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Cutting Edge

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# Cutting Edge: Alum Adjuvant Stimulates Inflammatory Dendritic Cells through Activation of the NALP3 Inflammasome

Mirjam Kool,* Virginie Pétrilli,† Thibaut De Smedt,‡ Aline Rolaz,‡ Hamida Hammad,*§ Menno van Nimwegen,* Ingrid M. Bergen,* Rosa Castillo,† Bart N. Lambrecht,[1,2]*§ and Jürg Tschopp[1,2]†

**Adjuvants are vaccine additives that stimulate the immune system without having any specific antigenic effect of itself. In this study we show that alum adjuvant induces the release of IL-1β from macrophages and dendritic cells and that this is abrogated in cells lacking various NALP3 inflammasome components. The NALP3 inflammasome is also required in vivo for the innate immune response to OVA in alum. The early production of IL-1β and the influx of inflammatory cells into the peritoneal cavity is strongly reduced in NALP3-deficient mice. The activation of adaptive cellular immunity to OVA-alum is initiated by monocytic dendritic cell precursors that induce the expansion of Ag-specific T cells in a NALP3-dependent way. We propose that, in addition to TLR stimulators, agonists of the NALP3 inflammasome should also be considered as vaccine adjuvants.** ***The Journal of Immunology,*** **2008, 181: 3755–3759.**

Activationof dendritic cells (DCs)[3] is a crucial mechanism by which vaccine adjuvants stimulate protective adaptive immunity to cancer or microbial infection. Agonists to several TLRs are in clinical development as adjuvants (1). The most promising TLR ligand is an unmethylated CpG dinucleotide containing DNA that stimulates TLR9 in a pathway requiring the adaptor MyD88, leading to the activation of DCs (2, 3). Nonetheless, there is a debate as to whether TLR stimulation is essential for the Ab-enhancing effect of vaccine adjuvants like alum. Although initial studies showed that MyD88- deficient mice are defective in mounting Th1 and B cell responses (4), this notion has been recently challenged (5). It has also become clear that an alternative pathogen detection system exists, in addition to TLRs, that relies on a family of intracellular receptors called nucleotide-binding oligomerization domain-like receptors (NLRs) (6–8). In addition to pathogen-associated molecular patterns, some of these NLRs also sense the presence of endogenous danger signals. The most studied NLR member is NALP3, which, together with Cardinal and apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), forms a caspase-1 activating complex, the so-called inflammasome (9). This complex is activated by multiple agonists including a bacterially derived muramyl dipeptide or endogenous ATP and uric acid, leading to the processing and release of IL-1β (10, 11). We therefore considered the possibility that NLRs, in addition to TLRs, are implicated in the effect of some adjuvants.

## Materials and Methods

### *Mice*

Wild-type (WT), ASC$^{-/-}$, IPAF$^{-/-}$ (gifts of V. Dixit, Genetech; IPAF is IL-1β-converting enzyme protease activating factor), MyD88$^{-/-}$, and NALP3$^{-/-}$ mice (12) (all mice in C57BL/6 background) were bred at the University of Lausanne, Lausanne, Switzerland and at the University Hospital, Ghent, Belgium. Male and female mice were used between the ages of 8 and 14 wk. All in vivo experiments were approved by the animal ethics committee of Ghent University.

### *Isolation and activation of mouse primary cells*

Peritoneal macrophages were isolated 3 days after an i.p. injection with 10% thioglycollate and primed in vitro with LPS as previously described (13). Uric acid, uricase, and cytochalasin D were bought from Sigma-Aldrich and crystals were generated as previously described (12).

### *Ags and adjuvant*

OVA was purchased from Worthington Biochemical. In Ag-tracking experiments, OVA-Alexa Fluor 647 (Molecular Probes) was used. Imject Alum (Pierce Biochemicals; hereafter simply called alum) is a mixture of aluminum hydroxide and magnesium hydroxide and was mixed at a 1:20 ratio with a

*Department of Pulmonary Medicine, Erasmus University Medical Centre, Rotterdam, The Netherlands; †Department of Biochemistry, University of Lausanne, Epalinges, Switzerland; ‡TopoTarget Switzerland, Lausanne, Switzerland; and §Laboratory of Immunoregulation, University Hospital Ghent, Ghent, Belgium

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[1] B.N.L. and J.T. shared supervision over the work.

