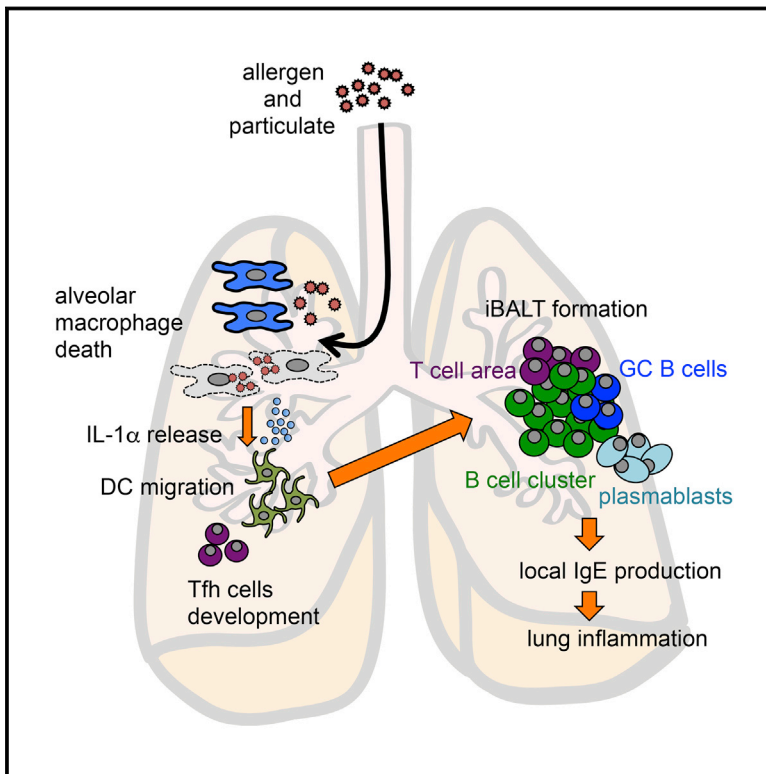


Immunity

Inhaled Fine Particles Induce Alveolar Macrophage Death and Interleukin-1 α Release to Promote Inducible Bronchus-Associated Lymphoid Tissue Formation

Graphical Abstract



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In Brief

Inhaled fine particulates are known to induce allergic responses, but the exact cell types and immunological factors that initiate the lung-specific immune responses remain unclear. Kuroda et al. (2016) show that particulates trigger tertiary lymphoid organ formation in the lung as a consequence of alveolar macrophage death and IL-1 α release.

Highlights

- Particulates act as a long-lasting adjuvant to subsequent antigen inhalation
- Particulates induce alveolar macrophage death and IL-1 α release in the lung
- IL-1 α release in the lung is involved in iBALT formation
- Tfh cells induced by direct airway sensitization are required for iBALT formation



Inhaled Fine Particles Induce Alveolar Macrophage Death and Interleukin-1 α Release to Promote Inducible Bronchus-Associated Lymphoid Tissue Formation

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SUMMARY

Particulate pollution is thought to function as an adjuvant that can induce allergic responses. However, the exact cell types and immunological factors that initiate the lung-specific immune responses are unclear. We found that upon intratracheal instillation, particulates such as aluminum salts and silica killed alveolar macrophages (AMs), which then released interleukin-1 α (IL-1 α) and caused inducible bronchus-associated lymphoid tissue (iBALT) formation in the lung. IL-1 α release continued for up to 2 weeks after particulate exposure, and type-2 allergic immune responses were induced by the inhalation of antigen during IL-1 α release and iBALT formation, even long after particulate instillation. Recombinant IL-1 α was sufficient to induce iBALTs, which coincided with subsequent immunoglobulin E responses, and IL-1-receptor-deficient mice failed to induce iBALT formation. Therefore, the AM-IL-1 α -iBALT

axis might be a therapeutic target for particulate-induced allergic inflammation.

INTRODUCTION

The numbers of patients with allergic asthma or rhinitis have recently increased, especially in developed countries. The reason is unclear, but many studies have suggested that particulate pollution might exacerbate allergic responses (Ichinose et al., 2008; Løvik et al., 1997). Several nanometer- to micrometer-sized particulates, such as particulate matter 2.5 (PM_{2.5}), which is less than 2.5 μ m in diameter, can enter the respiratory tract and settle deep in the lungs, causing chronic pulmonary inflammation, including asthma (Granum and Løvik, 2002). In an epidemiological study, increased particulate matter in the air was significantly related to increased hospitalization for asthma (Schwartz et al., 1993). Particulates stimulate the immune system to generate inflammatory responses; aluminum salts (referred to as alum) and silica crystals particularly induce type 2 responses, characterized by eosinophil accumulation at the injection sites and elevated antigen-specific serum immunoglobulin E (IgE) levels

in vivo (Kool et al., 2011; Kuroda et al., 2011; Mancino et al., 1983; Marichal et al., 2011). Most particulates, including particulate pollutants, are thought to function as immune adjuvants enhancing allergen-specific type 2 responses. However, the basis for the adjuvant activity of these particulates and the underlying mechanisms by which they induce type 2 responses are still unclear.

Recent studies have demonstrated that innate immunity is required for the effective induction of adaptive immunity (Akira, 2011). In general, pathogen-derived factors, known as pathogen-associated molecular patterns, are recognized by innate immune cells via pattern-recognition receptors (PRRs). In addition, factors released from dying or stressed cells, damage-associated molecular patterns (DAMPs) (or “alarmins”), also contribute to inflammation, and are recognized by PRRs or cytokine receptors on innate immune cells. DAMPs can be critical for adjuvant activity and the induction of sterile inflammation (Bianchi, 2007; Desmet and Ishii, 2012; Rock et al., 2011; Willart and Lambrecht, 2009). Several studies have demonstrated that particulates stimulate innate cells to release DAMPs and that DAMP-dependent innate immune responses are required for the induction of adaptive immune responses (Kool et al., 2011; Marichal et al., 2011; McKee et al., 2013). Some small cytotoxic chemicals also function as adjuvants, inducing antigen-specific IgG1 and IgE production (Jacobson et al., 2013; Tonti et al., 2013). Therefore, particulate-induced DAMPs might participate in the induction and exacerbation of allergic responses.

Inducible bronchus-associated lymphoid tissues (iBALTs) are a tertiary lymphoid organ (TLO) that is not preprogrammed, but is induced in the lung by chronic inflammation, infections, or autoimmune diseases (Neyt et al., 2012; Rangel-Moreno et al., 2006). Lymphoid clusters are also frequently found in the lungs of patients with asthma and in a mouse model of asthma, and are currently regarded as iBALTs (Chvatchko et al., 1996; Elliot et al., 2004; Foo and Phipps, 2010). iBALTs are found in the lung parenchyma. It supports T and B cell proliferation and enhances immunoglobulin class switching in its germinal centers (GC) and also contributes to the survival of plasma cells in the lung (Foo and Phipps, 2010; Randall, 2010). iBALTs are reported to offer protective immunity against pathogens and induces virus-specific antibody production (GeurtsvanKessel et al., 2009; Moyron-Quiroz et al., 2004). However, iBALTs are sometimes considered detrimental, and is reported to correlate with disease severity in patients with rheumatoid arthritis (RA) and chronic obstructive pulmonary disease (COPD) (Neyt et al., 2012; Rangel-Moreno et al., 2006). Thus, iBALTs play important roles in the immune responses that are specifically occurring in the lung.

Some environmental particles such as sand dust are composed of mineral elements, including silicon and aluminum (Ichinose et al., 2008). Therefore, we established a particulate-induced lung inflammation model using the intratracheal (i.t.) instillation of particulate alum or silica. In this model, we found that the direct administration of particulates to the airway induced unique immune responses in the lung, involving interleukin-1 α (IL-1 α) release after particulate-induced alveolar macrophage death, and the subsequent formation of iBALT. Therefore, the release of DAMPs, triggered by exposure to particulates, and TLO formation might be important adjuvant effects in the induction of particulate-pollution-induced allergic inflammation.

RESULTS

Alveolar Macrophages Specifically Induce IL-1 α in Response to Particulates

When lungs are exposed to particulates such as alum or silica, the particulates are thought to be engulfed by phagocytes, such as alveolar macrophages, to be cleared from deep in the lung (Ling and van Eeden, 2009; Oberdörster et al., 2005). Therefore, we first examined the effects of particulates on alveolar macrophage function. We prepared in-vitro-differentiated alveolar macrophages (referred to as “cultured alveolar macrophages,” in Figure S1A), stimulated them with alum in vitro, and then measured the typical proinflammatory factors produced by macrophages. Alum stimulated alveolar macrophages to produce IL-1 α , but no other inflammatory factors were detected (Figure 1A). In a similar experiment using alveolar macrophages freshly isolated from mouse lungs, alum-stimulated cells produced IL-1 α (Figure 1B). Crystalline silica also stimulated cultured alveolar macrophages to produce IL-1 α . Both alum and silica induced cell death (Figure 1C). However neither aluminum oxide nor hydroxyapatite induced IL-1 α production or cell death.

A previous report shows that particulates, such as alum and monosodium urate crystals, induce IL-1 α release by bone marrow (BM)-derived dendritic cells (BMDCs) or BM macrophages that were primed with lipopolysaccharide (LPS) (Gross et al., 2012). However, we used alveolar macrophages without LPS priming. We also found that *Myd88*^{−/−} alveolar macrophages released IL-1 α after stimulation with alum (Figure 1D). A previous study shows that IL-1 α production is partly associated with the activation of apoptosis-associated speck-like protein containing CARD (ASC) in BMDCs and keratinocytes (Yazdi et al., 2010). However, particulates induced similar IL-1 α levels in wild-type (WT) and *Asc*^{−/−} alveolar macrophages (Figure 1E). These results indicate that both MyD88 (Toll-like receptor [TLR] signaling) and ASC (inflammasome) were not essential for the induction of IL-1 α in alveolar macrophages in response to particulates.

