Alum Induces Innate Immune Responses through Macrophage and Mast Cell Sensors, But These Sensors Are Not Required for Alum to Act As an Adjuvant for Specific Immunity[1]

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To understand more about how the body recognizes alum we characterized the early innate and adaptive responses in mice injected with the adjuvant. Within hours of exposure, alum induces a type 2 innate response characterized by an influx of eosinophils, monocytes, neutrophils, DCs, NK cells and NKT cells. In addition, at least 13 cytokines and chemokines are produced within 4 h of injection including IL-1β and IL-5. Optimal production of some of these, including IL-1β, depends upon both macrophages and mast cells, whereas production of others, such as IL-5, depends on mast cells only, suggesting that both of these cell types can detect alum. Alum induces eosinophil accumulation partly through the production of mast cell derived IL-5 and histamine. Alum greatly enhances priming of endogenous CD4 and CD8 T cells independently of mast cells, macrophages, and of eosinophils. In addition, Ab levels and Th2 bias was similar in the absence of these cells. We found that the inflammation induced by alum was unchanged in caspase-1-deficient mice, which cannot produce IL-1β. Furthermore, endogenous CD4 and CD8 T cell responses, Ab responses and the Th2 bias were also not impacted by the absence of caspase-1 or NLRP3. These data suggest that activation of the inflammasome and the type 2 innate response orchestrated by macrophages and mast cells in vivo are not required for adjuvant effect of alum on endogenous T and B cell responses. ***The Journal of Immunology,*** **2009, 183: 4403–4414.**

Aluminum salts, referred to collectively as alum in this study, have been used for almost a century to enhance Ab responses in animals and humans with very little understanding about how they mediate their effects on the immune system (1). In human vaccines, aluminum hydroxide, aluminum phosphate, and aluminum sulfate are used, and the choice of the formulation depends on how well it adsorbs the protein components of the vaccine. Traditionally, all of the adjuvant effects of alum have been attributed to its ability to prolong Ag exposure to the immune system. It is now clear, however, that alum can be recognized by the innate immune system leading to multiple downstream effects including, perhaps, its ability to act as an immunological adjuvant.

Alum elicits a type 2 inflammatory response, characterized by the accumulation of eosinophils at the site of injection in vivo. This may contribute to the effects of alum on specific immune responses (2). The eosinophils increase MHC class II levels and signaling in B cells via IL-4 and promote early IgM responses. Alum also induces the conversion of monocytes into Ag-presenting dendritic cells (DCs)[3] (3).

A number of studies have investigated how alum achieves its inflammatory effect. It is known that engagement of TLRs by their ligands, such as LPS, stimulates innate immunity and has powerful adjuvant effects on specific immune responses (4). However, several studies have shown that ability of alum to act as an adjuvant does not depend on either MyD88 or TRIF, adaptor molecules in the signaling pathways that act downstream of TLR ligation (5, 6).

Other pattern recognition receptors such as the NOD-like receptors can also promote innate responses that, in turn, stimulate specific immunity (4, 7–10). Upon activation, members of the NOD-like receptor family, such as NLRP3, form complexes with adaptor protein ASC and pro-caspase-1. The complex formed by these molecules is referred to as the inflammasome. The NLRP3 inflammasome is activated by a number of materials, including alum (11–15). Its activation may be caused directly by the material in question, or indirectly via the products of cell damage induced by the material. Such products include uric acid crystals or enzymes released by lysosomes in damaged cells (14, 16). Whatever the cause of inflammasome activation, the consequences include production of active caspase-1, thus conversion of inactive precursor cytokines of the IL-1 family, including IL-1β, IL-18, and IL-33 to their active forms (17). Because these cytokines are potent stimulators of adaptive responses in some contexts (7–10), the NLRP3 inflammasome is an attractive candidate as and an intermediary of the adjuvant effects of alum.

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[3] Abbreviations used in this paper: DC, dendritic cell; LN, lymph node; MIG, monokine-induced by γ-IFN; IP-10, 10-kDa IFN-induced protein; KC, keratinocyte-derived chemokine; WT, wild type.

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Several reports have in fact come to that conclusion, showing that the ability of alum to induce migration of Ag-loaded DCs to the lymph nodes (LN) (3), to increase Ab production (12, 13), or even to induce cellular infiltrates (3, 12) is greatly reduced in animals that lack NLRP3 or other components of the inflammasome, ASC or caspase-1. Moreover, some studies have shown that uricase inhibits the effect of alum on Ag presentation and T cell priming (3), suggesting that uric acid is involved in the activation of the NLRP3 inflammasome by alum. However, although it is universally agreed upon that the ability of alum to induce IL-1β production depends on the NLRP3 inflammasome (3, 12–15), some studies have found no role for NLRP3 in the ability of alum to enhance IgG Ab responses (3, 15). Such results are supported by the early findings we mentioned suggesting that MyD88, a required intermediary in the signaling pathways of IL-1-related cytokines, is not needed for alum to improve Ab responses (5, 6).

In this study, we decided to study the effects of alum on innate and specific immunity in more depth, to find out which cells respond most immediately to the introduction of alum into the body and which of the effects of alum depend on the NLRP3 inflammasome. Our results show that many proinflammatory cytokines and chemokines are rapidly produced in vivo after exposure to alum. Eosinophils, neutrophils, monocytes, NK cells, NKT cells, and DCs are rapidly recruited to the site of injection. Simultaneously, mast cell, macrophage, and B cell numbers at the site of injection fall. The recruitment of eosinophils was promoted by both macrophages and mast cells, the latter cells via their alum-induced production of IL-5 and histamine, but did not require the inflammasome component caspase-1.

Alum not only induced enhanced endogenous CD4 T cell responses and Ab production but also enhanced CD8 T cell priming. None of these effects of alum on the specific immune response required macrophages, mast cells, or eosinophils or, most significantly, the inflammasome components NLRP3 or caspase-1. Only IL-1β production was affected by the absence of inflammasome activity. Thus, although mast cells and macrophages are early sensors of alum, and despite the fact that these cells make many stimulatory substances in response to alum, they are not required for the adjuvant effects of alum. Furthermore, although the NLRP3 inflammasome is required for optimal IL-1β production following exposure to alum in vivo, alum promotes its effects on CD4 and CD8 T cell priming and enhancing Ab responses by mechanisms that are independent of the inflammasome.

Materials and Methods

Mice

Wild-type (WT) C57BL/6 (B6), WT BALB/c, CCR3$^{-/-}$, 5-Lipoxygenase$^{-/-}$, 15-Lipoxygenase$^{-/-}$, GATA1Δ, and *W*/+ and *W*^{v}/+ breeder mice were purchased from The Jackson Laboratory. B6 IL-4 reporter (4Get) mice were obtained from Dr. R. Locksley (University of California, San Francisco, CA). Phil transgenic mice were provided by Dr. J. Lee (Mayo Clinic, Scottsdale, AZ). BLT1$^{-/-}$ mice developed by Dr. B. Haribabu (James Graham Brown Cancer Center, Louisville, KY) and IL-5$^{-/-}$ mice were obtained from Dr. E. Gelfand (National Jewish Medical Center, Denver, CO). Caspase-1$^{+/-}$ mice generated by Dr. R. A. Flavell (Yale University, New Haven, CT) were provided with permission by Dr. K. Rock (University of Massachusetts Medical Center, Boston, MA) and were bred in-house to generate caspase-1$^{+/-}$, caspase-1$^{+/+}$, and caspase-1$^{-/-}$ mice for experiments. NLRP3$^{-/-}$ mice, backcrossed over nine generations onto a B6 background, were obtained from Dr. R. Flavell (Yale University, New Haven, CT). All animals were housed and maintained at the Biological Resource Center, National Jewish Medical Center (Denver, CO) in accordance with the research guidelines of the Institutional Animal Care & Use Committee.

Abs and reagents

Alum was precipitated in the laboratory as previously described (18), and endotoxin levels in the material were determined to be <1 ng/ml using the *Limulus* amebocyte lysate assay (Endpoint Chromogenic LAL; Lonza). Alhydrogel was purchased from Accurate Chemical and Imject from Pierce, and endotoxin levels were determined to be <1 ng/injection using the *Limulus* amebocyte lysate assay. The following mAbs were purchased from BD Biosciences: PE anti-CD19 (1D3), PE anti-NK1.1 (PK136), FITC anti-CD11c (HL3), PE anti-Siglec F (E50-2440), PE-Cy7 anti-CD117 (2B8), AF647 anti-CCR3 (83103), PerCP anti-Gr1 (RB6-8C5), allophycocyanin anti-TCR-β (H57-597), allophycocyanin-Cy7 anti-CD4 (GK1.5), PerCP anti-CD8 (536.7), and FITC anti-CD62L (MEL-14). FITC anti-FcERI (MAR-1), Pacific blue-conjugated anti-F480 (BM8), Pacific blue-conjugated anti-B220 (RA3-6B2), and PE-Cy7 anti-CD44 (IM7) were purchased from eBioscience. PE-labeled α-galactosylceramide mouse CD1d tetramers were produced previously described in the laboratory of Dr. L. Gapin (National Jewish Health, Denver, CO) (19). PE-labeled IA^{b}/3K tetramer and allophycocyanin-labeled K^{b}/SIINFEKL tetramers were produced as described in our laboratory (20, 21). 3K-OVA was generated using the Imject Maleimide Activated OVA kit from Pierce and a cysteine linked 3K peptide (FEAQKAKANKAVDGGGC) purchased from Genemed Synthesis. Endotoxin free OVA protein was isolated as previously described (22) by and provided generously by R. Kedl (National Jewish Medical Center, Denver, CO). The Proteome Profiler Mouse Cytokine Array Panel A kit was purchased from R&D Systems. Cobra venom factor from *Naja naja kaouthia* was purchased from Calbiochem. Clodronate and PBS containing liposomes were synthesized using Cl_2MDP (clodronate), a gift of Roche Diagnostics, phosphatidylcholine, obtained from Lipoid, and cholesterol purchased from Sigma-Aldrich, as previously described (23). J113863 and UCB35625 were purchased from Tocris Bioscience. Neutralizing IL-5 mAb was purified from TRFK5 hybridoma supernatants (24). WEB2086 and CV-3968, were purchased from Biomol. Pyrilamine and famotidine, and G-200 Sephadex beads were purchased from Sigma-Aldrich. Diphtheria toxoid and toxin were both purchased from List Biological Laboratories.

Injections

A total of 2–5 mg of Alhydrogel or, in some experiments, alum precipitated in our laboratory were injected i.p. or i.m. We did not notice significant differences between the innate response induced by these two different formulations of alum at 18 or 24 h (data not shown). A total of 50 μl of the 7 mg/ml liposomal clodronate suspension was injected i.p. 24 h before alum or PBS injection. In some experiments mice were immunized i.p. or i.m. (into the hind calf muscle) with 10 μg of 3K-OVA or diphtheria toxoid precipitated in 2 mg of Alhydrogel or Imject alum as noted in each experiment. For treatment of mice with J113863 or UCB35625 inhibitors, mice were i.p. injected with 10 mg/kg each of inhibitor 2 h before injection with either PBS or alum. For in vivo neutralization of IL-5, we i.v. injected 500 μg of anti-IL-5 Ab 24 h before injection with PBS or alum. Complement depletion was induced by three i.p. injections of 4 U of cobra venom factor at 12-h intervals with the last injection 12 h before injection of alum. This treatment resulted in ~90% depletion of complement. Famotidine (200 μg/mouse) and pyrilamine (100 μg/mouse) were i.p. injected into mice 1 h before injection of PBS or alum. For inhibition of platelet activating factor, mice were i.p. treated for 1 h before PBS or alum treatment with WEB2086 (100 μg/mouse) or CV-3968 (100 μg/mouse).

Cell preparation

Peritoneal cells and in some experiments, spleens, were harvested into balanced salt solution. In some experiments blood was harvested by cardiac puncture and sera stored at −20°C until analysis. For analysis of CD4 and CD8 cells, spleens were harvested 9 days after immunization and processed into single cell suspensions using nylon mesh. Red cells were lysed using ammonium chloride and nucleated cells enumerated using a Coulter Counter.