[2] Address correspondence and reprint requests to Dr. Jürg Tschopp, Department of Biochemistry, Chemin des Boveresses 155, 1066 Epalinges, Switzerland. E-mail address: jurg.tschopp@unil.ch or Dr. Bart Lambrecht, Laboratory of Immunoregulation, University Hospital Ghent, Ghent, Belgium. E-mail address: bart.lambrecht@ugent.be

[3] Abbreviations used in this paper: DC, dendritic cell; ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain; IPAF, IL-1β-converting enzyme protease activating factor; MLN, mediastinal lymph node; MSU, monosodium urate; NLR, nucleotide-binding oligomerization domain-like receptor; ROS, reactive oxygen species; WT, wild type.

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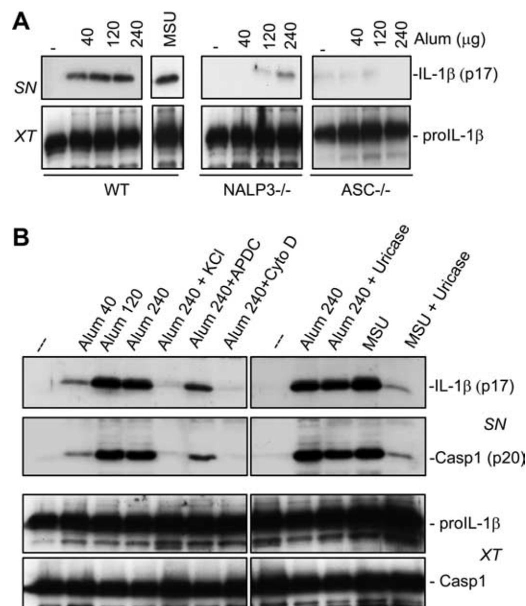


**FIGURE 1.** Role of NALP3 and ASC in alum-mediated IL-1$\beta$ production. *A*, Peritoneal macrophages from WT, NALP3$^{-/-}$, and ASC$^{-/-}$ mice were primed overnight with ultrapure LPS and then stimulated with different amount of Imject Alum (Pierce Biochemicals). MSU (150 $\mu$g/ml) was used as a control. Production of mature IL-1$\beta$ was measured by Western blotting in supernatants (SN) and cell extracts (XT). *B*, Alum-induced IL-$\beta$ secretion is dependent on phagocytosis, $K^+$ efflux, and ROS. Peritoneal macrophages from WZ mice were primed as described in *A* and treated with alum in the presence and absence of high concentrations of extracellular $K^+$ (130 mM), the ROS inhibitor (2*R*,4*R*)-4-aminopyrrolidine-2, 4-dicarboxylate (APDC) (100 $\mu$M), cytochalasin D (2 $\mu$M), and uricase (0.1 U/ml).

solution of OVA Ag in saline, followed by stirring for at least 1 h. For immunization, 500 $\mu$l of alum (crystalline magnesium hydroxide and amorphous aluminum hydroxide) suspension (1 mg) containing 10 $\mu$g of OVA (OVA-alum) was injected i.p. or, alternatively, 10 $\mu$g of OVA in 500 $\mu$l saline was injected.

*Detection of cellular influx in vivo after OVA-alum injection*

In WT and NALP3$^{-/-}$ mice, 2, 6, and 24 h after injection the peritoneal cavity was washed with 3 ml of PBS containing EDTA (peritoneal lavage) and the mediastinal lymph nodes were harvested and used for flow cytometry as previously described (14).

In experiments where the role of IL-1$\beta$ was examined, mice were treated with anti-IL-1$\beta$ mAb (200 $\mu$g/ms i.p.; BioLegend) 5 min before OVA-alum injection and sacrificed 24 h later. Detection of Ag-specific T cell responses was done as previously described (14).

*Statistical analysis*

For all experiments, the difference between groups was calculated using the Mann-Whitney *U* test for unpaired data (GraphPad Prism version 4.0). Differences were considered significant when $p < 0.05$.