Cultured alveolar macrophages include two different populations: CD11c⁺ sialic-acid-binding immunoglobulin-type lectins (siglec)-F⁺ cells and CD11c^{low} Siglec-F^{low} cells. We observed higher amounts of IL-1 α from cells expressing CD11c⁺ and siglec-F⁺, typical surface markers of alveolar macrophages (Figure 1F). Moreover, neither peritoneal macrophages, granulocyte-macrophage colony-stimulating factor (GM-CSF)-derived BM-macrophages, nor lung fibroblasts produced IL-1 α in response to alum or silica, whereas these particles caused the same degree of cell death in all the cell types we tested (Figure 1G and Figure S1B, S1C–S1E). We also examined the release of IL-1 α by small intestinal lamina propria macrophages (Uematsu et al., 2008), however IL-1 α was not released by the lamina propria macrophages in response to alum (Figure 1H). Therefore, particulate-induced IL-1 α release was a unique characteristic of alveolar macrophages.

Because IL-1 α is released from dying cells and functions as a DAMP (Cohen et al., 2010; Rider et al., 2011), we next examined the intracellular IL-1 α in the lysates of alveolar macrophages after three freeze-thaw cycles. We detected intracellular IL-1 α in unstimulated alveolar macrophages and the amounts of

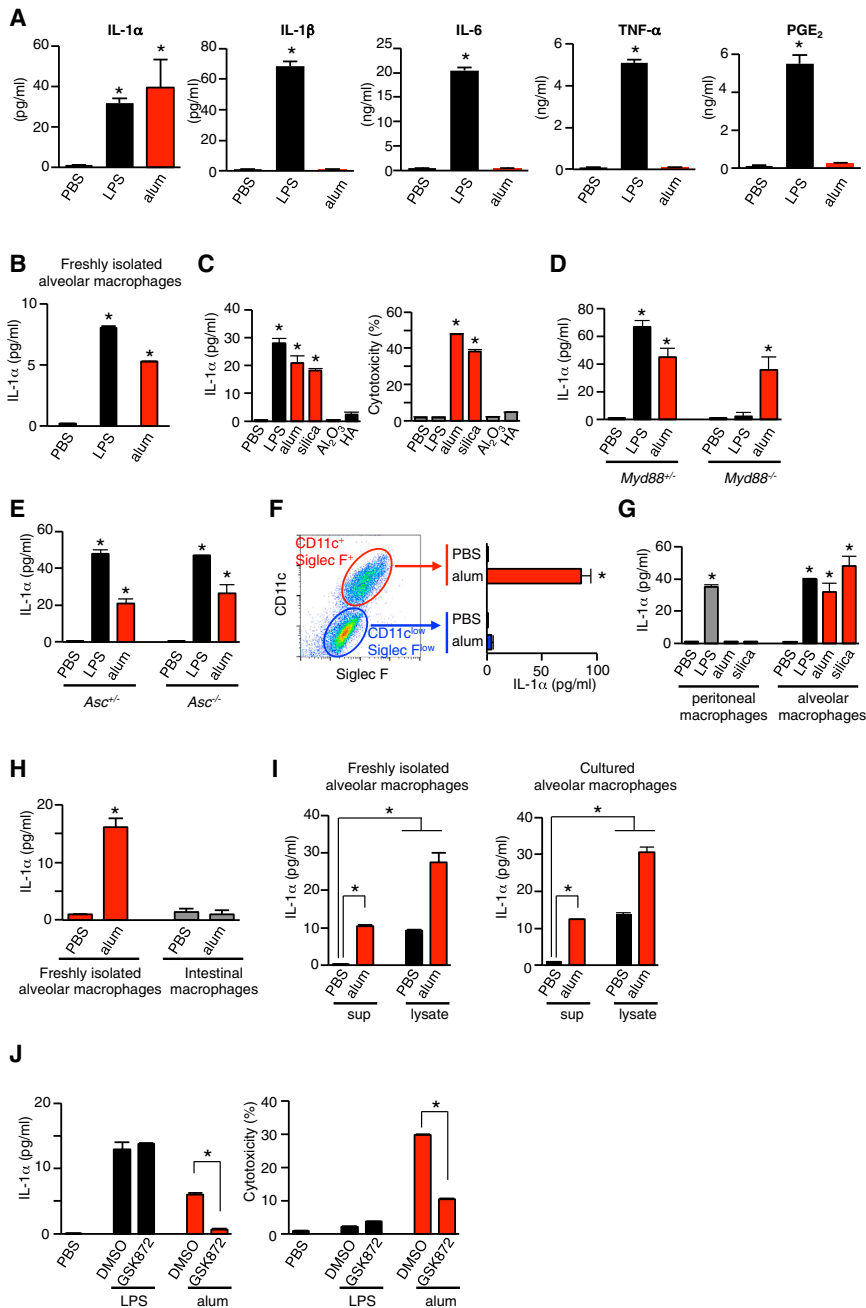


Figure 1. Alveolar Macrophages Release IL-1 α in Response to Particulate Adjuvants

(A) Cultured alveolar macrophages were stimulated with LPS or alum for 6 hr. Supernatants were analyzed for cytokines with ELISAs.

(B and C) Freshly isolated (B) and cultured (C) alveolar macrophages were stimulated with LPS or particulates as indicated for 6 hr. Supernatants were used for cytokine ELISAs or to test for lactate dehydrogenase (LDH) release. Al₂O₃, aluminum oxide; HA, hydroxyapatite.

(D) Myd88- or (E) ASC-deficient cultured alveolar macrophages were stimulated with LPS or alum for 6 hr.

(F) Cultured alveolar macrophages were sorted into CD11c⁺ siglec-F⁺ and CD11c^{low} siglec-F^{low} cells, and then the sorted cells were stimulated with alum for 6 hr.

(G) Peritoneal or cultured alveolar macrophages were stimulated with LPS, alum, or silica for 6 hr.

(H) Freshly isolated alveolar and lamina propria macrophages were stimulated with alum for 8 hr.

(I) Freshly isolated and cultured alveolar macrophages were stimulated with alum for 6 hr. The culture supernatants and cell lysates were used for an IL-1 α ELISA.

(J) Cultured alveolar macrophages were stimulated with alum in the presence of a RIPK3 inhibitor (GSK872). All macrophages were prepared from five to ten mice. Data are shown as mean $\pm$ SD and represent at least two independent experiments (* p < 0.05), except in Figure 1H, in which the experiment was performed once, but 50 mice were used for the preparation of the lamina propria macrophages. See also Figure S1.

Therefore, we suggest that alum-induced cell death was RIPK3-dependent but RIPK1-independent necroptosis (Moriwaki and Chan, 2016).

Particulates Induce IL-1 α Release after Cell Death, Resulting in the Generation of B Cell Clusters

We next examined whether alveolar macrophages in the lung release IL-1 α in vivo. An immunohistochemical analysis of lung tissues showed that some of the F4/80⁺ cells expressed IL-1 α after the intratra-

cheal (i.e.) administration of alum (Figure 2A), whereas we could not observe IL-1 α in other types of cells. Alveolar macrophages constitutively expressed intracellular IL-1 α , but at very low levels (picograms/ml). In contrast, LPS-stimulated alveolar macrophages released IL-1 α at picogram levels, but expressed 100-fold greater amounts of intracellular IL-1 α (Figure S2A), and the intracellular IL-1 α was easily detected with flow cytometry (Figure S2B). However, it was difficult to detect intracellular IL-1 α in unstimulated or alum-stimulated alveolar macrophages with flow cytometry (Figure S2B). We detected a weak signal for intracellular IL-1 α in some of the F4/80⁺ cells in bronchoalveolar lavage (BAL) cells from mice after the i.t. administration of alum (Figure S2C).

The mechanisms by which particulates induce the cell death of alveolar macrophages are unclear. We found that GSK872, a receptor-interacting serine/threonine protein kinase-3 (RIPK3) inhibitor that suppresses necroptosis, significantly blocked both IL-1 α and cell death, indicating that alum-induced cell death was necroptotic (Figure 1J) (Silke et al., 2015). However, necrostatin did not suppress IL-1 α release or cell death (Figure S1F).