Flow cytometry

Peritoneal cells were incubated with 2.4G2 hybridoma supernatant (anti-FcγRI/II) and stained using Abs against the cell surface markers indicated in the figure legends. Splenocytes were stained as previously described (18) with PE-labeled IA^{b}/3K and allophycocyanin-labeled K^{b}/SIINFEKL tetramers, allophycocyanin-Cy7 anti-CD4, Pacific blue-conjugated anti-B220, Pacific blue-conjugated anti-F480, and PE-Cy7 anti-CD44. Wells were washed and analyzed on a Cyan Flow Cytometer using Summit Software (DakoCytomation). After data acquisition, data were analyzed using FlowJo software (Tree Star).

Analysis of cytokines and chemokines

Mice were injected and peritonea washed with 1 ml of balanced salt solution. Cells were spun down and fluid was passed through a 0.2-μm

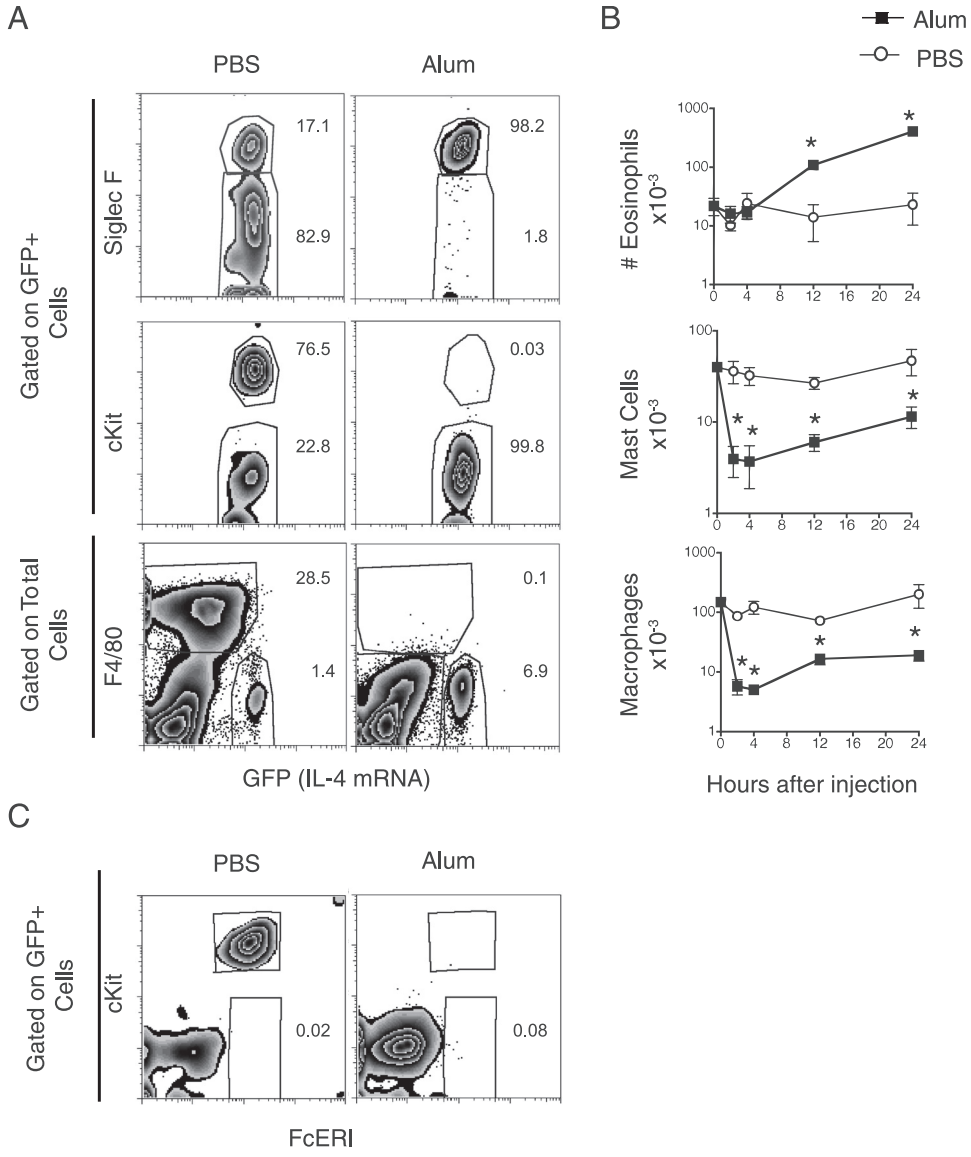

FIGURE 1. Characterization of the innate response 24 h after injection with alum. *A*, 4Get mice were injected with either PBS or alum i.p. and peritoneal cells harvested and stained 24 h later. IL-4 expressing (GFP+) cells from PBS- or alum-injected mice were analyzed for expression of the eosinophil marker, Siglec-F, and mast cell marker, c-*kit*. Total cells were analyzed for expression of F4/80. *B*, The kinetics of appearance of eosinophils, mast cells, and macrophages following injection of PBS (○) or alum (■) are shown. The total number of eosinophils was determined from the percentage of total cells that were Siglec-F$^+$/IL-4$^+$, the total number of mast cells was determined using the percentage of total cells expressing c-*kit* and IL-4, and the total number of macrophages was determine using the percentage of total cells expressing high levels of F4/80 as in *A*. *C*, Gated IL-4$^+$ cells from PBS- or alum-injected mice were analyzed for expression of c-*kit* and FcERI to determine whether ckit$^-$ FcERI$^+$ basophils were present. Scatter plots in *A* are representative of $n = 3$ individual mice. The number in scatter plot indicates the percentage of cells that fall in the indicated gates for the gated population in the sample shown. Points on line graph indicate the mean total number of each indicated cell type for $n = 3$ individual mice and error bars indicate SEM. ∗, $p < 0.05$ indicating a significant difference was detected between PBS- and alum-injected mice using a Student *t* test. The experiment was performed more than six times with similar results.

syringe filter. Fluid was analyzed using the Proteome Profiler kit (R&D Systems) or the histamine enzyme immunoassay (Immuno Biological Laboratories) according to the manufacturer's instructions.

ELISA analysis

The 96-well Immulon plates (Thermo) were coated with OVA or diphtheria toxin at 10 μg/ml in PBS. Plates were washed using ELx405 autoplate washer (Bio-Tek Instruments) and then blocked with 10% FCS/PBS for 2 h at room temperature. Plates were washed and Ab serum samples diluted in 10% FCS/PBS were added to the plates. To determine relative units, we used a positive control pooled serum sample from B6 and BALB/c mice that contained OVA or diphtheria toxin specific Th2 and Th1 Ab isotypes. The samples were incubated overnight at 4°C. Plates were washed and alkaline phosphatase-conjugated anti-IgG1 (X56), anti-IgG2a/c (R19-15), biotinylated anti-IgE (R35-119) (BD Pharmingen) or anti-κ (Southern Biotechnology Associates) detection Abs were added for 2 h at room temperature. For IgE alkaline phosphatase-conjugated streptavidin was added for 1 h at room temperature. Substrate (p-nitrophenyl phosphate) diluted in glycine buffer was added to each well and 405 nm absorbance values were collected on Elx808 microplate reader.

Statistics

Statistical significance between selected groups was determined using Student's two tailed *t* test and in some experiments, a one-way ANOVA was performed with Bonferonni post hoc test. All statistical analysis was done using GraphPad Prism software (version 4).

Results

Alum induces rapid accumulation of innate IL-4-expressing cells that are primarily composed of eosinophils

Alum usually generates Th2-related specific immune responses. In part this bias is caused by the ability of alum to induce production of IL-4. IL-4 produced in response to alum is not required to drive Th2 responses but rather, acts by suppressing Th1-related phenomena, leading to a more polarized immune response (18, 25). Because of this action, we tracked the appearance of type 2 innate cells after alum injection. To monitor inflammation following exposure to alum, we used a peritoneal model to be able to follow cell infiltrates and production of soluble mediators in the peritoneal cavity (18). Alum was injected into mice that express GFP from an internal ribosomal entry site immediately downstream of the IL-4 stop site (4Get mice) (26, 27). Previous experiments using these mice showed that Gr1^{int}IL-4 expressing cells are recruited to the peritoneal cavity in response to i.p. injection of alum by 24 h (18).

To characterize the nature of innate IL-4-expressing cells that respond to alum, 4Get mice were injected i.p. with alum and IL-4$^+$ (GFP-positive) cells at the site of injection and examined 24 h later for markers of eosinophils (Siglec F), mast cells (c-Kit), and basophils (FcERI) (Fig. 1*A*).

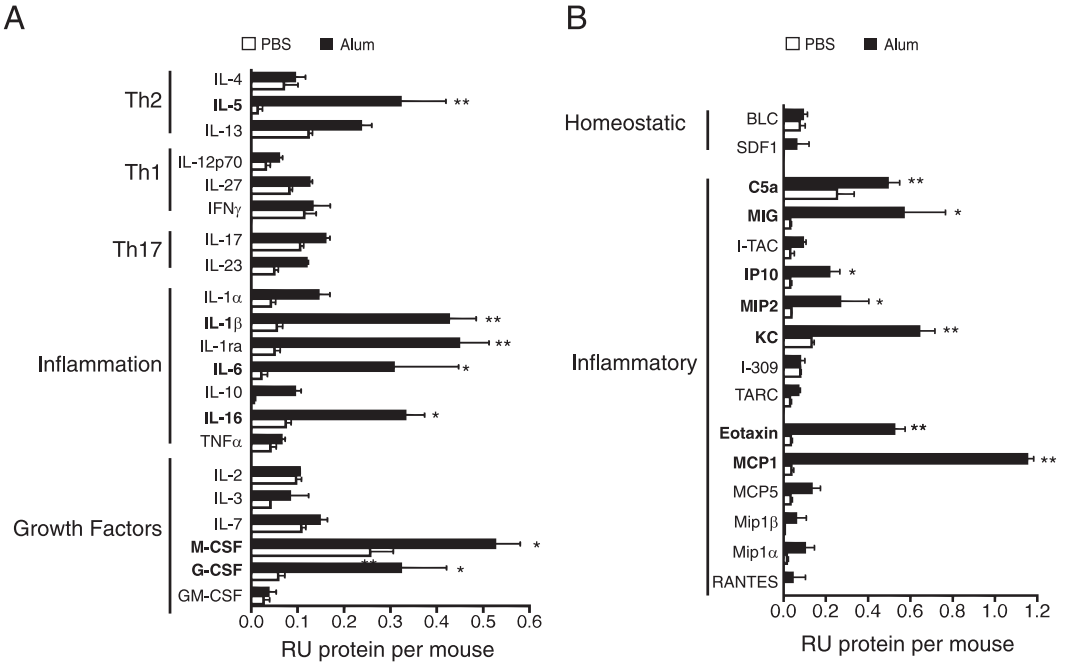

FIGURE 2. Cytokines and chemokines induced 4 h after exposure to alum. Mice were injected with either PBS (□) or alum (■) and peritoneal fluid harvested 4 h later by lavage and assayed for the presence of cytokines (*A*) and chemokines (*B*) as described in *Materials and Methods*. Bars indicate mean relative units of each factor detected for $n = 3$ individual mice per group. Error bars indicate SEM. Data are representative of two individual experiments. *, $p < 0.05$ and **, $p < 0.01$ indicate a significant difference detected between alum- and PBS-treated groups as determined by one-way ANOVA and Bonferonni post hoc test.

In PBS-injected mice, most of the IL-4-expressing cells in the peritoneal cavity were mast cells (Siglec F^-ckit^+) and only a few were eosinophils (Siglec F^+ckit^-) (Fig. 1*A*, *left column*, and 1*C*). The mast cells dropped in numbers after alum injection (Fig. 1*A*, *right column*, and 1*B*). In alum-injected mice, almost all of the IL-4-expressing cells were eosinophils (Fig. 1*A*) and these cells coexpressed low levels of Gr1 (data not shown). Consistent with previous findings (2, 3), the number of eosinophils rose after alum administration, beginning their increase a few hours after the disappearance of the mast cells (Fig. 1*B*). Basophils also express IL-4 and have been described to play a role in Th2 responses (28–32). We did not find any basophils ($FcER1^+ckit^-$) in the peritoneum before or following injection of alum (Fig. 1*C*). Thus, almost all of the innate IL-4^+ cells in alum-induced inflammation had staining characteristics of eosinophils (Siglec F^+ckit^-) (Fig. 1*A*, *right column*).

