailed in experiments. For the experiment in Fig. 1D, DC were infected with live or heat-killed *Francisella tularensis* at a multiplicity of infection of 100. All experiments were repeated at least three times using cells derived from different donors.

The stably transfected HeLa-TLR4/MD-2 cell lines was previously described (20). To obtain mouse DCs, the femur/tibiae were removed and freed of muscles and tendons. Bone ends were cut with scissors and bone marrow cells were flushed out of the bone cavity with Dulbecco PBS using a syringe with a 25-gauge needle. The cell suspension was filtered through a 70- $\mu$ m cell strainer, washed once and resuspended in RPMI 1640/10% FCS supplemented with penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), and recombinant murine GM-CSF (20 ng/ml; R&D Systems). The cell suspension was plated at  $1 \times 10^6$  cells/ml in 100-mm petri dishes. The medium was replaced on day 4 and suspension cells collected and stimulated on day 8. This procedure routinely results in  $>80\%$  CD11c<sup>+</sup> cells. The use of animals for these experiments was approved by the University of Tennessee Animal Care and Use Committee.



**FIGURE 2.** Alum adjuvants activate caspase-1. Human PBMC were stimulated with LPS in the presence or absence of Alum adjuvants (6  $\mu$ l/0.5 ml culture) or 5 mM ATP for 20 h. *A*, Cleaved caspase-1 p20 subunit was detected in conditioned supernatant by immunoblot. The *top blot* shows a shorter exposure of the membrane. *B*, Pro-IL-1 $\beta$  and mature IL-1 $\beta$  were detected in cell lysates (*top*) or conditioned supernatants (*bottom*) by immunoblot. AIPO, Aluminum phosphate; AIHy, Alhydrogel; Z, Z-YVAD.

#### RNase protection assay

Total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies). RNase protection assay was performed using 4  $\mu$ g of total RNA using BD Pharmingen Riboquant kit as previously described (20). The human CK-2b, human CK-5, multiprobe template sets were used.

#### Cytokine measurements

Cytokine levels in conditioned supernatants were measured by ELISA using the following paired Abs kits: human IL-1 $\beta$ , IL-8, IL-10, IL-18, and IFN- $\gamma$ -inducible protein (IP-10) and murine IP-10 (R&D Systems), IL-1 $\beta$ , and IL-6 (eBioscience).

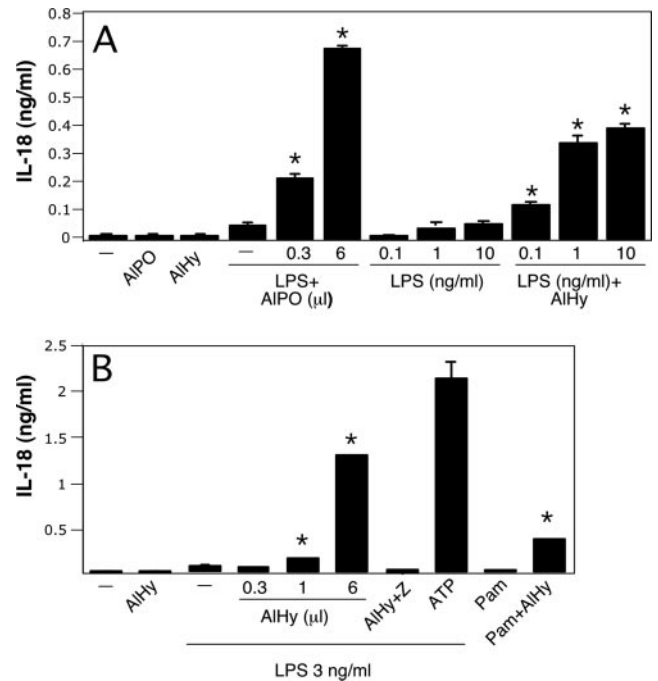
#### Statistical analysis

All data were expressed as mean  $\pm$  SEM. Comparison of groups for statistical difference were done by using Student's two-tailed *t* test. Significance was set at *p* < 0.05.