## Results

*Alum adjuvant activates the NALP3 inflammasome*

We first exposed peritoneal macrophages obtained from WT, NALP3$^{-/-}$, and ASC$^{-/-}$ mice to various amounts of alum (see *Materials and Methods* for composition**)**. Even at low doses, a substantial amount of processed IL-1$\beta$ was detected in the cellular supernatant, which compared well with the quantities of this cytokine observed with crystalline monosodium urate (MSU), one of the most potent activators of the NALP3 inflammasome (12) (Fig. 1*A*). IL-1$\beta$ was almost completely absent in supernatants of macrophages from NALP3- and ASC-deficient mice, indicating a crucial involvement of NALP3 in caspase-1-mediated, pro-IL-1$\beta$ processing.

Activation of caspase-1 occurs not only upon assembly of the NALP3 but also upon that of the IPAF inflammasome (15). However, a contribution of this complex in alum-triggered caspase-1 activation is unlikely, because alum-induced IL-1$\beta$ secretion from macrophages derived from IPAF-deficient mice was still observed (data not shown). Also, alum adjuvant continued to activate caspase-1 in the absence of MyD88, excluding a major role of TLRs in alum-mediated inflammasome activation (Ref. 16 and data not shown).

*Mechanisms of NALP3 inflammasome activation by alum and uric acid crystals are similar*

We next investigated the mechanisms by which alum activates the NALP3 inflammasome. Recent studies have shown that NALP3 inflammasome activation is dependent on $K^+$ efflux (17, 18) and the generation of reactive oxygen species (ROS) (17). $K^+$ efflux can be blocked by the addition of high concentrations of $K^+$ (130 mM) to the extracellular medium. The blockade of $K^+$ efflux resulted in the inhibition of alum-induced caspase-1 activation and IL-1$\beta$ secretion (Fig. 1*B*). Activation of the inflammasome was also reduced when the ROS inhibitor (2*R*,4*R*)-4-aminopyrrolidine-2, 4-dicarboxylate (APDC) was added (Fig. 1*B*).

We could not exclude the possibility that activation of the inflammasome by alum occurred indirectly through one of the two endogenous danger signals known to activate NALP3, namely ATP and uric acid (12, 19). ATP, which is thought to be released from necrotic cells, potently activates the inflammasome via binding to the P2X7 receptor (19), whereas uric acid can form crystals that activate the inflammasome by an as yet poorly understood mechanism. We have recently reported that extracellular ATP triggers inflammation by activating DCs (20) and that in vivo injection of alum into the peritoneal cavity leads to uric acid (MSU) release (14). It was therefore possible that MSU or ATP or both, were released from alum-treated cells, leading to the subsequent activation of the NALP3 inflammasome. However, we obtained no evidence for this hypothesis. Uricase, which catalyzes the conversion of uric acid to allantoin (14), had only a minor effect on IL-1$\beta$ processing in vitro (Fig. 1*B*). Alum also still activated the inflammasome in macrophages of P2X7-deficient mice (data not shown).

Despite the fact that both ATP and MSU activate the NALP3 inflammasome, the initial pathways leading to inflammasome activation are not identical. MSU is a particulate matter and requires endocytosis (12). In fact, endocytosis seems to be required for particles in general, including asbestos and silica, to activate the inflammasome (13). It was therefore expected that alum also required endocytosis. The effect of alum, but not ATP (data not shown), was abrogated by the actin-destabilizing drug cytochalasin D (Fig. 1*B*). Taken together, NALP3 inflammasome activation in vitro by alum requires endocytosis of the particle, $K^+$ efflux, and generation of ROS, but not release of ATP or uric acid.