Further examination of the inflammation induced by alum confirmed that, as previously described (3), the number of peritoneal macrophages ($F4/80^{high}$) fell following alum injection (Fig. 1, *A* and *B*), and with the same kinetics as the mast cells (Fig. 1*B*). As predicted (3), the number of neutrophils ($Gr1^{high}SSC^{high}$), Gr1 intermediate ($Gr1^{int}$) $Ly6C^{high}SSC^{low}$ monocytes (see supplemental Fig. 1),[4] and DCs (see supplemental Fig. 2*B*) all increased in response to alum. In addition, NK cells and NKT cell also increased in number (see supplemental Fig. 2, *C* and *D*), whereas T cell numbers were unchanged and B cell numbers dropped modestly (see supplemental Fig. 2, *A* and *C*).

Induction of multiple chemokines and cytokines occurs within hours of exposure to alum

To characterize the soluble factors released during the inflammatory response induced by alum, we used a multiplex membrane bound ELISA to test the peritoneal fluid from mice injected previously with PBS or alum. A time course showed that the amounts of induced factors peaked 4 h after injection (data not shown). In alum-injected mice, we detected increased levels of 14 soluble factors including the Th2-associated cytokine IL-5 (Fig. 2*A*). No Th1- or Th17-associated factors were detected over background levels (Fig. 2*A*). In addition to IL-1β (Fig. 2*A* and 11), several other inflammation associated cytokines were elevated after alum injection, including IL-1ra and IL-6, whereas levels of IL-1α, TNF-α, and IL-10 remained unchanged (Fig. 2*A*). In addition to MCP-1, keratinocyte-derived chemokine (KC), and eotaxin, which have been shown to increase after alum injection (Fig. 2*B* and 11), alum-induced increased levels of additional cell-attractive proteins, including C5a, monokine-induced by γ-IFN (MIG), 10-kDa IFN-induced protein (IP-10), and MIP2 (Fig. 2*B*).

Macrophages and mast cells promote type 2 inflammation

The rapid disappearance of macrophages and mast cells after alum injection may result from activation, adherence to the peritoneal cell wall, mast cell degranulation, or cell death as has been observed in macrophages exposed to alum in vitro (14), and suggests that these cells may respond rapidly to alum and participate in the production of cytokines following exposure to alum. To test the idea, macrophage or mast cell-deficient mice were injected with alum and the appearance of cytokines and chemokines in the peritonea analyzed 4 h later. To deplete macrophages, WT B6 mice were injected with the macrophage-depleting agent, clodronate liposomes (33). As expected, liposomal clodronate was highly efficient at depleting macrophages, but not mast cells, DC, neutrophils, or eosinophils (see supplemental Fig. S3). To determine the role of mast cells, we used mast cell-deficient W/W^v mice.

Of the cytokines and chemokines that were elevated significantly above controls by alum injection (Fig. 2), the amounts of

[4] The online version of this article contains supplemental material.

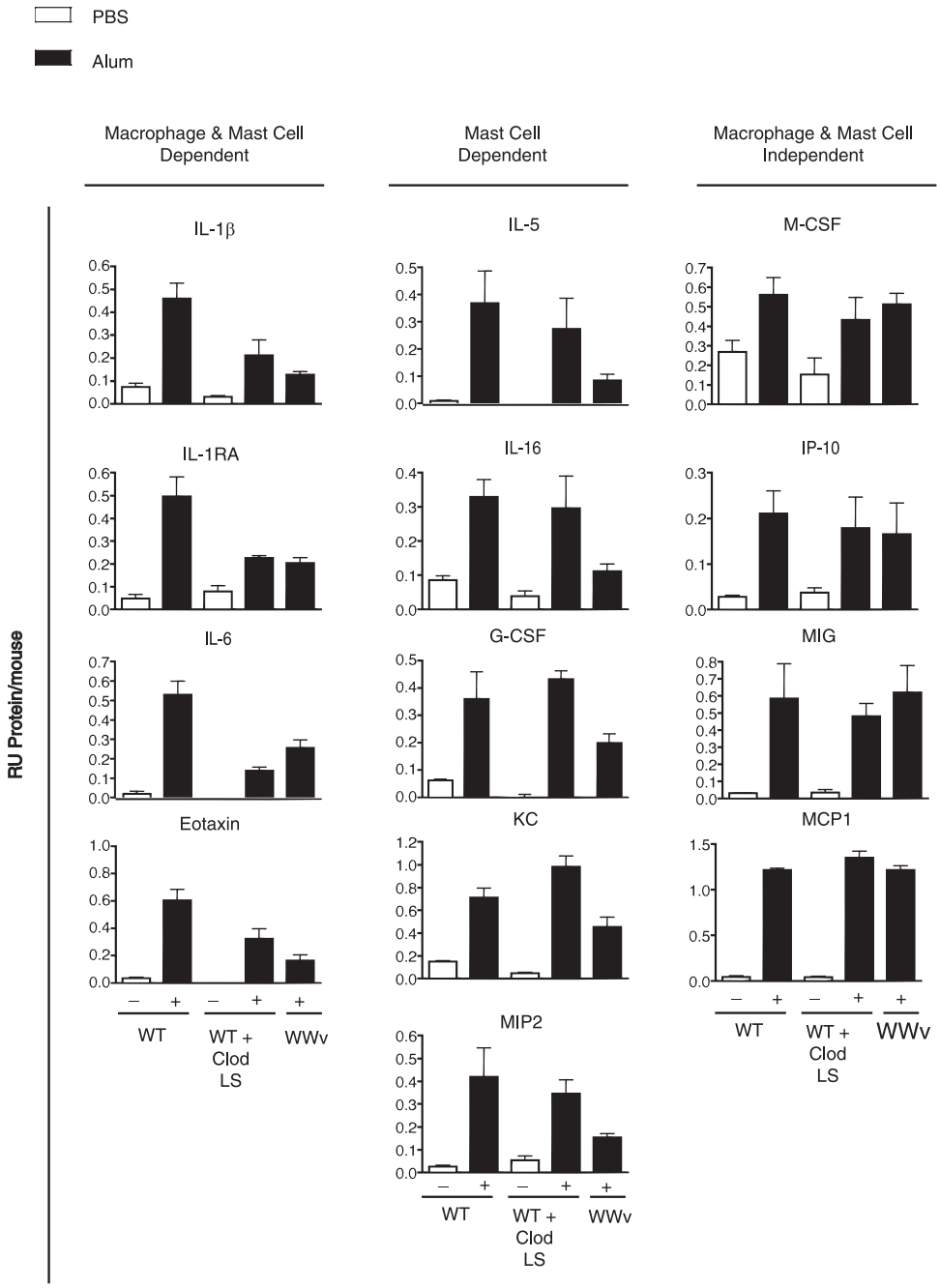

FIGURE 3. Macrophages and mast cells are sensors of alum in vivo. Cytokines shown to be induced by alum (Fig. 2) were analyzed in $Kit^{+/+}$ untreated mice (WT), $Kit^{+/+}$ mice pretreated with clodronate liposomes (WT+Clod LS) or mast cell-deficient mice (W/W^v) injected with either PBS (□) or alum (■). Cytokines whose levels in response to alum were significantly impaired (*left column*) in both mast cell-deficient and macrophage-depleted mice are shown. Alum-induced cytokines that were significantly impaired (*middle column*) in mast cell-deficient mice only are shown. Alum-induced cytokines that were unaffected (*right column*) by absence of either cell type are shown. Results show mean relative units of each factor for $n = 3$ individual mice and error bars indicate SEMs. Data are representative of two individual experiments. $p < 0.05$ for significant difference detected between similarly treated WT control mice using one-way ANOVA and Bonferoni post hoc test.

IL-1β, IL-1Ra, IL-6, and the chemokine, eotaxin, were all significantly decreased in clodronate liposome-treated mice (Fig. 3, *left column*). However, to our surprise, all of these factors were also decreased in alum-injected mast cell-deficient mice. This result suggests that these factors are potentially made by both macrophages and mast cells in response to alum or that interactions between these cell types promote optimal cytokine production. In contrast, levels of IL-5, IL-16, G-CSF, KC, and MIP2 were all decreased in mast cell-deficient but not macrophage-depleted mice (Fig. 3, *middle column*). Finally the levels of MIG, IP-10, KC, and MCP-1 were not significantly affected by absence of either macrophages or mast cells (Fig. 3, *right column*), suggesting that these factors may be made by other cell types including endothelial cells, fibroblasts, neutrophils or DC (34). These results suggest a complex inflammatory response to alum mediated by multiple cell types that culminates in the production of a wide range of soluble mediators.

Alum promotes mast cell-mediated recruitment of eosinophils via IL-5 and histamine

In mast cell-deficient mice, we found a partial reduction in the number of eosinophils recruited in response to alum, compared with the number in WT mice (Fig. 4*A*). IL-5, which is decreased in the mast cell deficient mice (Fig. 3), is known to play a role in the recruitment of eosinophils in response to other Th2 driving substances such as helminth eggs (35, 36). Eosinophil recruitment did not occur in IL-$5^{-/-}$ mice given alum (data not shown). However, IL-5 is needed for eosinophil development as well as optimal eosinophil movement (35, 37) and therefore lack of IL-5 could have reduced eosinophil accumulation because of a reduced number of the cells in IL-$5^{-/-}$ mice. To test this possibility, we transiently depleted IL-5 in vivo. Mice were injected with neutralizing anti-IL-5 Ab 1 day before the injection of PBS or alum, and the effect of these treatments on eosinophil accumulation was measured

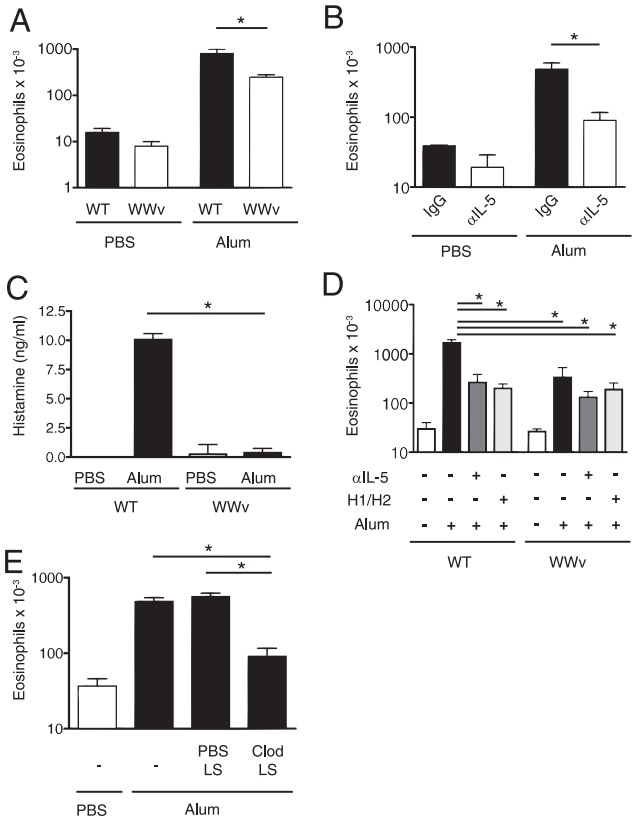

FIGURE 4. Mast cells and macrophages are needed for accumulation of eosinophils in response to alum. *A*, *W/W*^{v} mice (□) and Kit$^{+/+}$ (WT) littermate control mice (■) were injected with either PBS or alum and the total number of eosinophils recruited was determined as described in Fig. 1. *B*, B6 mice were treated neutralizing anti-IL-5 Ab (□) or rat IgG control Ab (■) and then injected with PBS or alum 24 h later. *C*, Kit$^{+/+}$ (WT) or *W/W*^{v} mice were injected with alum and peritoneal fluid was harvested 10 min later and tested for the presence of histamine as described in *Materials and Methods*. Graph shows mean concentration of histamine for $n = 3$ mice per group in one experiment of two conducted. *D*, Kit$^{+/+}$ (WT) or *W/W*^{v} mice were treated with either anti-IL-5 or antihistamines, as described in *Materials and Methods*, and injected with either PBS or alum. The total number of eosinophils recruited was determined as described in Fig. 1. *E*, B6 mice were treated i.p. with either PBS (−), PBS containing liposomes (PBS LS) or clodronate containing liposomes (Clod LS). At 24 h later, the mice were injected with either PBS (□) or alum (■). Data in *A* and *B* are from one representative experiment of three. Data in *D* are from one representative experiment of two. Results (*B, D, E*) equal mean number $\times 10^{-3}$ of each indicated cell type for $n = 3$ individual mice per group. Error bars indicate SEM. ∗, $p < 0.05$ indicates a significant difference determined from similarly treated control mice using a *t* test.