## Results

### Alum adjuvants induce IL-1 $\beta$ secretion in cells primed with pure TLR agonists

Although Alum adjuvants have been used for a long time, surprisingly little is known about the mechanisms by which they enhance the immune response. We decided to test the ability of Alum to activate caspase-1 and induce release of mature IL-1 $\beta$  and IL-18. Three different commercial Alum preparations were compared: Imject Alum (40 mg/ml), Alhydrogel (13 mg/ml), and Adju-Phos (2 mg/ml). Because the concentration of the active compounds in each preparation varies considerably, we compared the activity of equal volumes of each preparation rather than equal quantities of the active compound. Human PBMC were stimulated with pure TLR4 or TLR2 agonists (pure LPS or synthetic lipopeptide Pam<sub>3</sub>Cys) in absence or presence of increasing amounts of the Alum, and mature IL-1 $\beta$  was measured in culture supernatants (Fig. 1). Despite robust IL-1 $\beta$  mRNA induction (see below and Fig. 4A), stimulation with pure LPS or Pam<sub>3</sub>Cys alone was unable to induce release of an appreciable amount of mature IL-1 $\beta$  in culture supernatants (Fig. 1A), as previously demonstrated by our



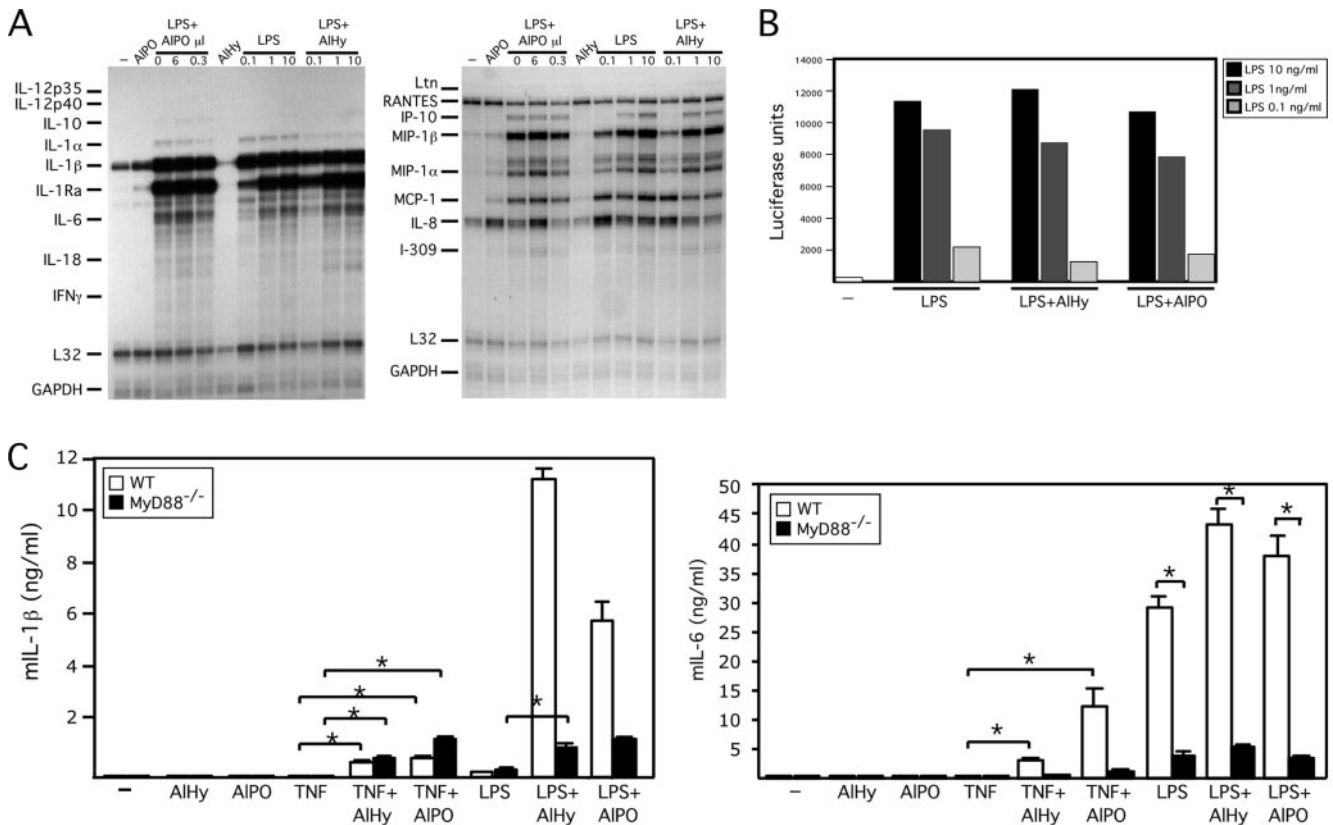
**FIGURE 3.** Alum adjuvants induce IL-18 release. Human PBMC were stimulated with pure TLR4 or TLR2 agonists at the indicated concentration in the presence or absence of Alum adjuvants (in the amount of microliters per 0.5 ml of culture). Where not indicated, LPS was used at 3 ng/ml, Pam<sub>3</sub>Cys at 1  $\mu$ g/ml, and Alhydrogel (AIHy) at 6  $\mu$ l/0.5 ml culture. IL-18 was measured in conditioned supernatants after 6 (*A*) or 24 h (*B*). Data are triplicate  $\pm$  SEM. \*, *p* < 0.05 vs LPS or Pam<sub>3</sub>Cys. AIPO, aluminum phosphate; Z, Z-YVAD.