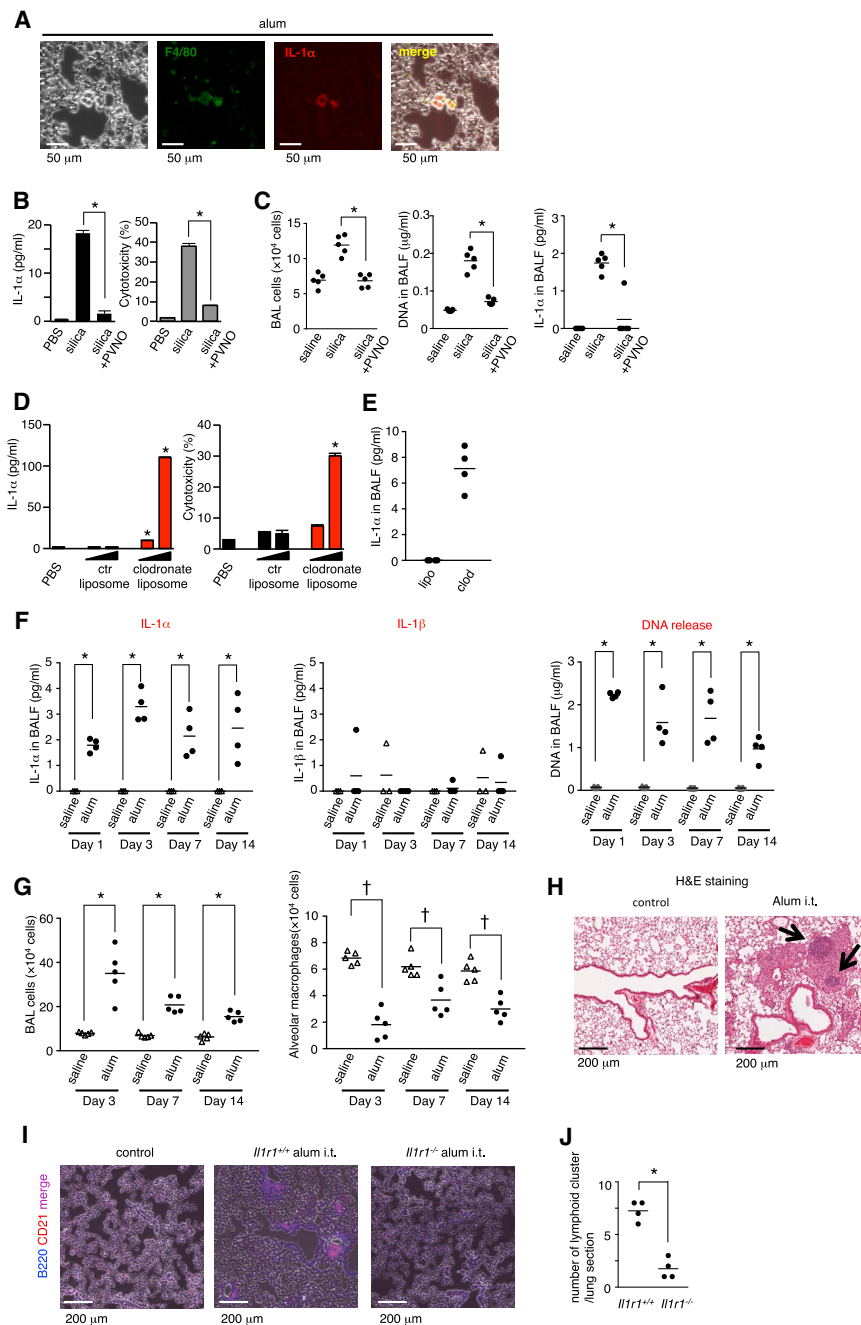


Figure 2. IL-1 α Is Released after Alveolar Macrophage Cell Death in the Lung

(A) Lungs ($n = 4$) were isolated 3 days after the i.t. instillation of alum. Frozen section of the isolated lung specimens were stained for F4/80 and IL-1 α , and analyzed with confocal microscopy. Scale bars represent 50 μ m.

(B) Cultured alveolar macrophages were stimulated with silica in the presence of PVNO. The supernatants were analyzed for cytokines with ELISAs or for LDH release.

(C) Silica was administered to mice ($n = 5$ mice, each group) by i.t. instillation with PVNO. BALF was harvested 3 days after particulate administration.

(D) Cultured alveolar macrophages were stimulated with empty or clodronate liposomes for 6 hr. (E) Clodronate (clod) or empty (lipo) liposomes were administered to mice ($n = 4$ mice, each group) by i.t. instillation. BALF was harvested 3 days after instillation and tested for IL-1 α with an ELISA.

(F) Alum was administered by i.t. instillation ($n = 4$ mice, each group). At 1, 3, 7, or 14 days after administration, BALF was harvested and analyzed for IL-1 α and IL-1 β with ELISAs or for DNA release.

(G) At 3, 7, or 14 days after i.t. alum administration, the total BAL cells and alveolar macrophages in the BALF were analyzed. ($n = 5$ mice, each group).

(H) Histological analysis (H&E staining) of the lungs of mice ($n = 4$ mice, each group) 14 days after i.t. instillation of alum. Scale bars represent 200 μ m. Arrows indicate lymphoid clusters.

(I and J) Lungs were isolated from *Il1r1*^{+/+} and *Il1r1*^{-/-} mice ($n = 4$ mice, each group) 14 days after the i.t. instillation of alum. (I) Frozen section of the isolated lung specimens were stained for B220 and CD21 (scale bars represent 200 μ m), and (J) the number of lymphoid clusters in the lungs of *Il1r1*^{+/+} and *Il1r1*^{-/-} mice. All alveolar macrophages were prepared from five to ten mice. Data are shown as mean $\pm$ SD and represent at least two independent experiments (*, $p < 0.05$). See also Figure S2.

IL-1 α released by macrophages in response to particulates seemed to be linked to cell death (Figures 1C and 1J). It is previously reported that treating macrophages with poly-2-vinylpyridine-N-oxide (PVNO), a lysosome-stabilizing agent, suppresses their silica-induced cell death (Kuroda et al., 2011; Mancino et al., 1983). Indeed, treatment with PVNO rescued alveolar macrophages from cell death and reduced their IL-1 α release in response to silica in vitro (Figure 2B). Similarly, the host DNA, an indicator of cell death, and IL-1 α released in the BAL fluids (BALFs) and bronchoalveolar lavage (BAL) cell recruitment in the lung after the i.t. instillation of silica were also significantly reduced by PVNO treatment in vivo (Figure 2C). These results

indicate that particulate-induced IL-1 α release was mediated by cell death. Clodronate liposomes are frequently used for the specific depletion of phagocytosis. Indeed, clodronate liposomes induced cell death and IL-1 α release in alveolar macrophages (Figure 2D). In an in vivo analysis, we observed the reduced number of alveolar macrophages in the BALF after the i.t. instillation of clodronate liposomes (Figure S2D). Furthermore, IL-1 α was released into the BALF after treatment with clodronate liposomes (Figure 2E). These results indicate that the direct administration of particulates to the airways induced IL-1 α in the lung via alveolar macrophage death.

We found that IL-1 α and DNA were released into the BALF for 14 days after the i.t. instillation of alum (Figure 2F), whereas IL-1 β was not detected in the BALF at any time point. The numbers of alveolar macrophages were also still reduced 14 days after the i.t. instillation of alum, regardless of the increased number of

inflammatory cells in the lung (Figure 2G and Figure S2E). These results suggest that alum-induced IL-1 α persisted for 2 weeks, which might be mediated by ongoing alveolar macrophage death.

We next performed a histological analysis of the lung after i.t. alum instillation. Hematoxylin-and-eosin (H&E) staining revealed that, in addition to the infiltration of inflammatory cells, lymphoid clusters were induced 14 days after the i.t. instillation of alum (Figure 2H). These clusters were mainly composed of B cells, and were characterized by areas of B220⁺ CD21⁺ B cells (Figure 2I). The number of clusters was clearly reduced in IL-1-receptor-deficient (*Il1r1*^{-/-}) mice (Figures 2I and 2J). These results suggest that the prolonged IL-1 α release caused by particulate-induced cell death was involved in the formation of lymphoid clusters in the lung.

IL-1 α Release and iBALT Formation Play Roles in the Delayed and Sustained Adjuvant Effects

Particulate-induced lymphoid clusters seem to be iBALTs. Therefore, antigen-specific immune responses might be induced by the administration of antigen into the lung during the release of IL-1 α and the formation of iBALT. To test this, mice were initially administered i.t. alum and were exposed to aerosolized ovalbumin (OVA) 3, 7, and 14 days later. Despite the lag time, the exposure to aerosolized OVA after alum instillation induced high OVA-specific serum IgG1 and IgE concentrations and increased BAL cell recruitment (Figure 3A). OVA exposure alone had no or a weak effect on antibody responses and BAL cell recruitment. The antibody responses were detected in mice exposed to OVA even 14 days after the administration of alum, which correlated with the release of IL-1 α and the formation of iBALT (Figures 2F and 2I). These data suggest that immune responses were activated even when the antigen and adjuvant were administered separately, and that delayed exposure to the antigen long after particulate inhalation affected lung inflammation and the IgE response. We administered alum intraperitoneal (i.p.) on day -7, and then injected OVA i.p. twice, but no OVA-specific serum antibody responses were detected (Figure 3B). Therefore, the immune responses induced by the direct administration of particulates into the lung were regulated by different mechanisms than those induced by their i.p. injection. Crystalline silica and nickel oxide (NiO) nanoparticles also have strong adjuvant activity and induce type 2 immune responses (Kuroda et al., 2011). Mice were administered i.t. alum, silica, or NiO and then exposed to aerosolized OVA 7 days later. We observed significant increases in OVA-specific serum IgG1 and IgE concentrations and in the number of BAL cells (Figure S3A). A similar experiment using other noninflammatory particulates, aluminum oxide and hydroxyapatite, induced weak IgG1 and no IgE responses (Figure S3B). Therefore, not all particulates administered by i.t. instillation induced severe lung inflammation and IgE production. Elevated numbers of airway eosinophils and goblet cell hyperplasia (periodic acid-Schiff [PAS] staining) were also found in mice exposed to OVA 7 days after the i.t. instillation of alum (Figure 3C). We performed a similar experiment with house-dust-mite (HDM) allergen instead of OVA. Mice were administered i.t. alum, and after 7 days, were sensitized to HDM by the intranasal (i.n.) route. The i.t. injection of alum enhanced the HDM-specific antibody responses and the number of BAL cells (Figure 3D).

Il1r1^{-/-} mice displayed reduced OVA-specific IgG1 concentrations, and OVA-specific IgE was absent in the mice exposed to OVA 7 days after the i.t. instillation of alum (Figure 3E) or silica (Figure S3C). We performed similar experiments with IL-1 α -deficient mice, and OVA-specific IgG1 and IgE concentrations were significantly reduced in *Il1a*^{-/-} mice (Figure 3F). Many reports have shown that particulates stimulate DCs and macrophages to produce IL-1 β by activating the NLRP3 inflammasome (Eisenbarth et al., 2008; Hornung et al., 2008). However, NLRP3-, caspase-1-, and ASC-deficient mice exhibited significant amounts of serum IgG1 and IgE, similar to those in the WT mice (Figure 3G and Figures S3D and S3E). Thymic stromal lymphopoietin (TSLP) and IL-33, important molecules in allergic inflammation (Haenuli et al., 2012; Zhou et al., 2005), were not involved in particulate-induced IgE production (Figures S3F and S3G). Although interferon regulatory factor 3 (IRF3) and stimulator of interferon gene (STING) are involved in the adjuvanticity of alum (Marichal et al., 2011; McKee et al., 2013), neither IRF3 nor STING was involved in the antibody responses induced by i.t. instillation of alum (Figures S3H and S3I). These results indicate that the induction of IgE by direct airway sensitization with particulates involved mechanisms that differ from those reported previously.