*Recruitment of inflammatory cells to the site of alum injection is decreased in the absence of NALP3*

Intraperitoneal injection of alum is frequently used in mice to induce cellular Th2 immunity and humoral immunity to adsorbed protein Ags (21). To assess the role of the NALP3 inflammasome in alum adjuvanticity in vivo, we examined the

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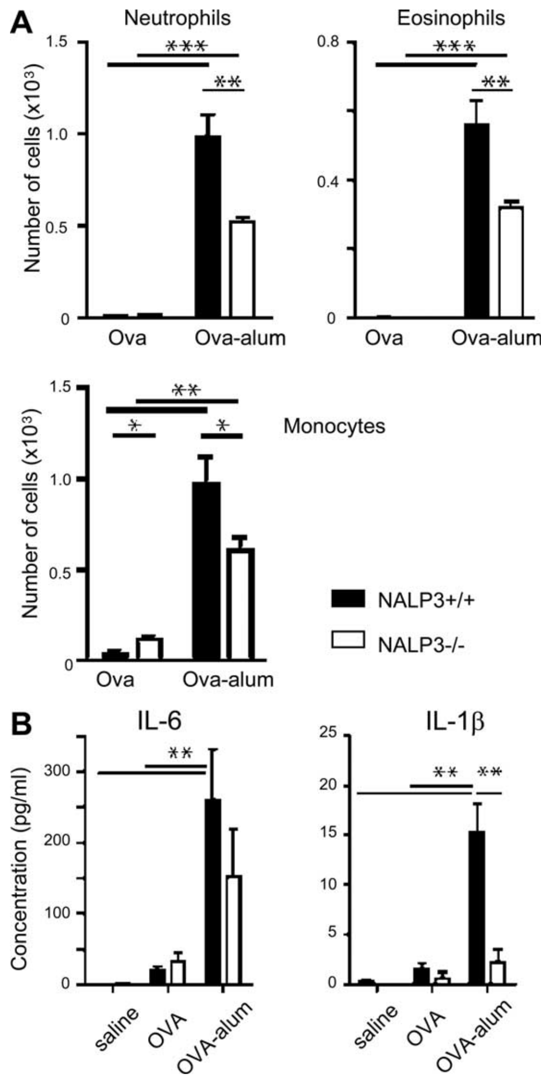


**FIGURE 2.** Innate inflammatory response induced by alum is NALP3 dependent. NALP3 $^{-/-}$ or WT mice were injected i.p. with OVA or OVA-alum. *A*, Twenty-four hours after injection, the peritoneal lavage was taken and the number of neutrophils ($CD11b^+Ly6C^+Ly6G^{high}F4/80^-$), eosinophils ($CD11b^+Ly6C^{int}Ly6G^{int}F4/80^{int}$), and monocytes ($CD11b^+Ly6C^{high}Ly6G^-F4/80^{int}$) (were "int" is "intermediate") was determined by flow cytometry. *B*, Two hours after OVA or OVA-alum injection the peritoneal lavage was taken and cytokine levels were determined in the supernatant by ELISA. Data shown are mean ± SEM; *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$; $n = 4–6$ mice per group.