group (21) and other groups (22). In contrast, stimulation with LPS or Pam<sub>3</sub>Cys in the presence of Alum adjuvants strongly triggered the release of IL-1 $\beta$ . Similar results were obtained using freshly purified human monocytes (data not shown). Among the three Alum adjuvants tested, Imject Alum repeatedly displayed the lowest potency despite containing the highest amount of active compound. IL-1 $\beta$  secretion induced by Alum occurred in a dose dependent fashion (Fig. 1, *A* and *B*). Mature IL-1 $\beta$  was not detected in the supernatants of cells treated with Alum adjuvants alone. However, isolation of PBMC using a different procedure (Isolymp instead of Ficoll density gradient) yielded cells that occasionally secreted IL-1 $\beta$  in response to Alum stimulation in absence of concomitant TLR stimulation. This response was likely due to activation of the PBMC during isolation with Isolymp.

Alum treatment was as efficient at inducing IL-1 $\beta$  release as ATP and MDP, two known caspase-1 activators and inducers of IL-1 $\beta$  secretion. However, the induction of IL-1 $\beta$  release by Alum occurred with a slower kinetic than that induced by ATP (Fig. 1C). This result, together with the finding that disruption of the actin cytoskeleton with cytochalasin B abrogated IL-1 $\beta$  induction by Alum but not ATP (Fig. 1C), suggests that activation of caspase-1 by Alum and ATP may proceed through distinct routes and may require phagocytosis of the Alum particles.

We recently demonstrated that the intracellular bacterium *F. tularensis* is able to induce release of a large amount of IL-1 $\beta$  from human or mouse DC by stimulating TLR2 and activating caspase-1 (21). In contrast, the heat-killed form of the bacterium, although still able to activate TLR2 and induce IL-1 $\beta$  mRNA, did not stimulate IL-1 $\beta$  release. This result was due to its inability to escape the phagosome and invade the cytoplasm where caspase-1 activation occurs. Therefore, we tested the effect of Alum on human DC stimulated with heat-killed *F. tularensis* and found that this treatment restored IL-1 $\beta$  production in these cells (Fig. 1D).





**FIGURE 4.** The effects of Alum adjuvants is not due to potentiation of TLR signaling and is independent of MyD88. *A*, Human PBMC were stimulated for 6 h with LPS (3 ng/ml) or 0.1, 1, and 10 ng/ml (as indicated) in the presence or absence of Alum adjuvants Alhydrogel (AlHy, 6  $\mu$ l) or aluminum phosphate (AlPO, in the indicated amounts as microliter per 0.5 ml of culture). Total RNA was extracted and used for RNase protection assay. *B*, HeLa-TLR4/MD-2 stable cell line was transiently transfected with ELAM-luciferase reporter construct, CMV-CD14 and CMV- $\beta$ -Gal (for normalization) and stimulated for 6 h with increasing amounts of LPS in the presence or absence of Alum adjuvants. NF- $\kappa$ B activation was measured by luciferase assay. *C*, Bone marrow DCs from wild-type and MyD88<sup>-/-</sup> mice were stimulated for 20 h with LPS (5 ng/ml) or TNF- $\alpha$  (40 ng/ml) in the presence or absence of Alum adjuvants (6  $\mu$ l/0.5 ml culture). Mature IL-1 $\beta$  or IL-6 was measured in conditioned supernatants. Data are triplicate  $\pm$  SEM. \*,  $p < 0.05$ . Z, Z-YVAD.