IL-1 α in the Lung Acts as a Potent iBALT Inducer and Is Involved in Local Immune Responses

We examined the particulate-induced iBALT structure in detail. Exposure to OVA 7 days after the i.t. instillation of alum induced many lymphoid clusters characterized by B220⁺ and CD21⁺ cell aggregates (Figure 4A), as did the i.t. instillation of alum without OVA exposure (Figure 2I). However, more and larger iBALT structures, around 300 μ m in diameter, were induced with OVA exposure than those without OVA exposure. Some of these clusters contained GL-7⁺ GC B cell areas (Figure 4B). Small T cell areas were observed and CD138⁺ cells (plasmablasts) were also located close to B cell clusters (Figures 4C and 4D). Areas of CD138⁺ cells were also stained for labeled OVA (Figure 4C). These results indicate that particulate-induced iBALTs functioned as TLOs in the lung and induced antigen-specific responses. The number of iBALTs were significantly reduced in *Il1r1*^{-/-} mice exposed to OVA 7 days after the i.t. instillation of alum (Figures 4E and 4F). OVA exposure without i.t. alum instillation did not induce iBALT structures in the lung (Figures 4E and 4F). Given that iBALT structures are reported to affect the local humoral immunity (GeurtsvanKessel et al., 2009), we measured the OVA-specific antibody responses in the BALF. OVA exposure after alum or silica instillation induced significant concentrations of OVA-specific IgG1 and IgE in the BALF, and these responses were absent in *Il1r1*^{-/-} mice (Figure 4G and Figure S4A). The i.t. instillation of IL-1 α plus OVA induced strong OVA-specific IgE responses, so IL-1 released in the lung played an important role in IgE production (Figure 4H). The direct instillation of IL-1 α into the airway also induced iBALT structures in the lung (Figure 4I and Figure S4B). The i.t. instillation of clodronate liposomes induced the release of IL-1 α in the lung in vivo (Figure 2E). OVA-specific IgG1 and IgE responses were detected in mice exposed to OVA 3 days after the i.t. instillation of clodronate liposomes (Figure 4J), and we also observed iBALT structures in the lung (Figure 4K and Figure S4C). These

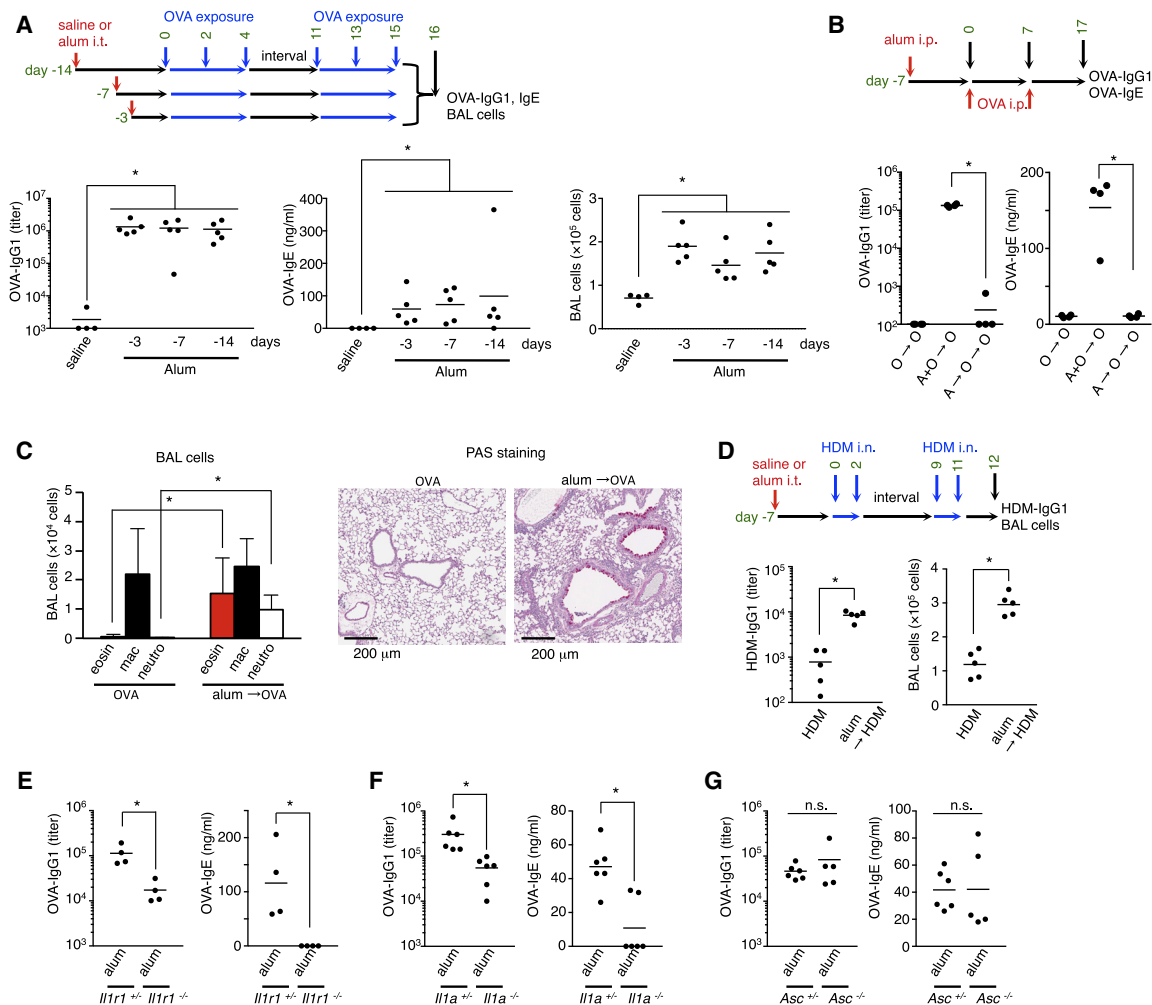


Figure 3. Delayed and Sustained Adjuvant Effect of Particulates Is Unique to the Lung

(A) Saline or alum was administered by i.t. instillation ($n = 4$ or 5 mice, each group). At 3, 7, or 14 days after its administration, the mice were exposed to aerosolized OVA. Sera and BALF were collected 24 hr after the last exposure.

(B) Alum was injected i.p. on day -7 and OVA was injected i.p. on days 0 and 7 (A→O→O) ($n = 4$ mice, each group). Injection of OVA alone (O→O) and alum mixed with OVA (A+O→O) were used as the controls.

(C) BAL cell and histological analyses (PAS staining) of the lungs from mice ($n = 4$ mice, each group) exposed to OVA 7 days after the i.t. instillation of alum. Scale bars represent 200 μm .

(D) Saline or alum was administered by i.t. instillation ($n = 5$ mice, each group). Seven days after its administration, the mice were sensitized with HDM by i.n. administration.

(E–G) Alum was administered to *Il1r1*^{-/-} (E), *Il1a*^{-/-} (F), and *Asc*^{-/-} (G) mice ($n = 4$ – 6 mice, each group). Seven days later, the mice were exposed to aerosolized OVA as in (A). Data are shown as mean $\pm$ SD and represent at least two independent experiments (* $p < 0.05$). See also Figure S3.

results indicate that the IL-1 α released by the death of alveolar macrophages was essential for the particulate-induced formation of iBALT and both the systemic and local IgE responses in the lung.

Particulate-Induced IL-1 α Regulates iBALT Formation through DC Migration

DCs play an important role in the formation and maintenance of iBALT structures (GeurtsvanKessel et al., 2009; Halle et al., 2009). The recruitment of DCs was observed in the lung after the i.t. instillation of alum and exposure to OVA, but less DC migration into the lung was observed in *Il1r1*^{-/-} mice (Figure 5A).

The i.t. instillation of IL-1 α also caused a marked increase in DC recruitment into the lung (Figure S5A). Thus, particulate-induced IL-1 α was involved in the recruitment of DCs to the lung. Next, we depleted lung DCs (as CD11c⁺ cells) in vivo using CD11c-diphtheria toxin receptor (DTR) transgenic mice. Diphtheria toxin (DT) was administered by i.t. instillation 1 day before the last exposure to OVA. Four days later, we analyzed the antibody responses and iBALT formation. After the administration of DT, the number of DCs was reduced in the lung (Figure S5B). The lung-CD11c⁺-cell-depleted mice displayed reduced antigen-specific antibody responses and the reduced number of iBALT structures (Figure 5B and Figure S5C). These results indicate

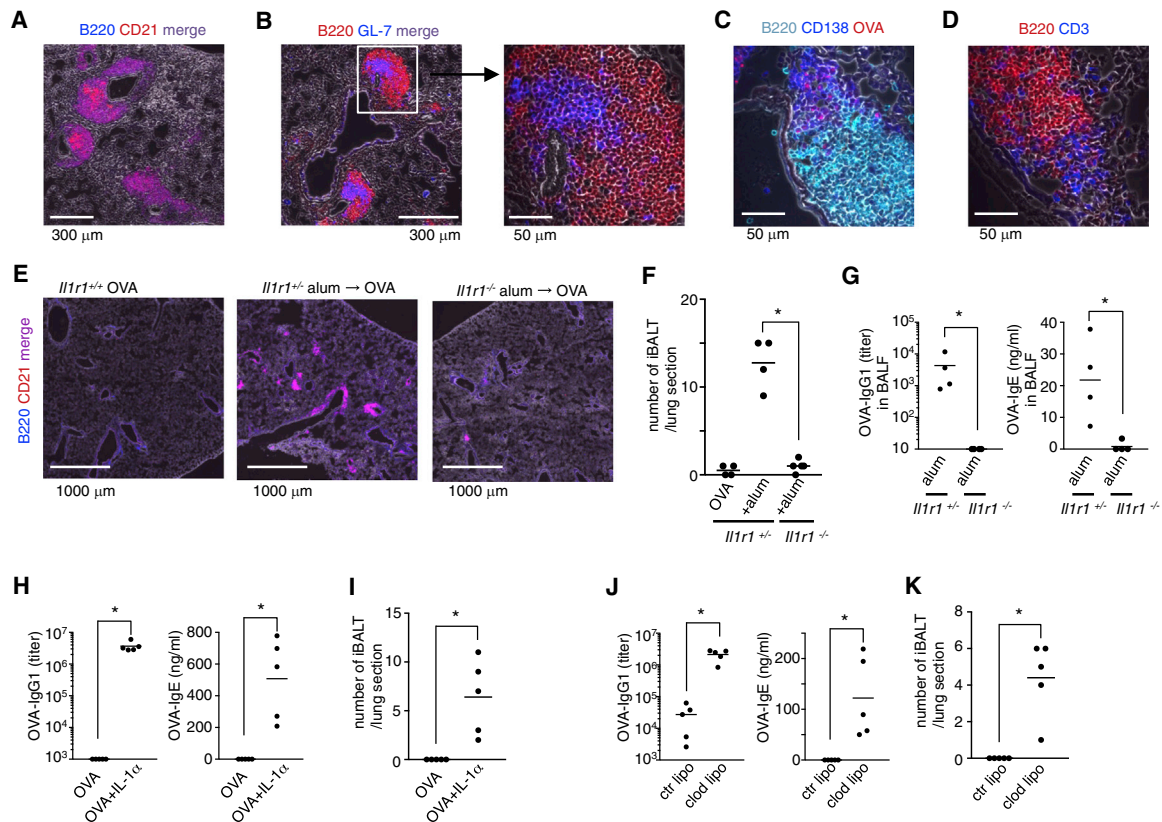


Figure 4. Exposure to Alum and Antigen Induces iBALTs in the Lung in an IL-1-Dependent Manner

(A–D) Frozen sections of lungs were isolated from mice ($n = 4$ mice, each group) exposed to OVA for 7 days after the i.t. instillation of alum. Frozen sections of specimens were stained for B220 and CD21 (A), B220 and GL7 (B), CD138, B220 and OVA (C), B220 and CD3 (D). All samples were analyzed with confocal microscopy. Scale bars represent 300 or 50 μm , as indicated.