24 h later. We found that blocking IL-5 resulted in a reduction in the accumulation of eosinophils in response to alum (Fig. 4*B*). In contrast, anti-IL-5 did not further reduce the small number of eosinophils that appear in response to alum in *W/W*^{v} mice (Fig. 4*D*). These data suggest that, like helminth eggs (36), alum attracts eosinophils by promoting mast cell-dependent IL-5 production.

Mast cell degranulation and the release of histamines have been shown to be involved in the recruitment of inflammatory cells in response to implanted biomaterial particles (38) and have been implicated in eosinophil recruitment as well (39). Accordingly, we were able to detect histamine release in the peritoneal fluid of alum-injected WT mice within 10 min of alum injection but not in similarly treated mast cell-deficient *W/W*^{v} mice (Fig. 4*C*). To evaluate whether histamine production had any impact on eosinophil recruitment, we used a combined treatment of famotidine and pyrilamine to block signaling through the H1 and H2 receptors. Treatment of mice with these antihistamines 2 h before exposure to alum resulted in a partial reduction in the total number of eosinophils recruited in response to alum particles in WT but not mast cell-deficient *W/W*^{v} mice (Fig. 4*D*). Treatment of mice with anti-IL-5 Ab together with antihistamines did not have an additive effect (Fig. 4*D*). Thus mast cells, IL-5, and histamine all promote eosinophil recruitment in response to alum and appear to be acting together.

Despite the reduced influx of eosinophils in the absence of mast cells, IL-5, or histamine receptor signaling, there still were an increased number compared with those in PBS-injected control mice, therefore an alternative pathway must promote eosinophil responses to alum. We observed a marked reduction of eosinophils in mice depleted of macrophages (Fig. 4*E*), suggesting that they also respond to alum by promoting eosinophil recruitment. Surprisingly, several factors expected to play a role, including eotaxin, platelet activating factor, complement, and leukotriene B4, were not required for the alum-induced eosinophil response (see supplemental Figs. 4 and 5).[4] Therefore, besides the mast cell/IL-5/histamine pathway, there are other pathways involved in the eosinophil response to alum. Macrophages may promote eosinophil recruitment through as yet undefined factors, or perhaps several soluble factors tested, may act redundantly.

Mast cells, macrophages, and eosinophils are not required for enhanced priming of T cells, Th2 bias, or Ab responses to alum

Our data suggest that mast cells and macrophages sense the presence of alum and respond by producing a number of inflammatory factors, chemokines and cytokines. Because soluble factors, such as IL-1β, have been implicated in the adjuvant effects of alum on B and T cells (11–13), it was possible that this is an important early step in the adjuvant activity of alum in vivo. To test this idea, we first tracked CD4 and CD8 T cell responses in untreated or clodronate liposome-injected WT (kit$^{+/+}$) or *W/W*^{v} mice. To avoid the artifacts that might occur if transferred T cells expressing transgenic TCRs are used (40, 41), we followed endogenous Ag-specific CD4 and CD8 T cell responses using peptide/MHC tetramers. To do this, we immunized mice with the 3K peptide (21, 42) conjugated to OVA protein (3K-OVA), and measured endogenous CD4 T cell responses with 3K/IA^{b} tetramers and CD8 T cell responses to an OVA peptide/K^{b} with SIINFEKL/K^{b} tetramers (21, 42). We found that injection of WT mice with 3K-OVA adsorbed to alum promoted endogenous CD4 T cell responses (18). Moreover, this Ag/adjuvant combination also, to our surprise, greatly enhanced CD8 T cell priming (Fig. 5, *B* and *C*). We found no difference in the percentage (Fig. 5, *A* and *B*) or total number (Fig. 5*C*) of Ag-specific CD4 or CD8 T cells primed by alum in mice lacking macrophages, mast cells, or both cell types. Likewise, absence of mast cells had no effect on the ability of alum to induce total Ig and IgG1 primary or secondary responses to OVA, or IgG2c secondary responses to the same Ag (Fig. 5*E*).

Similar experiments were performed in B6 or B6 4Get mice in which macrophages were depleted with clodronate liposomes. Lack of macrophages had no effect on the size of the CD4 and CD8 T cell response (Fig. 5*C*), the Th2 nature of the response (Fig. 5*D*) or the size and nature of the Ab response to 3K-OVA plus alum (Fig. 5*E*).

Previous results suggest that IL-4 is not required to initiate Th2 responses to alum, but may suppress Th1 responses (25). Treatment of mice with a Gr1-specific Ab had a similar but partial effect on the bias of the response (18). Although Gr1 is expressed at low levels on eosinophils, it is a nonspecific marker and may bind to both Ly6G- and Ly6C-expressing cell types. Thus, we wanted to

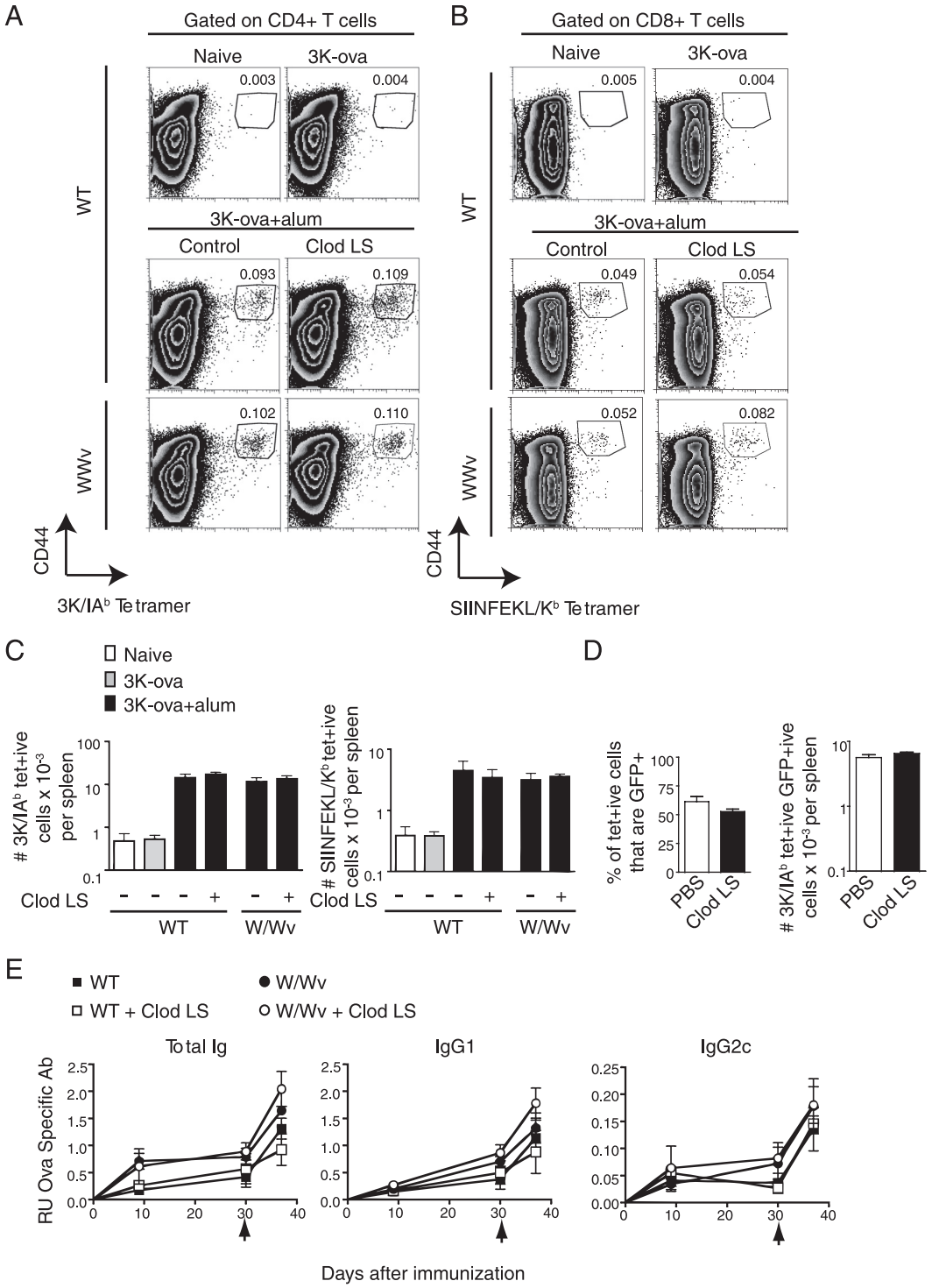

FIGURE 5. Mast cells and macrophages are not required for alum to enhance adaptive immunity. *A* and *B*, WT and *W*/*W*^{v} mice were immunized with either nothing (Naive), 3K-OVA, or 3K-OVA/alum. 3K-specific CD4 T cells and SIINFEKL-specific CD8 T cells in the spleen were analyzed by tetramer staining 9 days later. Number in scatter plots indicates the percentage of gated CD4 (*A*) or CD8 (*B*) T cells that fall within the indicated gates for the sample shown. Plots are representative of $n = 4$ individual mice. *C*, Results indicate the mean total number of IA^{b}/3K+CD44^{high} CD4^{+} cells or K^{b}/SIINFEKL^{+}CD44^{high} CD8^{+} cells detected in the spleen 9 days after injection for WT or *W*/*W*^{v} mice injected with either nothing (□), 3K-OVA (▨), or 3K-OVA/alum (■). *D*, B6 4Get mice were treated with clodronate liposomes i.p. and injected with 3K-OVA/alum and 3K/IA^{b} specific CD4 T cells in the spleen were analyzed 9 days after as described. Results show percentage and total number of 3K/IA^{b} tetramer-positive cells that express GFP in untreated (□) and clodronate liposome-treated (■) mice. *E*, WT (squares) or *W*/*W*^{v} (circles) mice were left untreated (solid symbols) or injected with clodronate liposomes (open symbols). At 24 h later, all mice were immunized and boosted on days 0 and 30 with OVA+alum (arrowheads). OVA-specific Ab isotypes in the serum were detected by ELISA. Value in graphs indicates mean value for $n = 4$ individual mice and error bars indicate SEM. Data in *A*–*C* are from one representative experiment of three and data in *D* and *E* are from one representative experiment of two conducted.

examine whether adaptive responses were affected in eosinophil-deficient mice immunized with alum. We first examined T cell priming and Ab responses in IL-5-deficient mice and found no significant effect on the total number of Ag-specific CD4 or CD8 T cells primed compared with WT control animals (Fig. 6*A*). In addition, we found no effect on the levels of total anti-OVA Ab in IL-5-deficient mice or in the levels of IgG1 or IgG2c (Fig. 6*B*), suggesting that eosinophils neither impact the ability of alum to act as an adjuvant nor the bias of the response induced. We also saw no decrease in the levels of OVA specific Ig, IgG1, or IgG2c Ab responses in eosinophil-deficient Phil mice (Fig. 6*C*). These mice express diphtheria toxin under the control of the eosinophil peroxidase promoter and are congenitally deficient in eosinophils (43). Finally, we saw no decrease in OVA specific Ig, IgG1, IgG2a, and IgE levels in eosinophil-deficient GATA1Δ mice that contain a mutation in GATA1 that prevents eosinophil differentiation (44) compared with WT controls (Fig. 6*D*). The only effect observed was an overall increase in IgE levels in the GATAΔ mice (Fig. 6*D*).

Thus, macrophage and mast cell sensors appear to play a role only in the inflammatory response to alum and do not participate in the ability of alum to act as an adjuvant for the specific immune response in vivo. Although the induction of eosinophils by alum has been shown to promote early IgM responses to alum and alter MHC class II-mediated intracellular signaling (45), they are unlikely to plan an important role in Ab responses relevant to immunization.