#### Alum adjuvants activate caspase-1

IL-1 $\beta$ -release in cells treated with Alum reflected caspase-1 activation as it was blocked by cotreatment with Z-YVAD-FMK, a caspase-1 specific inhibitor (Fig. 1A). Caspase-1 activation was confirmed by detection of the cleaved p20 subunit in the conditioned supernatant of PBMC treated with Alum (or ATP) but not LPS alone (Fig. 2A). Interestingly, Alum induced the secretion of the p20 caspase-1 subunit even in absence of concomitant TLR stimulation suggesting that caspase-1 activation by Alum occurs independently of TLR stimulation. This notion was also supported by the finding (Fig. 2B) that intracellular pro-IL-1 $\beta$  was equally induced by LPS regardless of the presence or absence of Alum. In contrast, the mature form of IL-1 $\beta$  was detected only in the supernatant of cells treated with both LPS and Alum.

#### Alum adjuvants induce secretion of IL-18

As expected for genuine caspase-1 activators, Alum adjuvants were also able to stimulate PBMC to release IL-18 (Fig. 3), a cytokine belonging to the IL-1 superfamily and whose secretion similarly requires caspase-1 activation.

#### Alum adjuvant effect is not due to potentiation of TLR signaling and is independent of MyD88

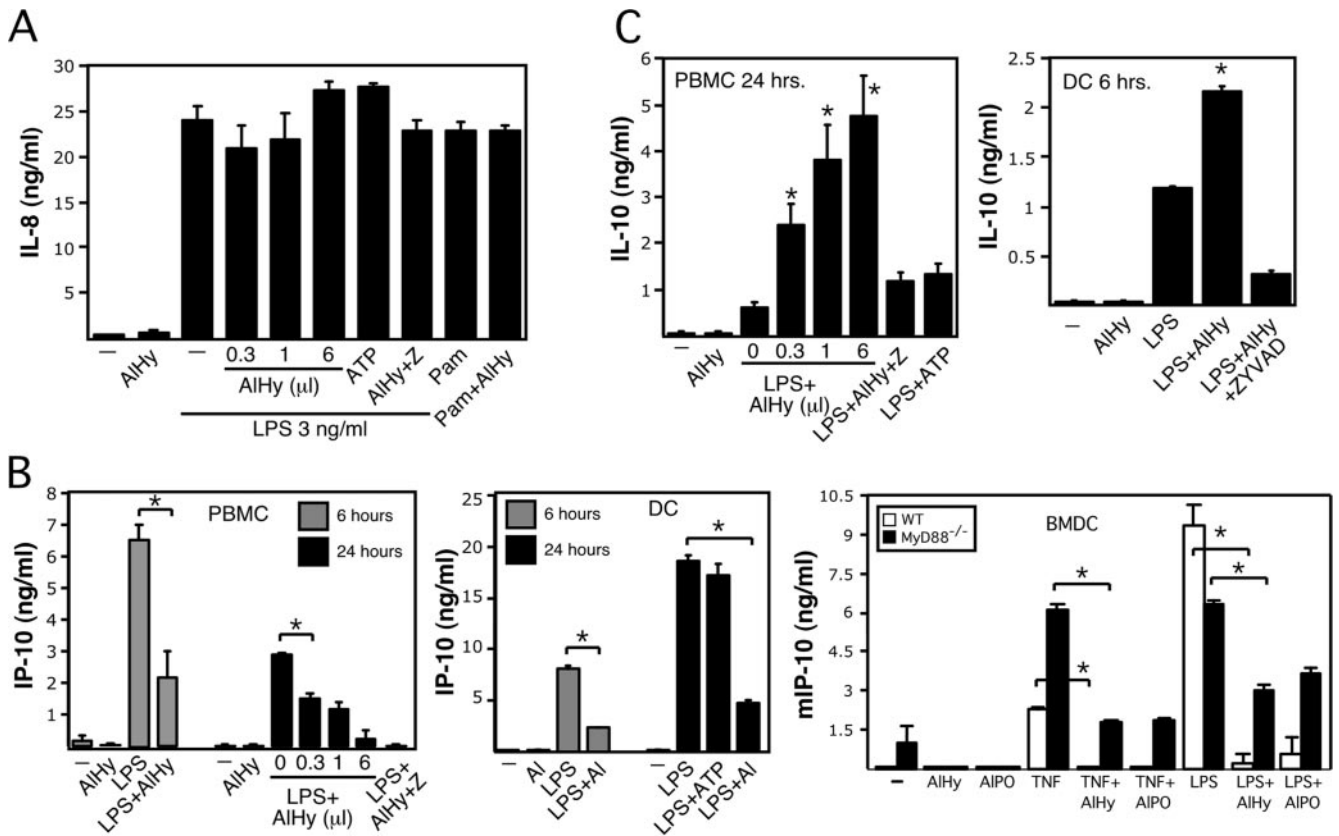
The results shown in Fig. 4A demonstrate that the effect of Alum adjuvants on IL-1 $\beta$  production was not at the level of mRNA induction. Alum alone was unable to induce the transcript of IL-1 $\beta$