(E and F) iBALT formation in *Il1r1*^{-/-} mice ($n = 4$ mice, each group). Immunohistochemical analysis of iBALT formation in the lungs (E) and the number of iBALT structures in lung sections (F) from WT and *Il1r1*^{-/-} mice. Scale bars represent 1,000 μm .

(G) Antibody responses in BALF from mice ($n = 4$ mice, each group) exposed to OVA after i.t. alum instillation.

(H and I) OVA or IL-1 α +OVA was administered to mice ($n = 5$ mice, each group) by i.t. instillation and the mice were then exposed to aerosolized OVA. At 24 hr after last exposure, the sera and lungs were analyzed for antibody responses (H) and the number of iBALT structures in the lung sections (I).

(J and K) I.t. instillation of clodronate liposomes induces immune responses in the lung. Empty (ctr lipo) or clodronate (clod lipo) liposomes were administered to mice ($n = 5$ mice, each group) by i.t. instillation Three days after their administration, the mice were exposed to aerosolized OVA. Sera and lungs were analyzed for antibody responses (J) and the number of iBALT structures in the lung sections (K). Data represent at least two independent experiments (* $p < 0.05$). See also Figure S4.

that DCs were required for the particulate-induced formation of iBALT.

Lymphotoxin (LT) and several homeostatic chemokines, including CXCL12, CXCL13, CCL19, and CCL21, are important in controlling the formation of iBALT (Foo and Phipps, 2010; GeurtsvanKessel et al., 2009; Moyron-Quiroz et al., 2004; Randall, 2010; Rangel-Moreno et al., 2011). We observed that LT- β , but not other chemokines, was mainly expressed by DCs (Figure S5D). We next examined the role of LT- β in the formation of iBALT induced by the i.t. instillation of alum and exposure to OVA. The administration of the LT- β receptor-Fc fusion construct had no effect on the antibody responses (Figure 5C). Furthermore, the iBALT structures were slightly reduced, although they were still observed in the LT- β receptor-Fc-treated mice (Figure 5C and Figure S5E).

These data indicate that although DCs were required, unique factors, other than LT- β , participated in the particulate-induced formation of iBALT.

Exposure of Airways to Particulates Induces Unique Immune Responses in the Lung

Next, we examined whether the IL-1 α -iBALT axis explains the unique features of the lung allergic responses to inhaled antigens. It has been reported that i.p. or footpad immunization with OVA plus alum induces OVA-specific IgE responses independently of MyD88 (Schnare et al., 2001). However, a recent report demonstrates that the inhalation of OVA plus alum induces OVA-specific IgE responses that is totally dependent on MyD88 (Matsushita and Yoshimoto, 2014). Consistent with previous reports, the OVA-specific antibody concentrations were similar in WT and *Myd88*^{-/-} mice immunized i.p. with OVA plus alum (Figure 6A). In sharp contrast, OVA-specific antibody responses were significantly reduced in *Myd88*^{-/-} mice exposed to OVA 7 days after the i.t. instillation of alum (Figure 6B). This response was not mediated by TLRs or IL-18 (Figures S6A–S6C). The antibody responses in the serum (systemic) and BALF (local) seemed to be differentially regulated. Mice were

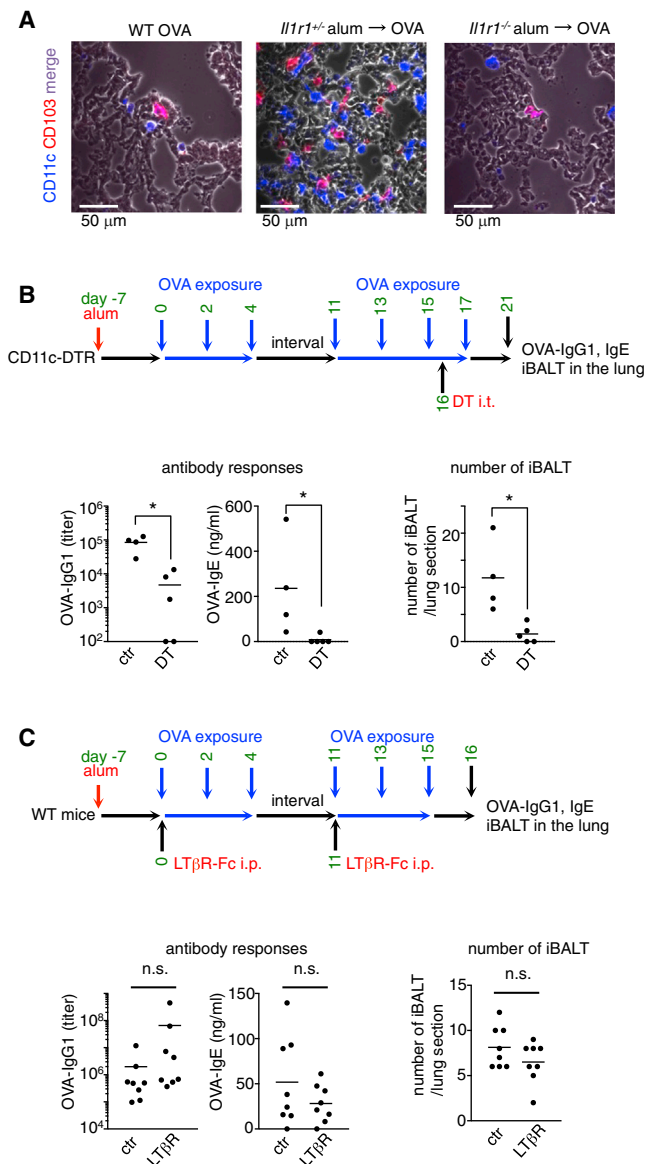


Figure 5. Particulate-Induced iBALT Formation Is Mediated by DC Recruitment

(A) Frozen sections of lungs isolated from WT and *Il1r1*^{-/-} mice ($n = 4$ mice, each group) exposed to OVA after i.t. alum instillation were stained for CD11c and CD103. Scale bars represent 50 μm, as indicated.
 (B) CD11c-DTR mice ($n = 4$ or 5 mice, each group) were administered i.t. alum, and were exposed to aerosolized OVA 7 days later. DT was administered by i.t. instillation 1 day before the last OVA exposure. Four days after the last OVA exposure, the sera and lungs were analyzed for antibody responses and the number of iBALT structures in the lung sections.
 (C) Alum was administered by i.t. instillation, and the mice were exposed to aerosolized OVA 7 days after its administration ($n = 8$ mice, each group). LTβR-Fc was administered by i.p. twice. Serum antibody responses and the numbers of iBALT structures in the lung sections were analyzed. Data represent at least two independent experiments (* $p < 0.05$). See also Figure S5.

immunized twice (days 0 and 7) with alum plus OVA by the i.p. or i.n. route, and then sensitized three times (days 14, 16, and 18) with OVA by the i.n. route. The serum (systemic) OVA-specific antibody responses were similar after i.p. and i.n. immunization

(Figure 6C). However, strong antibody responses in the BALF (local) were only observed in mice immunized by the i.n. route (Figure 6D). iBALTs especially affected the local immune responses (Figure 4G and Figure S4A). A histological analysis revealed that the lungs of mice immunized i.p. displayed very few, very small lymphoid clusters. In contrast, many large clusters were found in the lungs of mice immunized by the i.n. route (Figure 6E). We next examined the antigen-specific responses in regional lymph-node cells. Cells from the mediastinal lymph nodes secreted Th2 cytokines in response to OVA in mice that were immunized with i.p. alum plus OVA and then exposed to OVA (Figure 6F). However, the lymph-node cells of mice immunized with alum plus OVA by the i.t. route produced lower amounts of Th2 cytokines (Figure 6F). We observed no IFN-γ production in either case (data not shown). The infiltration of eosinophils and goblet cell hyperplasia might be moderate in mice directly sensitized to alum in the airway (Figure 3C) because smaller amounts of Th2 cytokines were produced by the lymph-node cells.

Recent studies have reported that IL-4 production by follicular helper T (T_{fh}) cells, but not conventional Th2 cells, is involved in the class-switch recombination to IgE (Harada et al., 2012; Vijayanand et al., 2012). Therefore, we examined the role of T_{fh} cells in particulate-induced lung inflammation, using *Cd4-cre Bcl6*^{flox/flox} mice as the T_{fh}-deficient mouse model (Onishi et al., 2015). The antibody responses were significantly reduced in the *Cd4-cre Bcl6*^{flox/flox} mice (Figure 6G). Moreover, the number of iBALT structures was also reduced by T_{fh}-deficiency (Figure 6H and Figure S6D). Thus, T_{fh} cells were required for both the IgE responses and iBALT formation.

Taken together, these results suggest that the direct exposure of the airway to particulates and antigens was more physiologically relevant than a model that was sensitized by i.p. and then exposed to antigen, and induced unique immune responses in the lung, which differed from those induced by i.p. sensitization.

DISCUSSION

Particulates, including particulate pollutants, probably increase the risk of allergic inflammation. Here, we have demonstrated that a DAMP, such as IL-1α, induced by particulates triggered lung immune responses through the generation of iBALT.