The inflammatory response to alum does not require caspase-1

Recent work by others (11, 12, 15, 46, 47), and our detection of IL-1β in peritoneal exudates (Fig. 3), suggests that alum can activate caspase-1 rapidly in response to alum. Other work has indicated that products of this activation, such as IL-1β family members might contribute to the downstream consequences of alum injection perhaps via effects on DCs (11, 12), whereas others have found no role for the inflammasome in the adjuvant activity of alum (15). To test whether the inflammasome promotes recruitment of inflammatory cells including those likely to be required for the adjuvant effects of alum, we injected caspase-1$^{-/-}$ mice with PBS or alum and examined the number of resident and inflammatory cells in their peritoneal cavities 18 h later. Absence of caspase-1 had no impact on the ability of alum to reduce the number of macrophages and mast cells in the peritoneal lavage, or on the alum-induced increase in the number of eosinophils, neutrophils, monocytes, or DCs in the peritoneal cavity (Fig. 7*A*). This was observed even though IL-1β production in response to alum was drastically reduced in these same animals (Fig. 7*B*). This result was surprising, considering the previous report that accumulation of granulocytes, monocytes, and DC are dependent upon NLRP3, an adaptor which is thought to mediate its effects by promoting activation of caspase-1 (11).

Together, these data strongly suggest that the inflammatory response does not require pathways dependent on caspase-1. The discrepancies between these results and ours are not clearly understood, although they may suggest that another pathway downstream of NLRP3 could be playing a role in early inflammatory responses to alum (11).

The adjuvant effects of alum on T and B cell responses are not dependent on NLRP3 or caspase-1

To examine, in our own hands, the issues surrounding the controversy about the role of the NLRP3 inflammasome in alum-induced responses (11–13, 15), we immunized NLRP3$^{-/-}$ and control WT mice and caspase-1$^{+/+}$ and caspase-1$^{+/-}$ littermate control mice

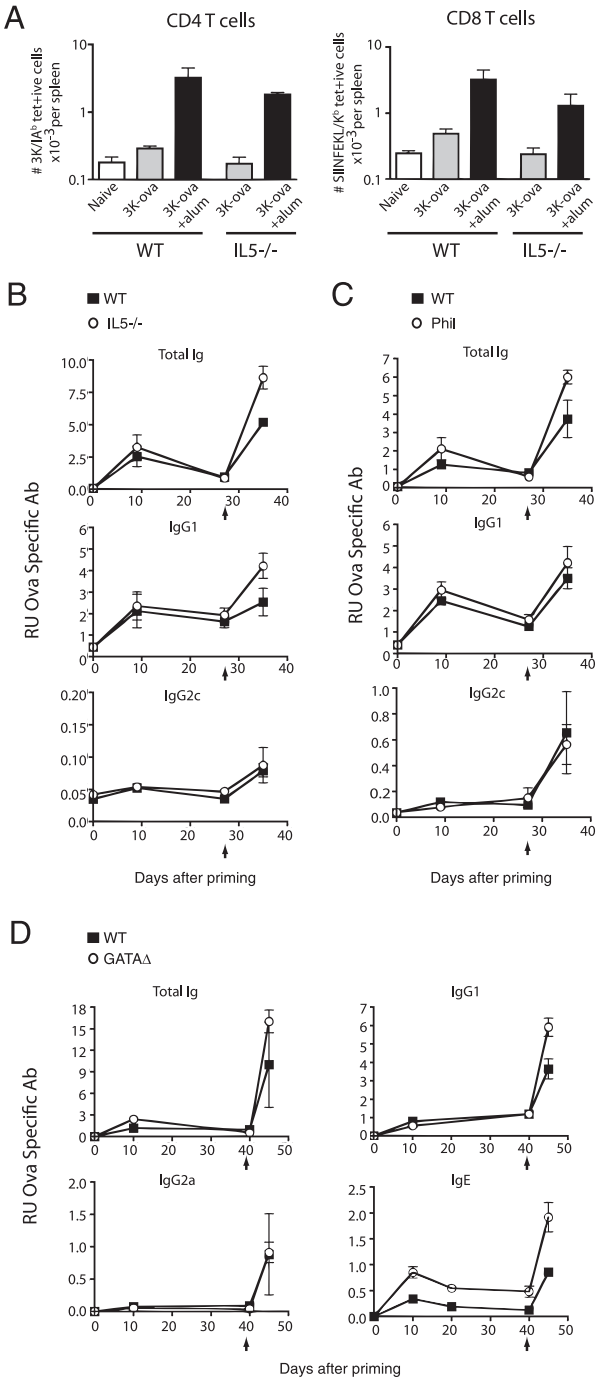

FIGURE 6. Eosinophils have no effect on the ability of alum to enhance T cell priming and Ab levels. *A*, WT or IL-5$^{-/-}$ mice were injected with either nothing (Naive) (□), 3K-OVA (▨) or 3K-OVA/alum (■) and splenocytes were analyzed for tetramer staining as in Fig. 5. Results indicate the mean total number of IA^b/3K+CD44^{high} CD4$^+$ cells or K^b/SIINFEKL$^+$CD44^{high} CD8$^+$ cells detected in the spleen 9 days after injection for $n = 3$ individual mice. Data are representative of two experiments. *B*, WT (■) or IL-5$^{-/-}$ (○) mice immunized and boosted on days 0 and 27 with OVA+alum (arrowheads). OVA-specific Ab isotypes in the serum were detected by ELISA. *C*, Eosinophil-deficient Phil (Phil) transgenic mice (○) or WT (WT) littermate controls (■) were injected and Ab responses analyzed as in *B*. *D*, WT BALB/c (■) or eosinophil-deficient Gata1Δ (○) mice were immunized and boosted with OVA/alum on days 0 and 40 and Ab responses analyzed by ELISA. Value in graph indicates mean value for $n = 4$ individual mice in *B* and *C* and for $n = 5$ individual mice in *D*. Error bars indicate SEM.

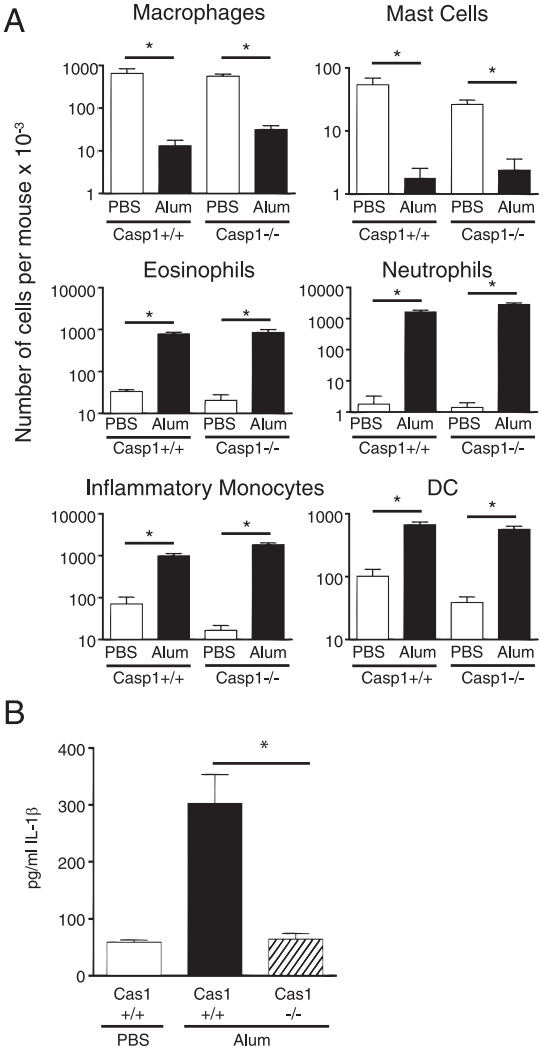

FIGURE 7. Induction of eosinophil recruitment by alum in caspase-1-deficient mice is normal. WT ($Cas1^{+/+}$) and caspase-1-deficient ($Cas1^{-/-}$) mice were injected with either PBS (□) or alum (■). The total number of macrophages, mast cells, eosinophils, neutrophils, monocytes, and DC was determined by flow cytometry as in Fig. 1 (also see supplemental Figs. S1 and S2). *B*, WT ($Cas1^{+/+}$) (■) or caspase-1-deficient ($Cas1^{-/-}$) (▨) mice were injected with either PBS (□) or alum (■) and IL-1β levels were determined from the peritoneal fluid obtained from the mice 4 h later by ELISA. Results are equal to the mean number $\times 10^{-3}$ of cells detected in the peritoneal cavities of $n = 3$ individual mice per group. Error bars indicate SEM. Data are representative from one experiment of two conducted. *, $p < 0.05$ indicates a significant difference as determined by *t* test.

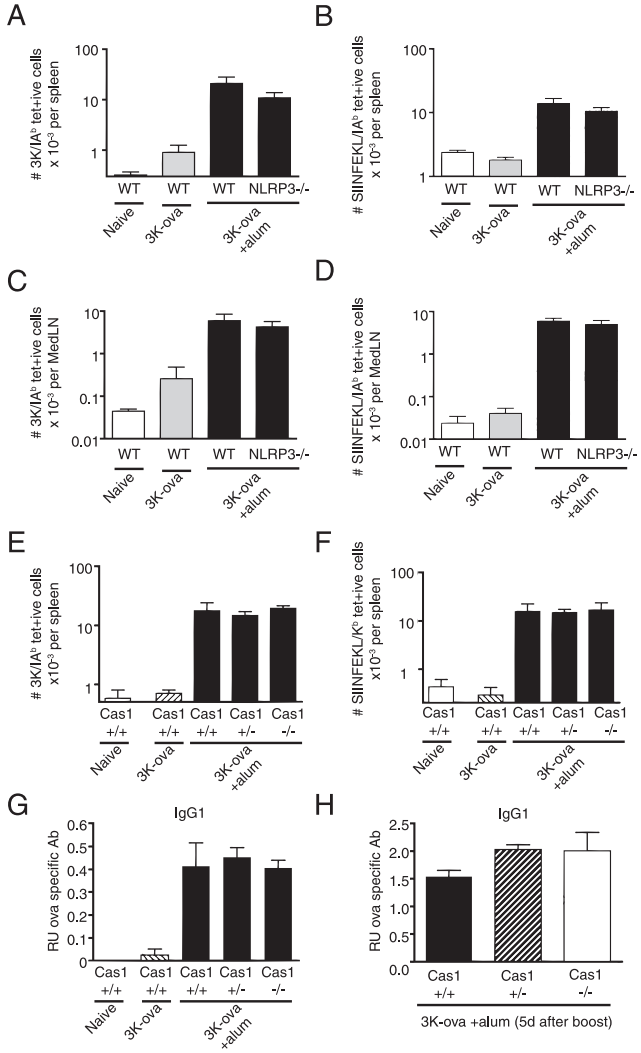

FIGURE 8. NLRP3 and caspase-1 are not required for alum to enhance endogenous CD4, CD8 T cell priming and Ab responses to adsorbed Ag. *A–D*, Graphs show total numbers of 3K/IA^b CD4 (*A* and *C*), and SIINFEKL/K^b CD8 (*B* and *D*) $CD44^{high}$ T cells in the spleen and mediastinal LN 7 days after injection of nothing (Naive), 3K-OVA, or 3K-OVA/alum (■) into WT or $NLRP3^{-/-}$ mice. *E* and *F*, WT ($Cas1^{+/+}$), heterozygous ($Cas1^{+/-}$), and homozygous caspase-1-deficient mice ($Cas1^{-/-}$) were injected with either nothing (□), 3K-OVA (▨), or 3K-OVA adsorbed to alum (■). Results indicate the mean total number of IA^b/3Ktet$^+$$CD44^{high}$ $CD4^+$ cells or K^b/SIINFEKLtet$^+$$CD44^{high}$ $CD8^+$ cells detected in the spleen 9 days after injection. *G*, The amount of OVA-specific IgG1 detected in mice in *A* and *B*. *H*, WT ($Cas1^{+/+}$), heterozygous ($Cas1^{+/-}$) and homozygous caspase-1-deficient mice ($Cas1^{-/-}$) were injected and boosted with alum on days 0 and 10 with OVA/alum. The amount of OVA-specific IgG1 detected on day 20. Data are mean ± SEM for $n = 4$ individual mice per group. Data shown in *A–D* are from a representative experiment of two. Data shown in *E–G* are from a representative experiment of three conducted.

i.p. with 3K-OVA adsorbed to alum and followed their endogenous Ag-specific CD4 and CD8 T cell responses in the spleen and mediastinal LN (the LN that drains the peritoneal cavity (3)), using MHC/tetramers (Fig. 8, *A–F*). Alum improved responses to its accompanying Ag because, as expected, T cell responses were much smaller in animals given Ag without alum (Fig. 8, *A* and *B*). The absence of NLRP3 or caspase-1 had no effect on the magnitude of CD4 T cell and CD8 T cell responses to Ag plus alum, either in spleen (Fig. 8, *A*, *B*, *E*, and *F*) or the mediastinal LN (Fig. 8, *C* and *D* and data not shown). In addition, we assayed Ag-specific Abs in the sera of these mice and found that caspase-$1^{-/-}$ mice had levels of anti-OVA IgG1 Ab that were similar to those of heterozygous or WT mice (Fig. 8*G*). Levels of Ag-specific IgG2c were undetectable at this time in all the mice (data not shown). Because these experiments were done very early in the immune response to look at T cell priming, the possibility remained that although early IgG1 responses were unaffected, long-term Ab responses may be impacted in mice unable to activate caspase-1. However, when we followed Ag-specific Ab responses to OVA in OVA/alum-injected caspase-$1^{+/+}$, caspase-$1^{+/-}$, and caspase-$1^{-/-}$ mice at later time points and during the secondary response, we were unable to see any significant difference in the Ab response (Fig. 8*H*).