and of several other cytokines and chemokines, and did not enhance the induction of these mRNAs by LPS or Pam<sub>3</sub>Cys in human PBMC. Similarly, Alum did not potentiate TLR4-mediated NF- $\kappa$ B-driven luciferase production in HeLa cell lines stably transfected with TLR4/MD-2 (Fig. 4B).

Alum adjuvants were able to induce release of IL-1 $\beta$  even in mouse DCs primed with LPS or TNF- $\alpha$  (Fig. 4C). In further support of the notion that the effect of Alum on IL-1 $\beta$  secretion is not mediated in any way by TLR, Alum-induced IL-1 $\beta$  secretion was observed also in MyD88-deficient DCs concomitantly stimulated with TNF- $\alpha$  (which does not signal through MyD88), but not, as expected, in cells stimulated with LPS (which requires MyD88 for induction of most cytokines). Production of IL-6 in response to TNF- $\alpha$  stimulation was also increased by Alum cotreatment (Fig. 4C). This effect was dependent on MyD88.

#### Alum adjuvants affect IP-10 and IL-10 production in opposite ways

Secretion of other proinflammatory cytokines such as IL-8 was not affected by Alum treatments, again suggesting that Alum adjuvant does not potentiate TLR signaling (Fig. 5A). However, TLR-induced production of IL-10 and IP-10 was affected in opposite ways by Alum cotreatment. Production of IP-10 by human or mouse cells stimulated with LPS or TNF- $\alpha$  was severely inhibited by Alum costimulation (Fig. 5B). This effect was observed even in MyD88-deficient bone marrow DC and was not blocked by the



**FIGURE 5.** Alum adjuvants do not enhance TLR-mediated IL-8 production but affect IL-10 and IP-10 production in opposite ways. Human PBMC, DC, or mouse bone marrow DC were stimulated as described in Figs. 1A and 4C for the indicated time points, and IL-8 (A), IP-10 (B), or IL-10 (C) were measured in conditioned supernatants. If not indicated, LPS was used at 3 ng/ml, Alum Inject (Al) at 2  $\mu$ l/0.5 ml culture, and Alhydrogel (AlHy) at 6  $\mu$ l/0.5 ml culture. Data are triplicate  $\pm$  SEM. \*,  $p < 0.05$  vs LPS, or as indicated.

caspase-1 inhibitor Z-YVAD, indicating that it occurred independently of the autocrine action of IL-1 $\beta$  and suggesting that Alum may activate other signaling pathways that counteract IP-10 production. The effect of Alum on IP-10 appears to occur at the post-transcriptional level because the induction of IP-10 mRNA by LPS was not affected by Alum cotreatment (see Figs. 4A and 5B; RNA and supernatants are from the same cell culture). In contrast to the inhibition of IP-10, Alum adjuvants increased production of IL-10 in human cells (Fig. 5C). Up-regulation of IL-10 by Alum requires caspase-1 activity, suggesting that it may be mediated by the autocrine action of secreted IL-1 $\beta$  or IL-18. IL-10 mRNA, which is expressed at much lower level than IL-1 $\beta$  and is visualized only over prolonged exposure of the gel shown in Fig. 4A, was weakly super-induced by LPS plus Alum treatment (data not shown), suggesting that IL-10 up-regulation occurs at least in part at the transcriptional level.