IL-1 is an important cytokine in murine allergic asthma models (Nakae et al., 2003; Schmitz et al., 2003). Here, we found that particulates such as alum and silica induce alveolar macrophage death and the release of IL-1α as a DAMP. We also observed IL-1α release in the lung after the specific depletion of phagocytes by the i.t. administration of clodronate liposomes. These observations suggest that macrophages were the major source of IL-1α in the lung after the administration of particulates through cell death. Indeed, *in vivo* analysis revealed that IL-1α tended to be released in the lung simultaneously with the release of host cell DNA, an indicator of cell death. Unlike IL-1β, pro-IL-1α is bioactive when released from dying cells, and IL-1α is an important factor in particulate-induced acute lung inflammation (Yazdi et al., 2010). A recent study also demonstrates that LPS-induced acute lung inflammation is mediated by the death of alveolar macrophages and the release of pro-IL-1α in the lung (Dagvadorj et al., 2015). Indeed, we observed that noninflammatory particulates (aluminum oxide and hydroxyapatite)

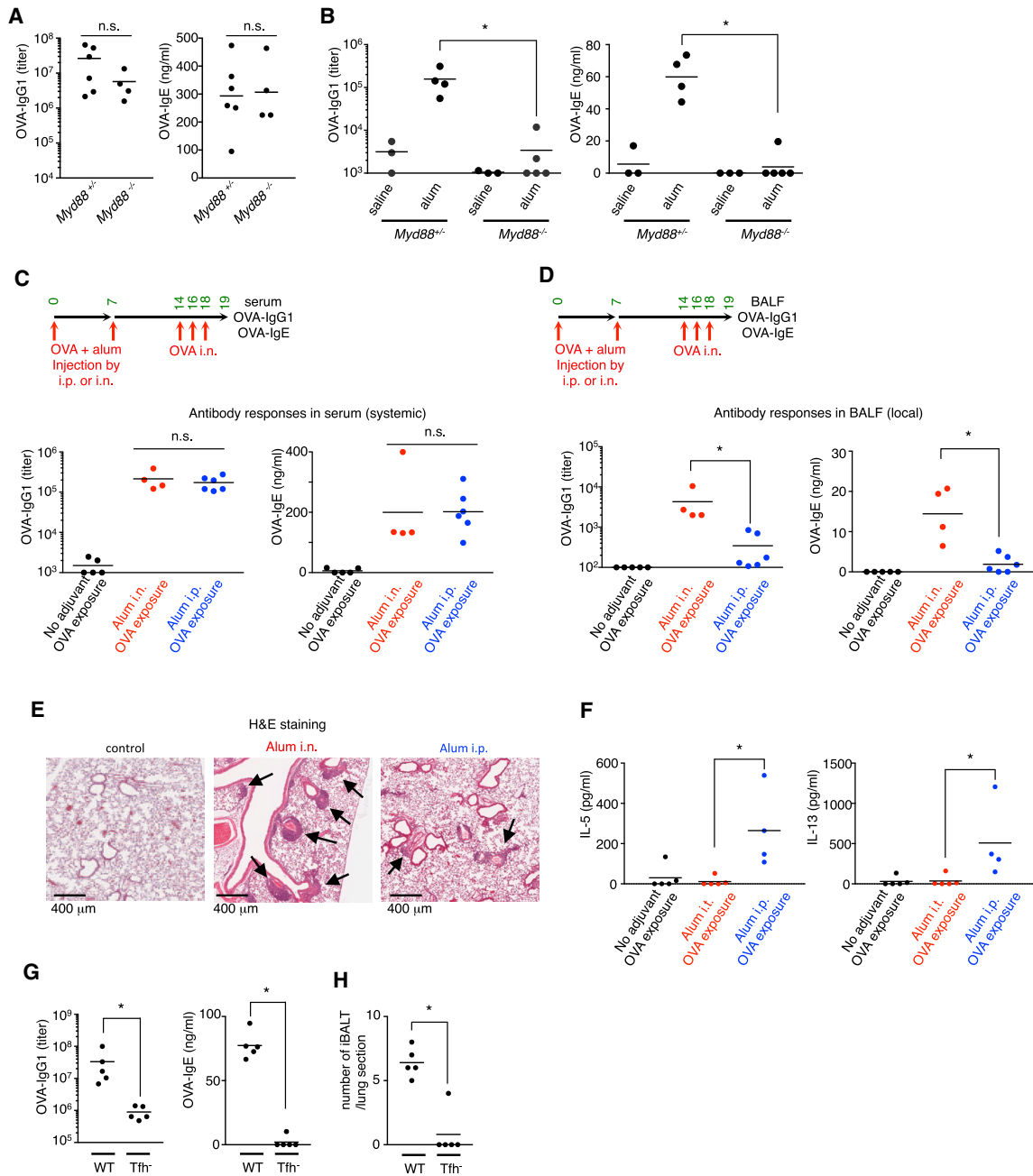


Figure 6. Direct Administration of Particulate Induces Unique Immune Responses in the Lung

(A) Alum+OVA was administered i.p. to *Myd88*^{-/-} mice, which were analyzed for serum OVA-specific IgE and IgG1 antibodies with ELISAs (n = 4–6 mice, each group).

(B) Saline or alum was administered to *Myd88*^{-/-} mice by i.t. instillation. Seven days after its administration, the mice were exposed to aerosolized OVA (n = 3–5 mice, each group), as in Figure 3A.

(C and D) Mice (n = 4–6 mice, each group) were immunized with alum+OVA twice (days 0 and 7) by the i.p. or i.n. route, and then sensitized three times with OVA by the i.n. route (days 14, 16, and 18). OVA-specific IgG1 and IgE levels in the serum (C) and BALF (D) were analyzed.

(E) Lungs were isolated from mice (n = 4) immunized with alum+OVA by the i.p. or i.n. route and then sensitized with OVA as in (C) and (D). The isolated lungs were analyzed with H&E staining. Arrows indicate lymphoid clusters.

(F) Mice (n = 4 or 5 mice, each group) were immunized with alum+OVA on day 0 by the i.p. or i.t. route, and then exposed to OVA for six times (days 7, 9, 11, 18, 20, and 22). Cells from mediastinal lymph nodes were cultured with OVA for 5 days. The culture supernatants were used for ELISA.

(G and H) *Cd4-cre Bcl6*^{flax/flax} mice Tfh⁻ were administered i.t. alum, and then exposed to aerosolized OVA as in Figure 3A (n = 5 mice, each group). The sera and lungs were analyzed for serum antibody responses (G) and the numbers of iBALT structures (H) in the lung sections. Data represent at least two independent experiments (*p < 0.05). See also Figure S6.

did not induce cell death and IgE responses. Thus, the cytotoxicity of particulates for alveolar macrophages and the consequently release of IL-1 α from these cells were considered to be critical in determining whether a particulate induces allergic inflammation. In addition, *in vivo* treatment with PVNO, which rescues the cell death induced by silica, reduced host DNA and IL-1 α levels after i.t. silica instillation. Therefore, controlling cell death or DAMP release could offer new options for the treatment of allergic inflammation. A recent study demonstrated that DCs and BM macrophages induce IL-1 α in response to particulates (Gross et al., 2012); however, they used LPS-primed cells. We observed that *Myd88*^{-/-} alveolar macrophages released IL-1 α in response to alum, indicating that LPS priming was not required for IL-1 α release from alveolar macrophages. Therefore, we suggest that IL-1 α release in response to particulates was a feature unique to alveolar macrophages and the specificity of tissue macrophages might reflect their tissue-specific immune responses. We are currently investigating which intracellular factors and events are associated with the release of IL-1 α and cell death by particulate-stimulated alveolar macrophages.

An important finding of this study is that the immune responses were activated even when the adjuvant (particulate) and antigen (OVA) were administered separately to the lungs. So far, adjuvants have been thought to enhance antigen-specific immune responses when administered concurrently with the antigens. However, antigen inhalation after the i.t. instillation of particulates enhanced the antigen-specific IgE responses. We have shown two possible mechanisms underlying the delayed and sustained adjuvant effects of particulates on the lung. The first is the continued release of IL-1 α . IL-1 α was released for at least 2 weeks after the i.t. instillation of alum, during alveolar macrophage cell death. Indeed, silica can be deposited in the lungs for 1 month *in vivo* (Yasuo Morimoto, personal communication). This release of IL-1 α was an extremely important event in the lung, bridging the innate and adaptive immune responses. The second important finding is that iBALT formation was a consequence of IL-1 α release. Therefore, antigen-specific IgE might be induced when the airway was exposed to antigens during the release of IL-1 α , with the subsequent generation of iBALT in the lung. The lung is not usually exposed to particulates and antigens concurrently, and we suggest that our separate exposure model is close to the natural course of allergen and particulate sensitization. Thus, once exposed to particulate pollution, the risk of allergen sensitization might persist for up to 2 weeks.

iBALTs are induced during chronic inflammation (Geurtsvan-Kessel et al., 2009; Halle et al., 2009; Moyron-Quiroz et al., 2004). In our model, iBALT formation was dependent on IL-1 signaling. IL-1 α played an important role in DC migration and chemokine expression. A recent report indicated that IL-17, produced by CD4⁺ T cells, is essential for the formation of iBALT (Rangel-Moreno et al., 2011), and IL-1 signaling is reported to induce IL-17-producing T cells (Chung et al., 2009; Sutton et al., 2009). The IL-1 α induced by particulates might be involved in the formation of iBALT through the induction of Th17, in addition to DC migration. Although we found that IL-1 α induced a strong antigen-specific IgE response, the detailed mechanism underlying the induction of IgE production is still unknown. A recent study reports that IL-1 directly stimulates CD4⁺ T cells to differentiate into Th2 cells, as well as Th17 cells (Ben-Sasson et al., 2009).

IL-1 α in the lungs might directly stimulate CD4⁺ T cells to induce IgE-producing B cells, as well as stimulating iBALT formation.