It has been suggested that different laboratories may find different requirements for the NLRP3 inflammasome because they use

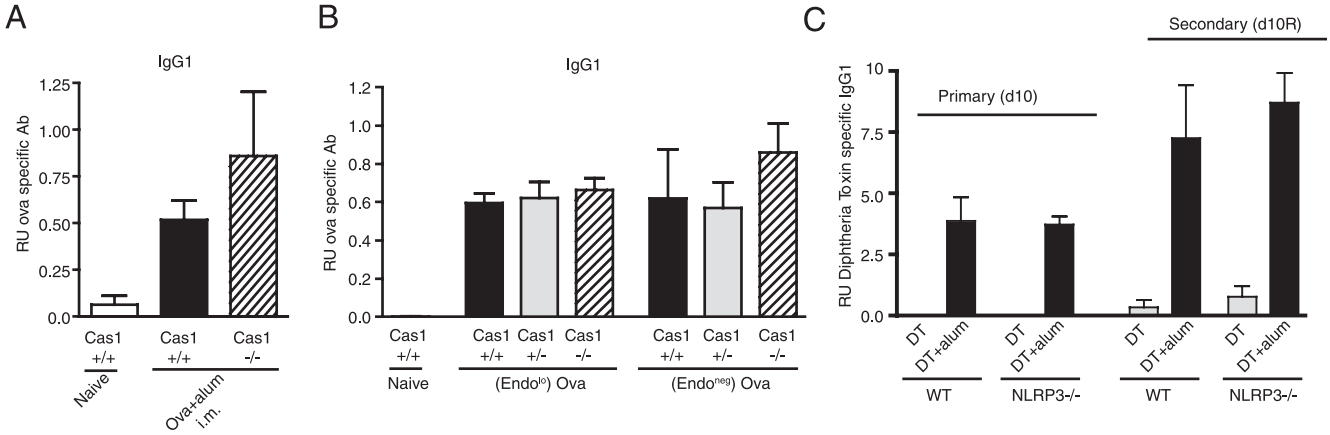

FIGURE 9. Enhanced T cell priming and Ab responses to alum adjuvant are normal in NLRP3-deficient mice. *A*, The amount of OVA-specific IgG1 detected in WT mice ($Cas1^{+/+}$) or caspase-1-deficient ($Cas1^{-/-}$) mice 14 days after i.m. injection with endotoxin free OVA adsorbed to alum and, for comparison, in uninjected WT mice. *B*, The relative levels of OVA-specific IgG1 detected in WT ($Cas1^{+/+}$), caspase-1 heterozygous ($Cas1^{+/-}$), and caspase-1 knockout ($Cas^{-/-}$) littermates injected with OVA that contains low levels of endotoxin ($Endo^{low}$ OVA) or endotoxin free OVA ($Endo^{-}$ OVA). *C*, Levels of circulating anti-diphtheria toxin Ab in WT or $NLRP3^{-/-}$ mice in mice immunized and boosted with diphtheria toxoid alone (▨) or adsorbed to alum (■) on day 10 of the primary response or on day 20 (10 days after a boost injection). Results indicate mean value and SEM for $n = 4$ mice per group. Data are from one representative experiment of two condcuted.

different alum formulations. To check this suggestion, we followed Ab responses to Ag adsorbed to Alhydrogel or Imject alum, and still found no requirement for caspase-1 (see supplemental Fig. 6A).[4] Likewise, the site of sensitization did not govern whether caspase-1 were required because responses to Ag plus alum were unaffected in mice immunized i.p. followed by intranasal challenge with Ag (see supplemental Fig. 6, A and B)[4] or after i.m. injection of Ag plus alum (Fig. 9*A*).

Although, consistent with published reports (5, 6), we found no effect on the ability of alum to increase either T cell priming or Ab production in MyD88-deficient mice (data not shown), it was possible that low levels of endotoxin present in our Ag preparation (<1.3 ng/injection) could influence our results, and could provide an explanation for the discrepant results regarding the role of the caspase-1 and NLRP3 in the adjuvant activity of alum. To test this possibility, we obtained OVA that had no detectible levels of endotoxin by the *Limulus* amebocyte lysate assay (endotoxin < 0.008 ng/injection) for immunization. Levels of IgG1 Abs induced against OVA were similar in WT and caspase-1-deficient mice injected with endotoxin free OVA plus alum (Fig. 9, *A* and *B*).

To check that our findings reflected the response to an Ag used in humans, we compared primary and secondary Ab responses to diphtheria toxin in WT or $NLRP3^{-/-}$ mice immunized with diphtheria toxoid adsorbed to alum. To control for any potential adjuvant properties of the toxoid alone, we also compared Ab responses induced in the absence of alum. Alum adsorbed diphtheria toxoid greatly enhanced Ab production against diphtheria toxin compared with injection of diphtheria toxoid alone. We confirmed that equivalent amounts of Ab that recognized diphtheria toxin were circulating in WT and NLRP3-deficient mice that had been immunized with diphtheria toxoid adsorbed to alum (Fig. 9*E*). Thus overall, we could not find any compulsory role for the inflammasome in the ability of alum to improve T and B cell responses to alum.

Discussion

Alum is a mysterious adjuvant and, despite a significant amount of work, many questions about the effects of this material remain. Others have previously shown that macrophages and DCs can respond directly to the presence of alum in vitro through activation of caspase-1 (11, 12, 47). The work described in this study confirms the finding that macrophages are targets in vivo and extends them to include responses of mast cells, suggesting that both these cell types have the machinery needed to detect alum particles. In addition, our results indicate that other resident cells, perhaps DCs or stromal cells, may also have this capacity because removal of both macrophages and mast cells from the site of alum injection did not completely ablate responses to the material.

How macrophages and mast cells detect alum is an intriguing question and in fact, alum may be detected in several, nonmutually exclusive ways. A number of in vitro experiments have shown that alum activates the NLRP3 inflammasome in macrophages, which in turn, activates caspase-1 and consequent production of IL-1β. In vitro, but not in vivo, the ability of alum to induce IL-1β production requires the additional stimulus of LPS (11, 12). The activation of the NLRP3 inflammasome in macrophages by alum requires intact phagocytic machinery (12) and may involve K^+ efflux (12) and may be initiated by so-called frustrated phagocytosis (48), or alternatively, by phagocytosis followed by fracture of lysosomes, release of lysosomal enzymes into the cytosol (14). Some data implicate uric acid in activating the inflammasome and production of IL-1β in vivo (3, 47).

The experiments described in this study show that eosinophil, neutrophil, DC, and monocyte cellular infiltrates induced in response to aluminum hydroxide are unaffected by absence of the enzyme caspase-1 needed to generate IL-1β. The accumulation of one cell type, the eosinophil is promoted in part by mast cell-derived IL-5 and histamine (37). Surprisingly, eosinophil accumulation did not require any of the many chemokines, including eotaxin, that are secreted in response to alum administration. Histamine is produced by activated mast cells and basophils, and because we found so few basophils in peritoneal cavities, this suggested an additional role for mast cells. Eosinophils respond directly to histamine via H4 histamine receptors, whereas H1 and H2 receptors are expressed by the endothelium (49). The experiments we described showed that eosinophil recruitment was inhibited by antagonists of histamine H1 and H2 receptors. Thus, in this case the histamine probably acted by promoting vascular leakiness, allowing eosinophil migration into the peritoneal cavity, rather than by affecting eosinophils directly.

The processes by which mast cells detect alum are not clear. It is known that alum activates several sets of serum enzymes, including those of the complement cascade (50) and these may activate cells such as macrophages and mast cells. However, although mast cells are activated to release histamine and other factors by complement fragments (51, 52), we show in this study that depletion of complement does not affect the appearance of eosinophil exudates in response to alum, suggesting that this is not the means whereby mast cells, at least, detect alum.

Despite the ability of mast cells and macrophages to respond to alum particles, their presence has no impact on the downstream adaptive responses initiated by alum. In addition, eosinophils are not required for T cell priming, and do not enhance the magnitude or change the overall nature of the Ab response to alum. Thus, although eosinophils express IL-4, they play no role in the suppression of Th1-associated isotypes that has been observed in IL-4-deficient or Gr1-depleted mice (18, 25). Perhaps T cells themselves, or basophils (28–32), are the important source of IL-4 that mediates this effect in vivo.

We, like some (15), but not all (3, 12, 13) have completely failed to find a connection between inflammasome activation and the adjuvant effects of alum. Our data suggest that this negative result is not due to contamination of our preparations with LPS, or with the type of alum we use, or with the timing or route of alum administration. Thus it seems that responses to Ag plus alum have a variable requirement (in our case, no requirement) for the NLRP3 inflammasome.

There are three, not mutually exclusive, explanations for the discrepant results. One is that subtle experimental differences, such as the precise status of the mice, allow the presence or absence of factors that can substitute for the products of the NLRP3 inflammasome. Assuming that are IL-1-related cytokines are responsible for mediating adjuvant effects, what could these compensating factors be? They are unlikely to be products of alum-activated macrophages such as IL-6 because macrophages, the major source of alum-induced IL-1β and IL-6 (Fig. 5), are not involved in the adjuvant activity of alum.

Another possibility is that alum enhances immune responses via redundant effects on DCs and is enhanced but not required by the activity of the NLRP3 inflammasome (3). Thus, in one study, alum enhanced trafficking of Ag-bearing monocytes and Ag presentation to a fairly large number of transferred TCR transgenic CD4 T cells in draining LNs, a process that was abrogated by neutralization of IL-1β (3, 11). Despite these effects on APCs, however, lack of NLRP3 had no negative effect on Ag-specific IgG levels in this study (11), a result that is consistent with our data. Perhaps the dependence of specific immune responses on the NLRP3 inflammasome depends on whether or not APC activity is limiting. In animals containing a large number of naive Ag-specific T cells, APCs may be limiting and the large immune response may then be dependent on NLRP3 activity for an optimal response. In animals containing a small number of endogenous Ag-specific T cells, NLRP3-mediated stimulation of APCs may not be needed and alum may act through additional pathways to support T cell priming and T dependent Ab responses.

Finally, assuming IL-1β or other related cytokines are the required product of the NLRP3 inflammsome, perhaps they can be produced via other redundant pathways. IL-1β cytokine can be produced by additional enzymes, which include proteases such as proteinase-3, elastase, and granzyme A (53). This latter possibility seems unlikely given that IL-1β levels are drastically reduced in alum-injected macrophage-deficient or caspase-1-deficient mice (Figs. 3 and 7), yet the adjuvant activity of alum in these animals is unabated.