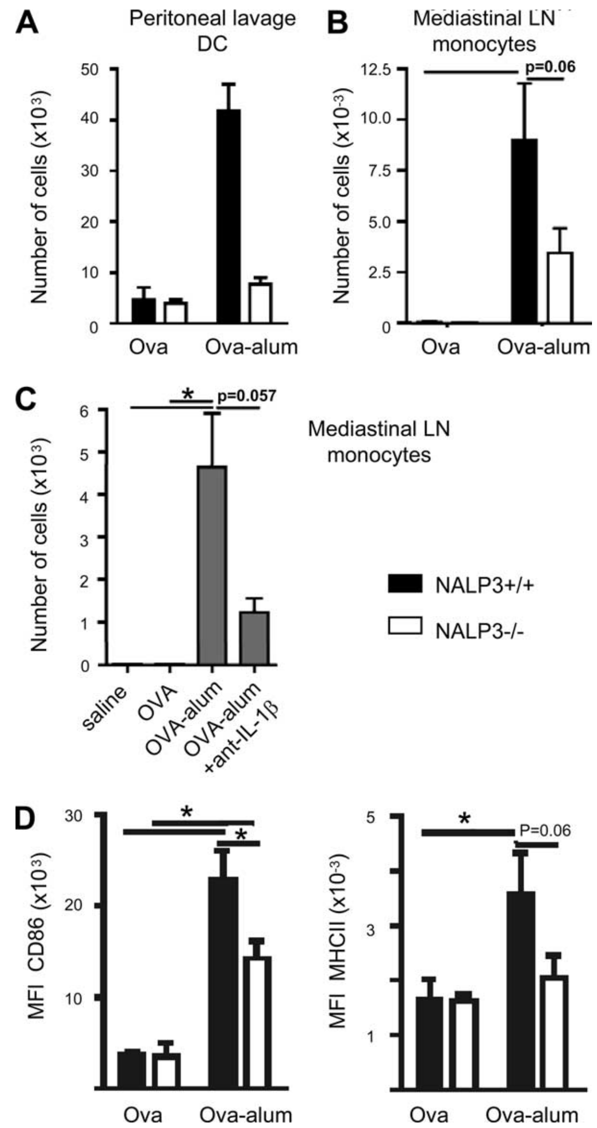


**FIGURE 3.** Activation of DCs by alum is NALP3 dependent. *A*, $NALP3^{-/-}$ or WT mice were injected i.p. with OVA-Alexa Fluor 647 or OVA-AF647-alum. Twenty-four hours after injection the number of $OVA^+$ resident $Ly6C^-CD11c^+$ DCs was determined in the peritoneal lavage by flow cytometry. *B*, Twenty-four hours after injection the number of $CD11c^+OVA^+$ monocytes in the MLN was measured. *C*, Mice were treated with anti-IL-1β mAb before OVA-alum injection. The influx of $CD11c^+OVA^+$ monocytes into the MLN was measured 24 h after injection. *D*, CD86 and MHC II expressions were measured on the $OVA^+$ and $OVA^-$ monocytes in the MLN 24 h after the injection of OVA-alum in WT or $NALP3^{-/-}$ mice. Data shown are mean ± SEM; *, $p < 0.05$; $n = 4–6$ mice per group.

innate inflammatory response caused by alum upon i.p. injection of OVA adsorbed to alum. Twenty-four hours after injection there was a massive recruitment of neutrophils ($Ly6G^+$, $F4/80^-$, and $CD11b^+$ cells), eosinophils ($F4/80^{dim}$, $Ly6G^+$, and $CD11b^+$ cells), and inflammatory mononuclear cells ($CD11b^+$, $F4/80^+$, and $Ly6C^+$ cells) into the peritoneal cavity that was not seen when OVA was injected without alum (Fig. 2*A*). This innate response was severely reduced in NALP3-deficient animals but nevertheless still present. Compared with injection of saline or OVA alone, injection of OVA-alum induced a rapid increase in the peritoneal cavity of the innate cytokines IL-6 and IL-1β (Fig. 2*B*). Strikingly, in $NALP3^{-/-}$ mice the increase in IL-1β was completely reduced to the level seen in mice receiving saline or OVA, whereas the level of IL-6 was reduced but not abolished (Fig. 2*B*).

*Activation of DCs is IL-1β and NALP3 dependent*

DCs are seen as nature's adjuvant and have a unique potential to induce adaptive immunity. We have recently reported that the induction of T cell adaptive immunity in vivo following the injection of alum depends on uptake and transport of Ag from the peritoneal cavity to the mediastinal LN by both resident $CD11c^+$ DCs and inflammatory $CD11b^+Ly6C^+$ monocytes that become $CD11c^+$ DCs upon migration to the draining mediastinal lymph node (MLN) (14). To address the contribution of inflammasome activation to this process, we measured the uptake of fluorescent OVA in resident $Ly6C^-CD11c^+$ DCs following the injection of OVA or OVA-alum. Adsorption of