In this study, we found that the lymph-node responses in mice sensitized with i.t. treatments differed from those in i.p.-sensitized mice. The responses of lymph-node cells were weak in the mice sensitized by the i.t. administration of alum plus OVA. However, we found iBALT formation in the i.t.-sensitized mice and observed higher concentrations of antibody responses. Given that iBALT formation is reported to occur in lymph-node-deficient (lymphotoxin-deficient) mice (Moyron-Quiroz et al., 2004), these results suggest that sensitization directly via the airway mainly activated iBALTs, which functioned as lymph nodes. In addition, we observed the importance of Tfh for iBALT formation. It is demonstrated that IL-4 from Tfh cells is involved in class-switch recombination to IgE and IgG1, whereas conventional Th2 cells are associated with eosinophil activation (Harada et al., 2012). We found that the i.t. administration of particulates induced milder infiltration of eosinophils and milder goblet cell hyperplasia in the lung, corresponding to lower levels of Th2 cytokines released from the cells of the mediastinal lymph node. These findings suggest that direct sensitization of the airway preferentially activated Tfh cells rather than conventional Th2 cells. Indeed, the number of iBALT structures was significantly reduced in Tfh-deficient mice. The importance of Tfh cells in iBALT formation was reported by the transfer Tfh cells into T cell-deficient mice (Rangel-Moreno et al., 2011). Taken together, our findings suggest that Tfh cells were primed by DCs that have been induced to migrate into the lung by the release of IL-1 α and were then involved in iBALT formation and IgE production through Tfh-derived IL-4 production. Thus, both Tfh and CD11c⁺ cells were required for iBALT formation, and the Tfh-DC interaction might be important for the maintenance of the iBALT structure in the lung.

In conclusion, in investigating particulate-induced pulmonary inflammation, we found that lung-specific immune responses, including the release of IL-1 α and the formation of iBALT, were important in the induction of the local and systemic humoral responses (Figure S6E). To develop novel treatments for pulmonary inflammation, we must understand in detail the tissue-specific immune responses, the generation of iBALT, and the characteristics of the particulates themselves.

EXPERIMENTAL PROCEDURES

Animals

Female C57BL/6 mice were purchased from CLEA Japan Inc. (Osaka, Japan). Information about the gene-deficient and mutant mice is described in the [Supplemental Information](#). All the animal experiments were performed according to the Guidelines for the Care and Use of Animals approved by Osaka University and the National Institutes of Biomedical Innovation, Health and Nutrition.

Cell Preparation

Freshly isolated alveolar macrophages were prepared from BAL cells as adherent cells. Cultured alveolar macrophages were prepared from lung homogenate. Briefly, the lungs were isolated and diced with scissors, incubated with DNase I and Liberase at 37°C for 1 hr and homogenized by a gentle MACS™ Dissociator (Miltenyi Biotech, Gladbach, Germany). The cell suspensions were washed and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 5 μ g/ml insulin, but without antibiotics. After 7 days in culture, macrophages proliferated on the fibroblastic cell. Macrophages were easily detached with reciprocal

shaking (170 strokes/min) for 2 hr at 37°C. Floating cells were used for cultured alveolar macrophages. Cultured macrophages were > 95% F4/80⁺ as determined with flow cytometry. We described detailed method for preparation of cultured alveolar macrophages in the [Supplemental Information](#).

In Vivo Experiments

Particulates were administered by i.t. instillation. In brief, mice were anesthetized and hung on a board by their incisors. A 1 mL syringe fitted with a thin plastic needle was inserted into the trachea and 50 μ L of particulate solution was injected directly into the lungs.

We administered 50 μ L of particulates by i.t. instillation, according to the standard protocol used in this study. We used alum (100 μ g), silica (250 μ g), NiO (100 μ g), aluminum oxide (100 μ g), or hydroxyapatite (100 μ g), and saline was used as the negative control. Seven days after i.t. instillation, the mice were exposed to aerosolized OVA on days 0, 2, and 4 (three times every 2 days). After 7 days, the mice were exposed to OVA again three times every 2 days (on days 11, 13, and 15). To expose the mice to OVA, an aerosol of 1% OVA in H₂O was administered for 10 min. The aerosolized OVA was generated with a nebulizer (NE-C28, Omron Corp. Kyoto, Japan), and the mice were exposed in a chamber box (25 $\times$ 25 $\times$ 30 cm³). Twenty-four hours after the last exposure, their sera and BALF were collected. In some experiments, OVA exposure was started 3, 7, or 14 days after the i.t. instillation of alum. Experimental design is briefly shown in [Figure 3A](#).

All other in vivo experimental protocols are described in the [Supplemental Information](#).

Histological Analysis

To stain the lung tissues, we inflated the lungs with OCT Tissue-Tek (Sakura) diluted 1:4 in PBS, embedded in OCT and frozen in -80°C . Frozen sections (12 μ m) of the isolated lung specimens were prepared, fixed and permeabilized with the BD Cytfix/Cytoperm Plus Fixation/Permeabilization Kit (BD Biosciences), according to the manufacturer's instructions.

To detect iBALT, we stained lung specimens at 4°C with anti-B220, anti-CD21, anti-GL7, anti-CD138, and anti-CD3 antibodies (BioLegend or BD Biosciences), or OVA labeled with Alexa Fluor 555 (Molecular Probes, Thermo Fisher Scientific). We also measured the number of iBALT structures as the total number of B220⁺ and CD21⁺ clusters in five random area (1 mm²) in the lung.

Statistical Analysis

All the experiments were repeated at least twice and representative results are shown, except in [Figure 1H](#), which shows an experiment that was performed once, but 50 mice were used for the preparation of the lamina propria macrophages. The statistical analyses were performed with the Mann-Whitney U test in the GraphPad Prism software. Differences between groups were considered statistically significant at $p < 0.05$ and are indicated with an asterisk.

Other experimental procedures are described in the [Supplemental Information](#).

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.immuni.2016.11.010>.

AUTHOR CONTRIBUTIONS

E.K., K. Ozasa, B.T., K. Ohata, C.X.K., T. Kanuma, T. Kusakabe, S.K., N.T., S.U., and M. Hayashi performed experiments and data analysis. E.K., M. Horie, and Y.M. established in vivo model. S.K., K. Kabashima, S.F.Z., Y.I., W.I., T. Kurosaki, T.N., and J.K. provided mice and gave advice for in vivo experiment. E.K., T.A., K. Kobiyama, C.C., and K.J.I. established and helped experiment design. E.K. and K.J.I. performed and oversaw research and wrote the manuscript.

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Supplemental Information

Inhaled Fine Particles Induce Alveolar Macrophage

Death and Interleukin-1 α Release to Promote Inducible

Bronchus-Associated Lymphoid Tissue Formation

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Supplemental Figures

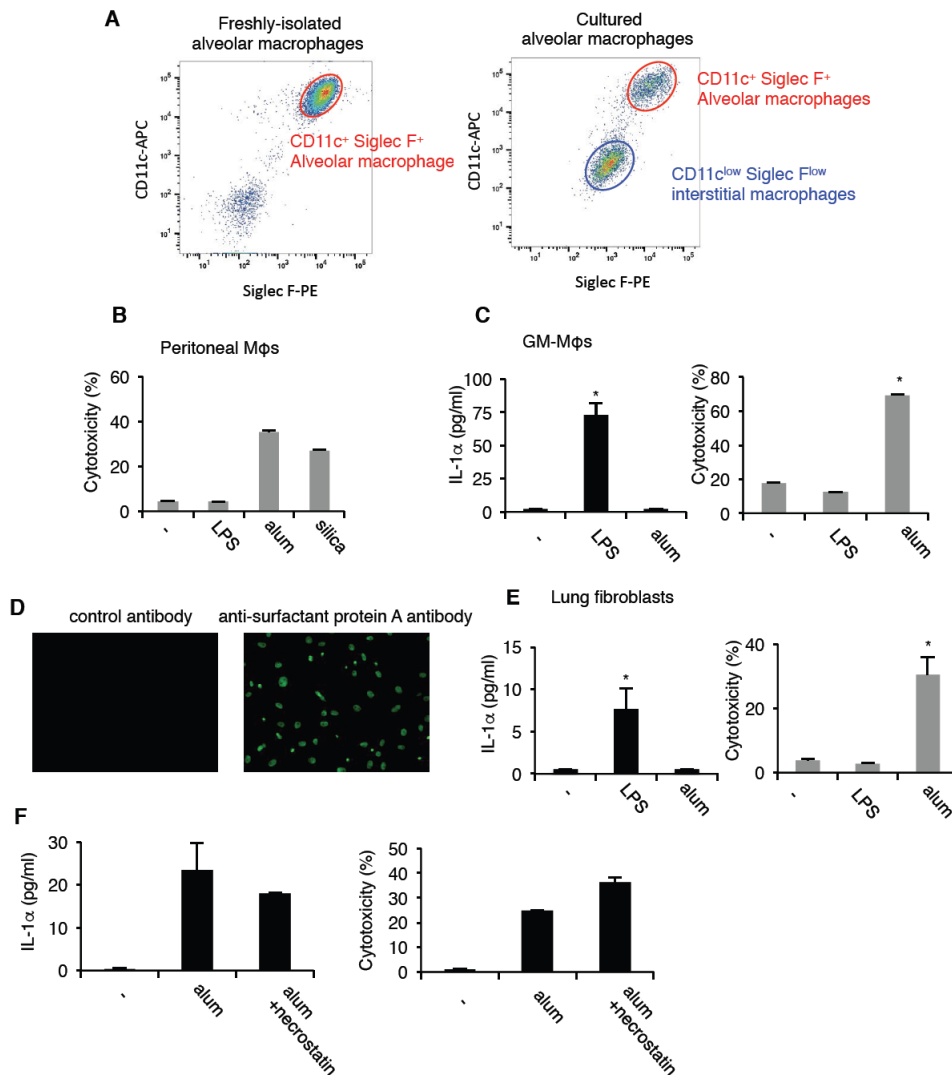


Figure S1 (related to Figure 1)

(A) Freshly isolated and cultured alveolar macrophages were obtained and stained with anti-CD11c and anti-Siglec-F antibodies. Cell-surface molecules were analyzed with flow cytometry (LSRII) (B and C) Thioglycolate-induced peritoneal macrophages and GM-CSF-derived macrophages were stimulated with LPS, alum or silica for 6 h. Supernatants were collected and analyzed for cytokines with ELISAs or for LDH release. (D) Lung fibroblasts were cultured on chamber slides (Mastunami, Osaka, Japan), fixed and permeabilized with the BD Cytotfix/Cytoperm™ Plus Fixation/Permeabilization Kit. Cells were stained overnight with anti-surfactant protein-A antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The cells were then stained with Alexa-Fluor-488-conjugated anti-rabbit IgG antibody (Cell Signaling Technology). (E) Lung fibroblasts were stimulated with LPS or alum for 6 h. Supernatants were collected and analyzed for cytokines with ELISAs or for LDH release. (F) Cultured alveolar macrophages were stimulated with alum in the presence or absence of necrostatin-1 (100 μM) for 6 hours. The culture supernatants were tested for IL-1α ELISA or for LDH release. DATA are shown as mean ± SD and represent at least two independent experiments (*p<0.05).