## Discussion

Our study revealed for the first time the ability of Alum adjuvants to induce caspase-1 activation and trigger IL-1 $\beta$  and IL-18 release, suggesting a highly plausible mechanism that explains Alum adjuvanticity. The ability of IL-1 $\beta$  to act as adjuvant is well documented. Several studies have demonstrated that IL-1 $\beta$  provides a necessary and sufficient signal to support T cell response to Ag in the absence of adjuvants (1, 15, 23, 24). In particular, IL-1 $\beta$  was found to specifically promote CD4 $^{+}$ , but not CD8 $^{+}$ , T cell responses, whereas the opposite was shown for IL-12. Furthermore IL-1 $\beta$  can activate Th2 cells and enhance Ab production (25). It is interesting to note that Alum is notoriously a poor stimulator of

cytotoxic responses while it successfully sustains Th2 types of responses. It is tempting to speculate that these features are related to the ability of Alum to promote IL-1 $\beta$  release. Indeed, it has been demonstrated that T cell response to Alum-adsorbed tetanus toxoid is partly dependent on IL-1 $\beta$  (26). IL-18 was originally identified as an IFN- $\gamma$ -inducing factor but later found to induce either Th1 or Th2 polarization depending on the immunologic context. Thus, although IL-18 cooperates with IL-12 to promote Th1 polarization, paradoxically, in absence of IL-12 it induces IgE expression and Th2 differentiation (27). Our finding that Alum stimulates production of IL-18 therefore is consistent with the Th2-polarizing activity of Alum and with a study that demonstrated a role for IL-18 in Alum-assisted vaccination (28). Finally, our discovery of the ability of Alum adjuvants to enhance production of IL-10, a Th2 cytokine, and inhibit that of IP-10, a chemokine specific for Th1 cells, also seems consistent with Alum support of humoral immunity. Despite the evidence of the involvement of IL-1 $\beta$  and IL-18 in Alum-assisted vaccination, it has been demonstrated that Alum retains some adjuvanticity in caspase-1-deficient mice (29), suggesting the existence of additional mechanisms.

It is unclear at present the mechanism that leads to caspase-1 activation by Alum, but some observations suggest that it may be different from the recently reported caspase-1 activator MSU. Although MSU induces DC maturation (18), costimulatory and MHC class II molecules are not up-regulated by Alum treatment (11 and our unpublished observations). In addition, MSU was shown to be a powerful inducer of Th1 responses (18), whereas Alum induces responses biased toward Th2. These disparities may suggest that the Nalp3 inflammasome, which mediates response to MSU (19),

may not be involved in Alum recognition. However, the NLR family of cytoplasmic PRR (16, 17), to which Nalp3 belongs, is composed of several proteins that can link pattern recognition to caspase-1 activation. Members of this family remain likely candidates for mediating (directly or indirectly) the effects of Alum.

An important question that remains to be answered is how the IL-1 $\beta$  mRNA is induced during vaccination with Alum adjuvants. Our results clearly show that Alum by itself is incapable of promoting IL-1 $\beta$  transcription. It is possible that Alum may act on cells that already contain traces of IL-1 $\beta$  messenger or that the vaccine injection itself may provide a stimulus for transcription of the IL-1 $\beta$  gene, whose promoter is highly responsive to several types of cellular stress. Interestingly, IL-18 is constitutively expressed at low levels by a variety of cells such as epithelial cells, macrophages, and DC, and is capable of inducing IL-1 $\beta$  (30). Thus, it is possible that Alum-induced IL-18 release at the injection site may occur in absence of classical proinflammatory stimulation. The released IL-18 would then be able to act on several cell types to induce IL-1 $\beta$  transcription leading to the amplification of Alum effects.

It is interesting to note that Alum powder resuspended in PBS at the same concentration found in Inject Alum was unable to stimulate IL-1 $\beta$  release (data not shown). This result should come as no surprise because it is known (4) that several physicochemical parameters, such as particle size distribution, electrical charge, and the hydrated colloid nature of the precipitate, affect the adjuvanticity of Alum preparations and were responsible for the batch-to-batch variability that was observed decades ago when Alum started to be manufactured in large scale. This effect may also explain why aluminum particles were reported to be unable to activate the inflammasome (19). It should also be mentioned that Alum treatment did not affect cell viability, as measured by trypan blue dye exclusion or a cytotoxic assay that measures LDH release, and did not induce apoptosis as judged by DNA fragmentation gel electrophoresis (data not show).

A deeper understanding of the mechanisms that determine the immunostimulatory properties of adjuvants is a prerequisite for the rational design of more sophisticated vaccines. Our study identifies for the first time activation of caspase-1 and stimulation of release of IL-1 $\beta$  and IL-18 as likely determinants of the action of Alum, the only adjuvants allowed for human use.

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## Disclosures

The authors have no financial conflict of interest.

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