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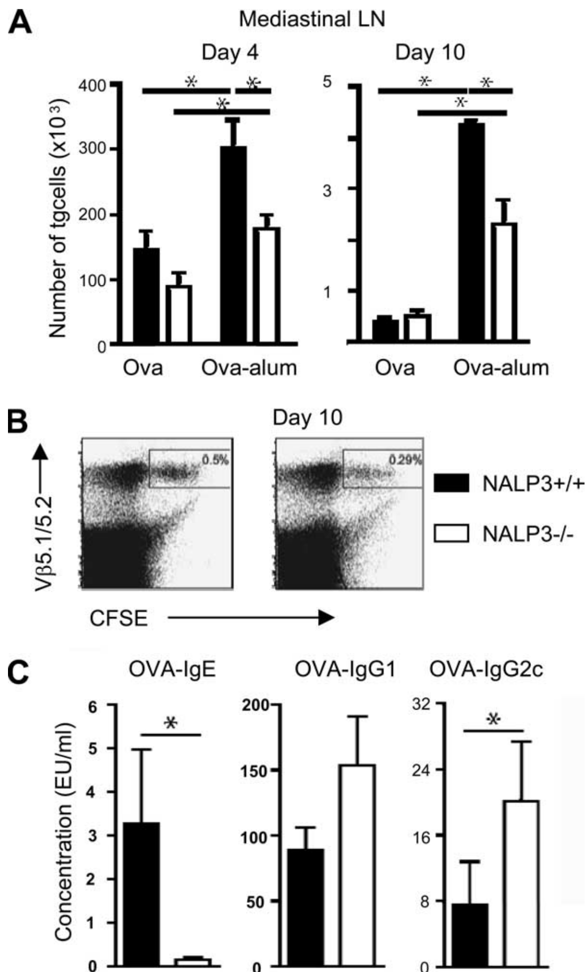


**FIGURE 4.** Induction of adaptive cellular immunity by alum is NALP3 dependent. *A*, NALP3 $^{-/-}$ or WT mice were injected with CFSE-labeled OT-2 OVA-TCR Tg cells 1 day before the i.p. injection of OVA or OVA-alum. Four and 10 days after the injection the MLN was analyzed for T cell proliferation with flow cytometry ($n = 4$ mice; experiment was performed two times). *B*, Representative CFSE profiles of adoptively transferred T cells on day 10 after injection. Data are shown as mean ± SEM; *, $p < 0.05$; $n = 5$–11 mice per group. *C*, On day 0, NALP3$^{-/-}$ or WT mice were injected i.p. with OVA-alum and boosted i.p. on day 7 with OVA. At day 14, serum samples were taken and OVA-specific IgE, IgG1, and IgG2c levels were determined by ELISA. Data are shown as mean ± SEM; *, $p < 0.05$; $n = 5$–11 mice per group.

OVA-Alexa Fluor 647 in alum led to a strong increase in DCs carrying antigenic cargo in WT mice but not in NALP3$^{-/-}$ mice (Fig. 3*A*). We also followed the appearance of OVA-laden Ly6C$^{+}$ inflammatory monocytes in the MLN (Fig. 3*B*). In WT mice there was a time-dependent increase in the number of inflammatory monocytes in the MLN at 24 h, and a majority of these carried OVA. In contrast, in NALP3$^{-/-}$ mice this increase was severely diminished. This deficiency was most likely explained by a defective generation of bioactive IL-1$\beta$ in NALP3$^{-/-}$ mice, as the alum-induced increase in OVA-laden CD11c$^{+}$ monocytes in the MLN was severely reduced when IL-1$\beta$ was neutralized in vivo, implying an important functional role for this cytokine in promoting monocyte migration and differentiation (Fig. 3*C*). Provision of costimulation is a necessary requisite for the induction of adaptive immunity. Injection of OVA-alum induced the up-regulation of the costimulatory molecule CD86 on inflammatory OVA$^{+}$CD11c$^{+}$Ly6C$^{+}$ monocytes in the MLN of WT mice, but to a much lesser extent in NALP3$^{-/-}$ mice (Fig. 3*D*). A similar effect was seen for MHC class II (Fig. 3*D*).

The reduced migration of OVA-laden Ly6C$^{+}$ monocytic cells with DC characteristics to the mediastinal nodes of NALP3$^{-/-}$ mice prompted us to study the activation of naive OVA-specific CD4$^{+}$ T cells. For this, mice first received a cohort of CFSE-labeled T cells obtained from OT-II TCR Tg mice followed by injection of OVA or OVA-alum, and accumulation of OVA-specific T cells was measured in the lymph nodes. In WT mice OVA-alum caused a marked increase in the number of Ag-reactive T cells in the mediastinal LN at days 4 and 10 postimmunization (Fig. 4*A*) compared with injection of OVA alone. In NALP3$^{-/-}$ mice, the number of T cells induced by OVA-alum was severely reduced but still higher than that obtained by the injection of OVA alone. These changes in the accumulation of T cells were mainly due to altered degrees of T cell division, as measured using CFSE dilution (Fig. 4*B*).