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Disclosures

The authors have no financial conflict of interest.

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Supplemental figure legends.

Figure S1. Alum induces rapid accumulation of monocytes and neutrophils.

F4/80^{neg}IL-4^{neg} cells were analyzed for Gr1 expression and side scatter characteristics. Total number of neutrophils and inflammatory monocytes was determined using the percent of total cells that were Gr1^{hi}SSC^{hi} and Gr1^{lo}SSC^{lo} respectively. Scatter plots are representative of 3 individual mice. Numbers on scatter plots indicate the percent of cells that fall in the indicated gates for the gated population in the sample shown. Bar graphs indicate the mean total number of each indicated cell type for 3 individual mice and error bars indicate SEM for 3. Asterisks indicate a significant difference was detected between PBS and alum injected mice ($p<0.05$) using t-test. The experiment was performed more than 6 times with similar results.

Figure S2. Effect of alum on B cells, DCs, T cells, NK cells, and NKT cells

Peritoneal cells from PBS (open bars) or alum (black bars) injected mice were analyzed using flow cytometry for the presence of different cell types. A. B cells were analyzed by staining total live cells with CD19. Total B cells were determined using the percent of total cells expressing CD19 in the indicated gate. B. To focus on potential antigen presenting cells that were neither B cells nor resident macrophages, we gated on F4/80^{neg}CD19^{neg} MHC II+ cells. A small percentage of these cells were CD11c^{hi} as shown in the FACS plots. Total numbers of CD11c+ DC and CD11c- MHCII+ cells were determined using the percent of total cells that fell in the indicated gates. C. To analyze T cells and NK cells we analyzed CD19^{neg}/F4/80^{neg} cells for expression of TCRβ and NK1.1 and determined the total number of NK cells (NK1.1+TCRβ^{neg}) and T cells (TCRβ+NK1.1^{neg}) by using the percent of total cells that fell in the indicated gates. D.

Determination of NKT cells was done using staining with TCRβ and αGalCer CD1d tetramers. The percent of total cells positive for both markers as shown on the FACS plot was used to determine total numbers of NKT cells. Numbers on FACS plots indicate the percent of cells that fall within the indicated gates for the gated population in the representative sample shown. Bars on graphs represent mean values for 3 mice per group and error bars indicate SEM. Asterisks indicate a significant difference was detected from PBS control mice ($p<0.05$) as determined by t-test. A, B, and C are from a representative experiment of 3. D is from a separate representative experiment of 3.

Figure S3. Effect of clodronate liposome treatment on different cell populations in the peritoneal cavity.

A. DCs, mast cells and macrophages were analyzed as described in figures 2 and S2 in mice injected with PBS containing liposomes (PBS, black bars) or clodronate containing liposomes (open bars). Asterisk indicates a significant difference was detected compared to PBS injected controls ($p<0.05$) using t-test. Data is from one representative experiment of 2. B. Mice were injected with either PBS (open bars) or alum (filled bars) to elicit eosinophils and neutrophils. 12 hours later mice were treated with either nothing (-, black bars), PBS lipsosomes (PBS LS, dark grey bars), or clodronate liposomes (Clod LS, light grey bars) and 24 hours later total eosinophils and neutrophils were analyzed as described in figure 2. Data in B is from one representative experiment of 2. Bars on graphs indicate mean values for three mice per group and error bars indicate SEM.

Figure S4. CCR3 and complement do not play a role in the innate response to alum.

A. WT Balb/c (open bars) and CCR3KO (black bars) mice were injected with either PBS or alum. B. B6 mice were treated with either nothing (-), vehicle alone (Veh), or the CCR3 inhibitor J113863, or the CCR1 and CCR3 inhibitor UCB3425 i.p. for 2 hours and then were injected with either PBS (open bars) or alum (black bars) i.p. C. B6 mice were treated with either nothing (black bars) or cobra venom factor (open bars) to deplete complement as described in methods. 12 hours later the mice were injected i.p. with PBS or alum. Total numbers of eosinophils were determined 18 hours after alum injection using markers described in Fig. 1. Bars on graphs are mean values for 3 individual mice per group. Error bars indicate SEM. A t-test was used to determine differences between alum injected KO vs WT mice or between vehicle or inhibitor treated mice and detected none.

Figure S5. Alum induced inflammation is independent of leukotriene synthesis and of PAF.

A. WT Balb/c (black bars) or BLT1 KO mice (open bars) were injected with either PBS or alum and eosinophils analyzed 18 hours later. B. WT B6 (black bars), 5-Lipoxygenase KO (5LO KO, grey bars), or 15-lipoxygenase KO mice (15LO KO, open bars) were injected with either PBS or alum and eosinophils analyzed 18 hour later. C. B6 mice were treated with the PAF inhibitors WEB2086 (grey bars) or CV-3968 (open bars) as described in methods and then injected with either PBS, alum or sephadex beads and compared to mice that did not receive a PAF inhibitor (black bars). Eosinophils were analyzed 24 hours later. Total eosinophil numbers in A-C were determined by flow cytometry using markers described in Fig. 1. Bars on graphs are mean values for 3

individual mice per group and error bars indicate SEM. Data in each panel is from one representative experiment of 2. A t-test was used to test for differences between alum injected WT and KO mice or in C, alum injected mice that were treated with inhibitors vs control mice. Asterisks indicate a significant difference was detected ($p<0.05$).

Figure S6. Caspase 1 deficiency has no impact on IgG1 responses in vivo.

WT (Cas1+/+), heterozygous (Cas1+/-) and knockout (Cas1-/-) littermates were sensitized with two ip injections of ova adsorbed to either Imject alum (A) or Alhydrogel (B) and challenged three times i.n. with ova in PBS as described previously(11). Ova-specific IgG1 in the serum was analyzed by ELISA at the indicated timepoints. Graphs show mean values for 4 individual mice per group and data is from one representative experiment of 2. Error bars indicate SEM.

Figure S1

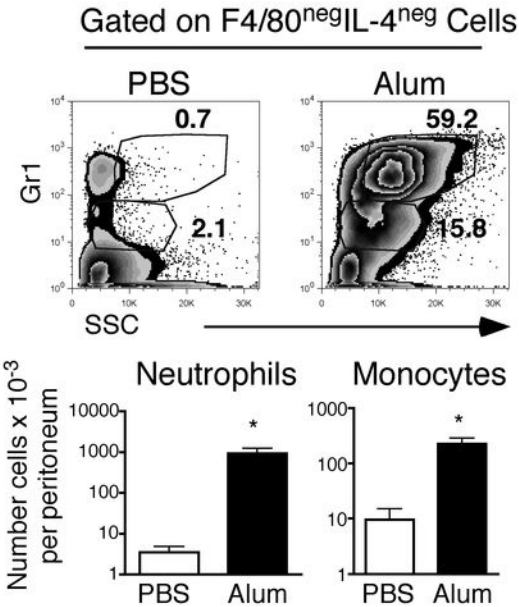

Gated on F4/80neg IL-4neg Cells
PBS
Alum
0.7
2.1
59.2
15.8
Gr1
SSC
Neutrophils
Monocytes
Number cells x 10-3 per peritoneum
PBS
Alum
PBS
Alum

Figure S2.

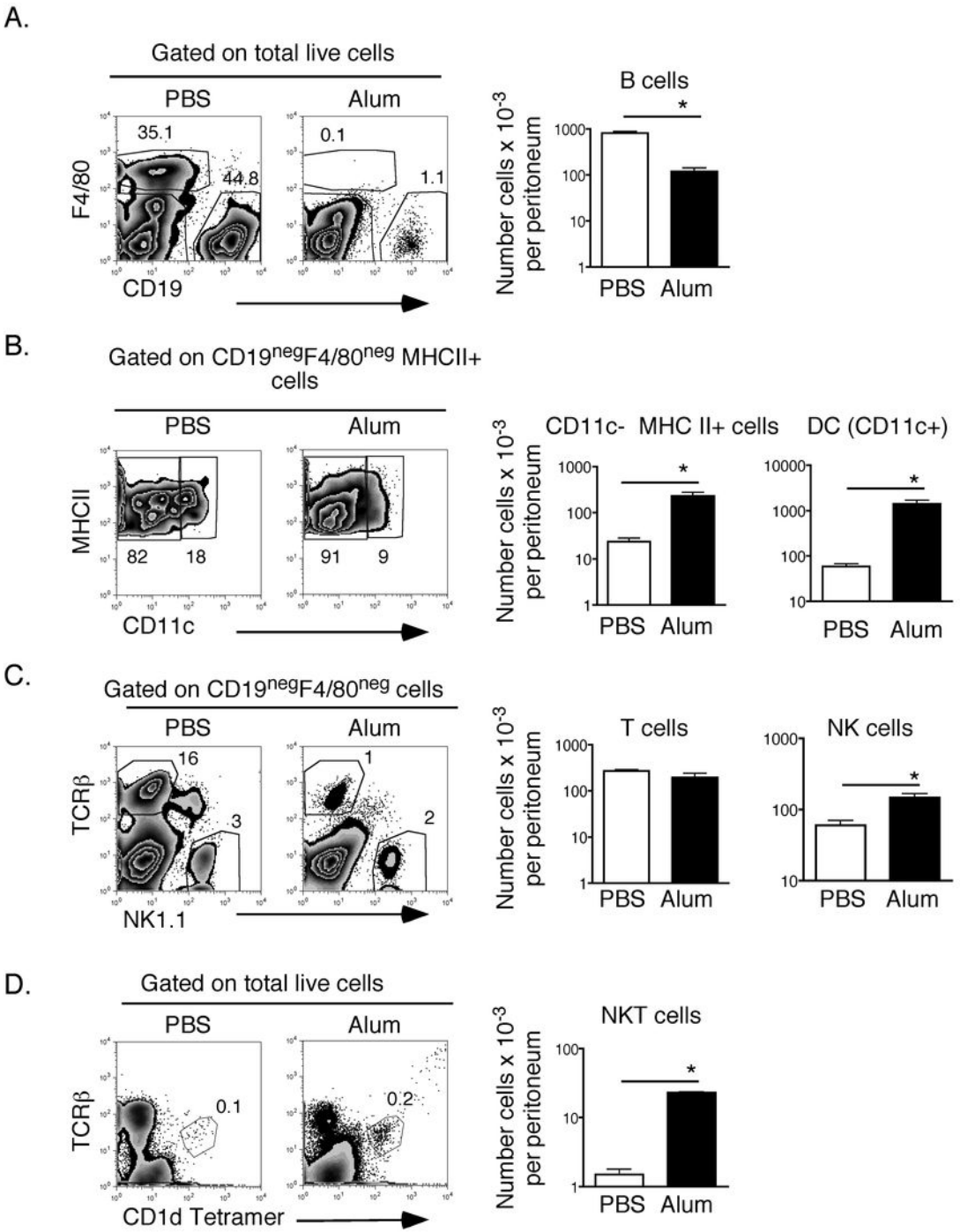

A.
Gated on total live cells
PBS
Alum
35.1
44.8
0.1
1.1
F4/80
CD19
B cells
Number cells x 10-3 per peritoneum
PBS
Alum
B.
Gated on CD19negF4/80neg MHCII+ cells
MHCII
82
18
91
9
CD11c
CD11c- MHC II+ cells
DC (CD11c+)
C.
Gated on CD19negF4/80neg cells
16
3
1
2
TCRβ
NK1.1
T cells
NK cells
D.
0.1
0.2
CD1d Tetramer
NKT cells

Figure S3.