We finally measured the effect of NALP3 deficiency on the type of immunoglobulins induced by OVA-alum cells (Fig. 4*C*). Mice receiving OVA alone have generally low Ab titers to OVA (data not shown). In WT mice, OVA-alum induced the production of IgE, IgG1, and somewhat lower levels of IgG2c. In NALP3$^{-/-}$ mice there was a significant decrease in OVA-specific IgE Abs and an increase in OVA-specific IgG2c Abs. Strikingly, IgG1 levels were unaffected.

## Discussion

In 1926, the adjuvant effect of aluminum compounds was first described by Glenny et al. (22). Despite this early discovery, to this day little is known about the mode of action of alum adjuvant. Adjuvants containing a pathogen-associated molecular patterns act as ligands for the TLRs. For example, TLR9 was shown to be essential for the adjuvant effect of CpG oligodeoxynucleotides (3). However, incubation of DCs with alum adjuvant fails to activate the DCs as indicated by the increased expression of MHC class II and costimulatory molecules (23); yet, several authors described an increased production of IL-1$\beta$ secretion (24, 25). Mice lacking TLR signaling components still mount a robust Ab responses if alum is given as an adjuvant (5). Thus, alum adjuvants, in contrast to bacteria-derived adjuvants, do not activate TLRs.

Entirely in agreement with two recent studies by Eisenbarth and Li and colleagues (16, 26), our data show that alum adjuvant triggers activation of the NALP3 inflammasome. The potential of alum to trigger the NALP3 inflammasome leads to early activation of the innate cytokine IL-1$\beta$ and an innate cellular immune response at the site of injection. Activation of the NALP3 inflammasome and the subsequent release of IL-1$\beta$ leads to the recruitment of immature monocytes and DCs. Production of IL-1$\beta$ also leads to the activation of inflammatory monocytes and their migration to the lymph nodes draining the peritoneum. This situation resembles the migration of skin Langerhans cells to the lymph nodes, which also requires IL-1$\beta$ for functional migration and maturation to occur (27). In OVA-alum-immunized mice, OVA-laden DCs and inflammatory monocytes take their cargo to the MLN and up-regulate the expression of critical costimulatory molecules like CD86 with the subsequent expansion of Ag-specific T cells (14). All of these steps are highly impaired in NALP3-deficient mice, indicating that activation of the NALP3 inflammasome contributes

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not only to the immediate inflammatory innate response at the site of injection of alum but also to the generation of adaptive cellular immunity.

Levels of serum Igs are also influenced in the NALP3-deficient mice, as a decreased level of OVA-IgE and increased levels of OVA-IgG2c are found. This pattern of Ig synthesis is most likely the result of a shift toward IFN-$\gamma$-secreting Th1-like helper cells necessary for Ig class switching to IgG2c and equally able to suppress IgE synthesis. Interestingly, we found that NALP3 does not influence IgG1 levels, whereas decreased levels of this subclass were found in the Eisenbarth report (16). This striking-difference may be due to a different experimental setup, as in their study Ab generation was determined after an intranasal challenge with OVA (16) whereas we boosted mice by injecting OVA i.p. The data are most consistent with the notion that NALP3-dependent IL-1$\beta$ production by DCs skews the adaptive cellular immune response toward a Th2 type of response, as recently proposed (25).

Strikingly, alum adjuvant activates the NALP3 inflammasome directly in vitro, in agreement with two reports showing IL-1$\beta$ secretion from alum-treated DCs (24, 25). We have recently found that alum also induces high-level production of uric acid in vivo and that this increased level of uric acid was required for the infiltration of inflammatory cells (14). Although how this is done and which cells generate or release uric acid upon alum administration are open questions, it suggests that the increased level of uric acid leads to an amplification of NALP3 inflammasome activation and, thus, IL-1$\beta$ secretion. Interestingly, uric acid was identified not only as one of the most potent danger signals released from dying cells, but also as an excellent adjuvant (28). Thus, we propose NALP3 as the newest member of a list of innate receptors that could be exploited for the design of new adjuvants.

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## Disclosures

The authors have no financial conflict of interest.

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