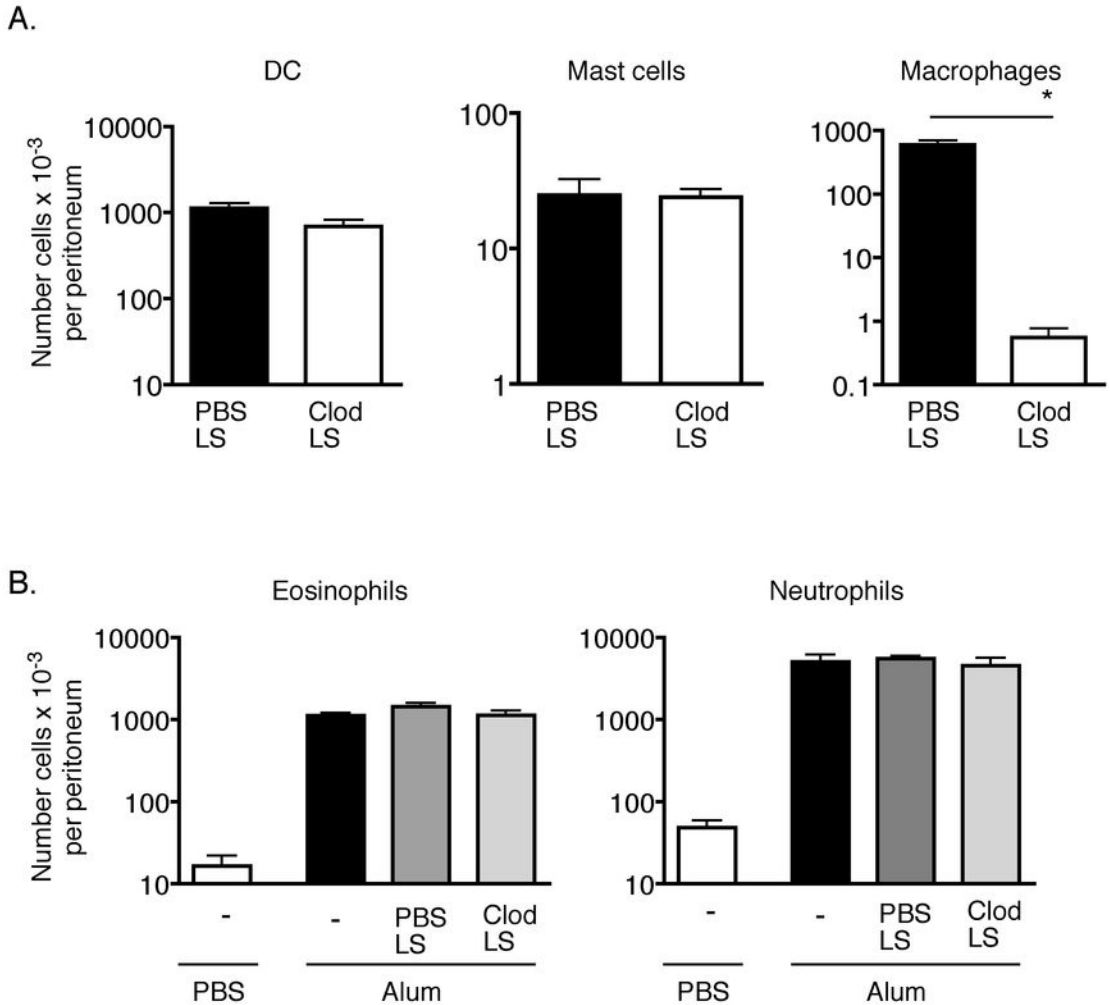

A.
DC
Mast cells
Macrophages
*
Number cells x 10-3 per peritoneum
10000
1000
100
10
100
10
1
1000
100
10
1
0.1
PBS LS
Clod LS
B.
Eosinophils
Neutrophils
10000
1000
100
10
-
PBS LS
Clod LS
PBS
Alum

Figure S4.

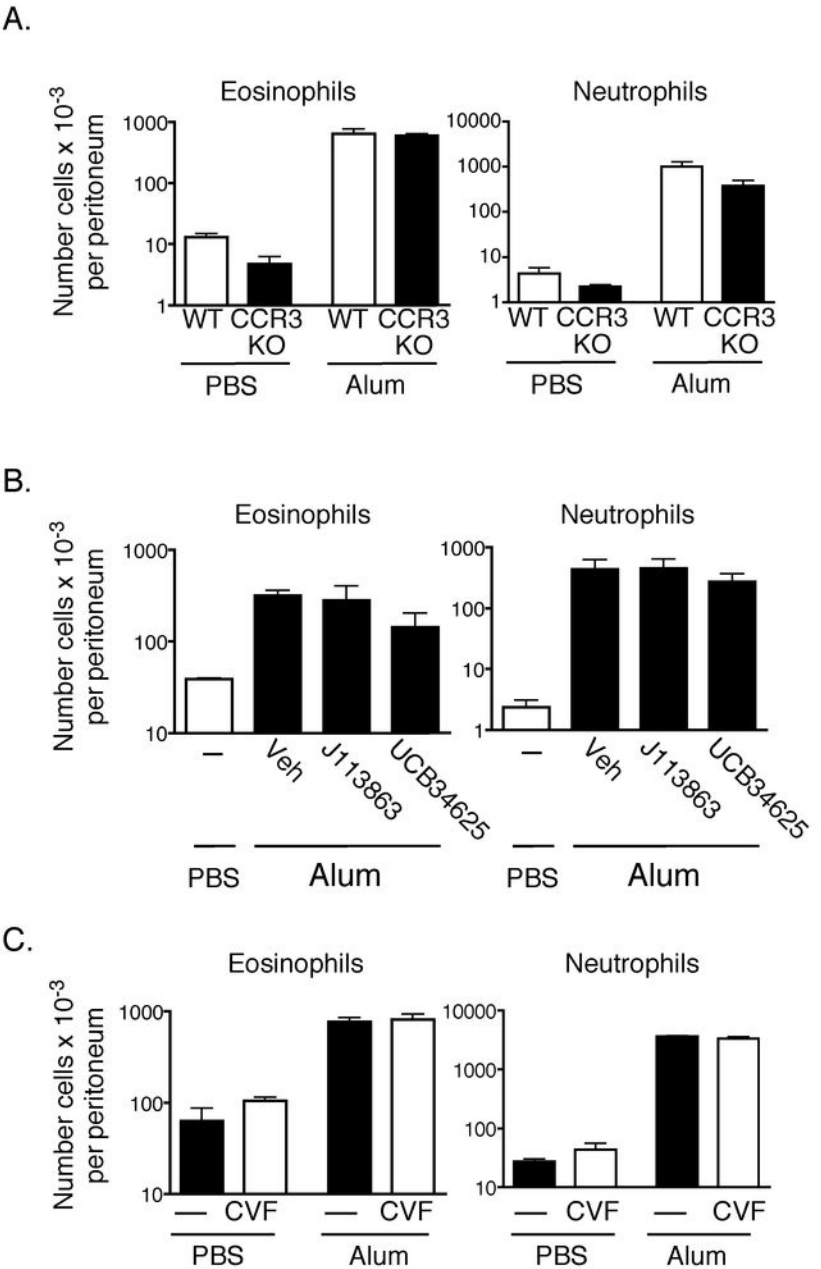

A.
Eosinophils
Neutrophils
Number cells x 10-3 per peritoneum
1000
100
10
1
10000
WT
CCR3 KO
PBS
Alum
B.
Eosinophils
Neutrophils
Number cells x 10-3 per peritoneum
—
Veh
J113863
UCB34625
PBS
Alum
C.
Eosinophils
Neutrophils
Number cells x 10-3 per peritoneum
—
CVF
PBS
Alum

Figure S5

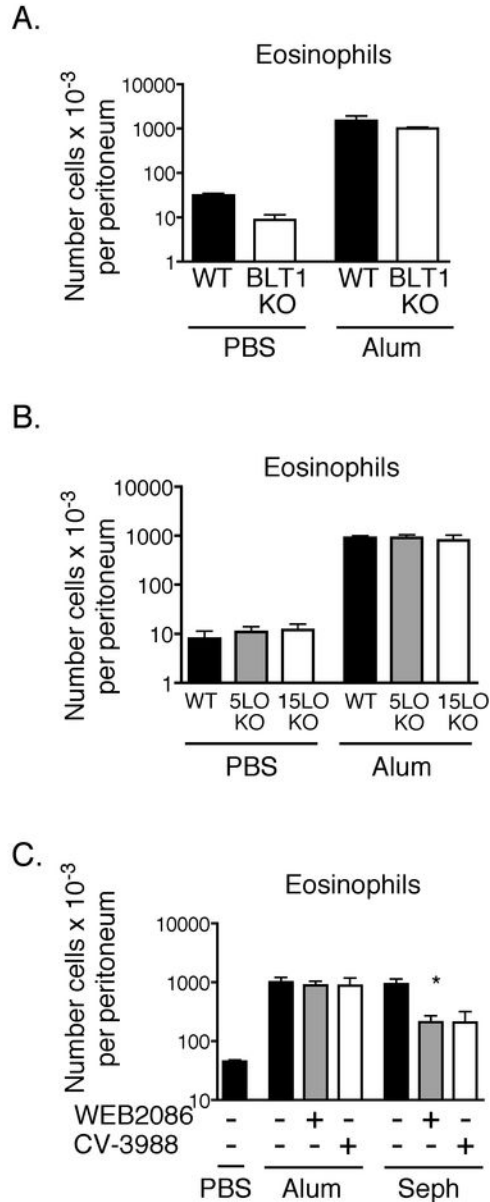
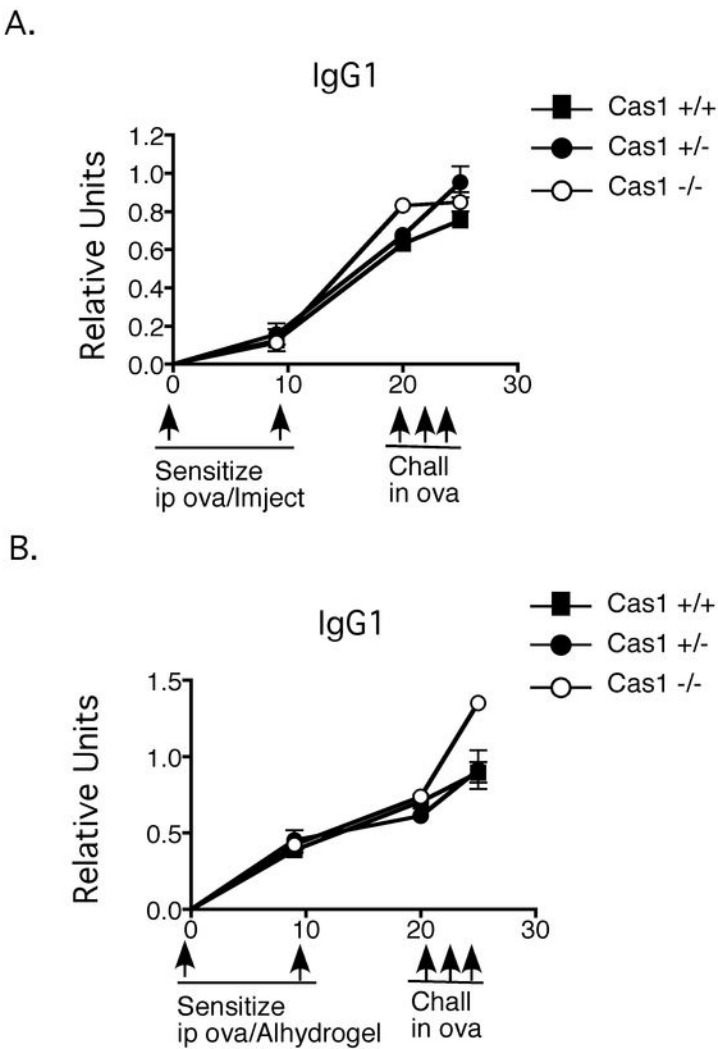

A.
Eosinophils
Number cells x 10-3 per peritoneum
10000
1000
100
10
1
WT
BLT1 KO
WT
BLT1 KO
PBS
Alum
B.
Eosinophils
Number cells x 10-3 per peritoneum
10000
1000
100
10
1
WT
5LO KO
15LO KO
WT
5LO KO
15LO KO
PBS
Alum
C.
Eosinophils
Number cells x 10-3 per peritoneum
10000
1000
100
10
*
WEB2086
CV-3988
PBS
Alum
Seph

Figure S6


A.
IgG1
Cas1 +/+
Cas1 +/-
Cas1 -/-
Relative Units
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
Sensitize
ip ova/Imject
Chall
in ova
B.
IgG1
Cas1 +/+
Cas1 +/-
Cas1 -/-
Relative Units
1.5
1.0
0.5
0.0
0
10
20
30
Sensitize
ip ova/Alhydrogel
Chall
in ova