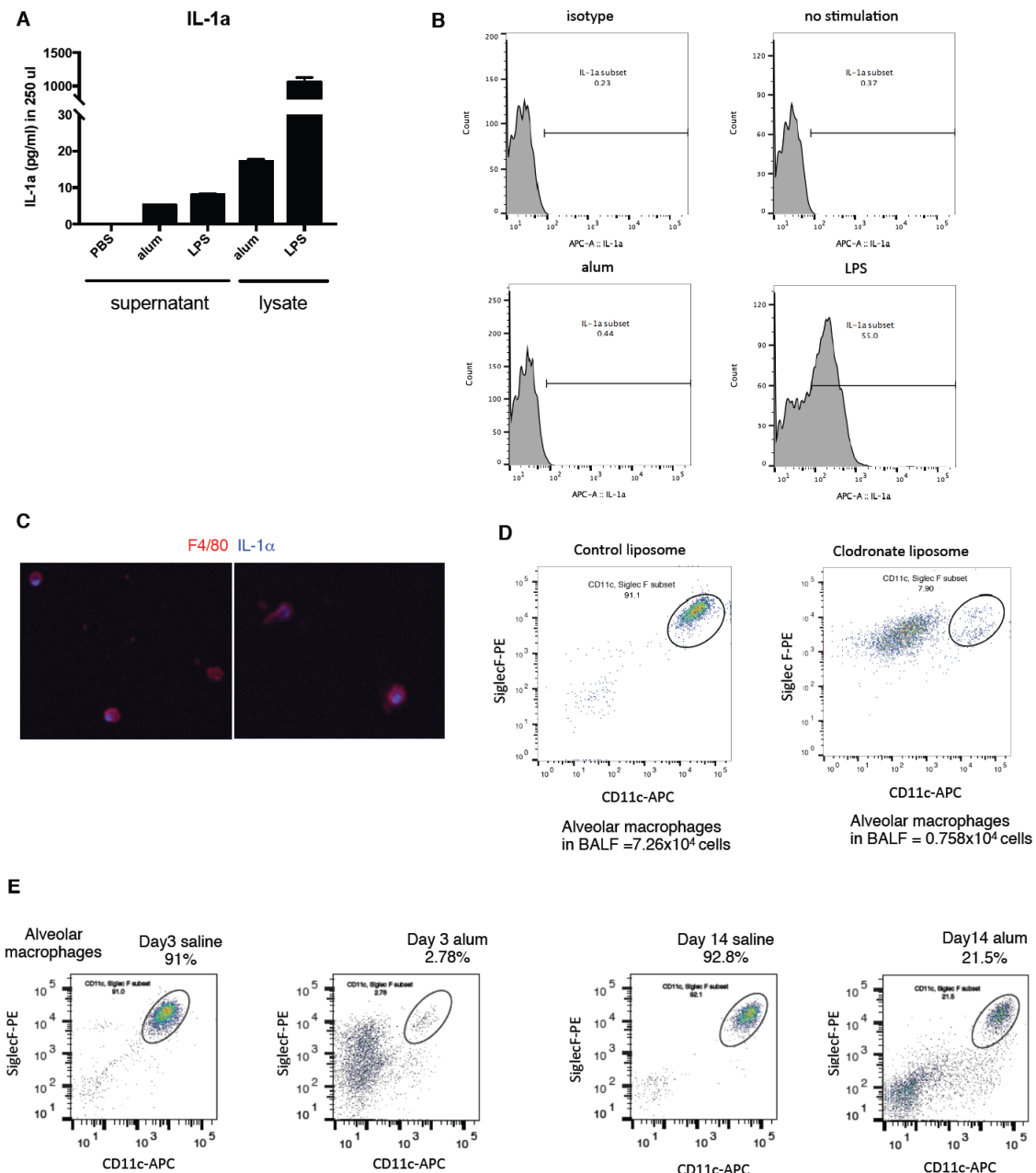


Figure S2 (related to Figure 2)

Analysis of intracellular IL-1 α in alveolar macrophages. (A) Cultured alveolar macrophages were stimulated with LPS or alum for 6 h. Supernatants were collected and analyzed for IL-1 α with an ELISA. Cell lysates were prepared with three freeze-thaw cycles and used for ELISAs. (B) Cultured alveolar macrophages were stimulated with or without alum or LPS for 6 h without brefeldin A. The cells were fixed and permeabilized with the BD Cytotfix/Cytoperm™ Plus Fixation/Permeabilization Kit, stained with anti-F4/80 and anti-IL-1 α , and analyzed with flow cytometry. (C) Alum was administered by i.t. instillation. Three days later, BALF was harvested and BAL cells were cultured on chamber slides as in Figure S1D. Adherent cells were fixed, permeabilized and stained overnight with anti-IL-1 α and anti-F4/80 antibodies. Images were analyzed with confocal microscopy. (D) Clodronate liposomes (50 μ l) or control liposomes were administered to mice by i.t. instillation. BAL cells were isolated 3 days after i.t. instillation, and the alveolar macrophages (CD11c^{high} and siglec F^{high} cells) were analyzed with flow cytometry. (E) At 3 or 14 days after i.t. alum or saline administration, the BALF was harvested and alveolar macrophages (CD11c^{high} and siglec F^{high} cells) were analyzed. DATA represent at least two independent experiments.

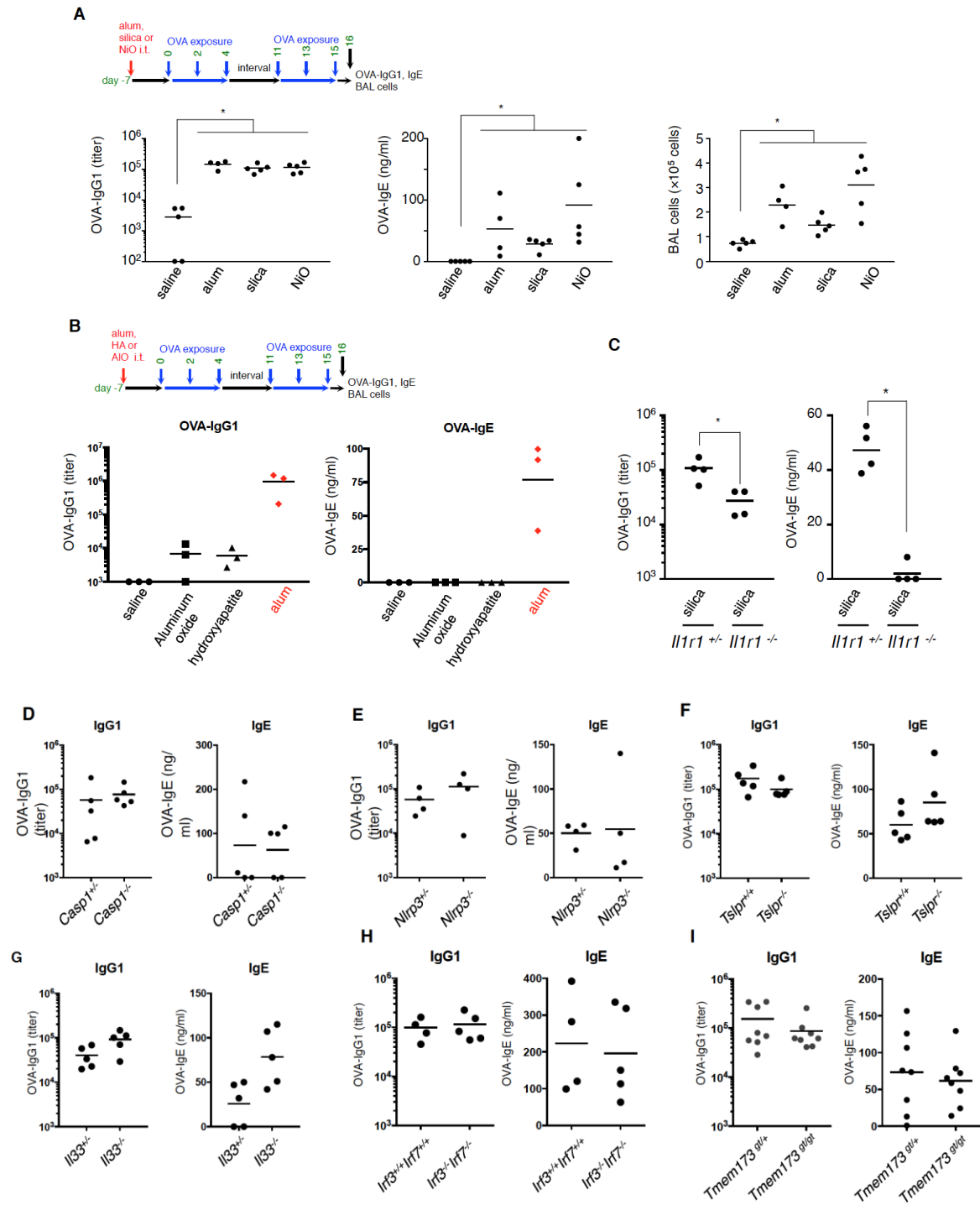


Figure S3 (related to Figure 3)

(A) Saline, alum (100 μ g), silica (250 μ g), or NiO (100 μ g) was administered to mice by i.t. instillation (n=5 mice, each group). Seven days later, the mice were exposed to aerosolized OVA, as in figure 3A. The sera were used for OVA-specific IgG1 and IgE ELISAs. (B) Saline, aluminum oxide (100 μ g) or hydroxyapatite (100 μ g) was administered to the mice by i.t. instillation (n=3 mice, each group), and the mice were then exposed to aerosolized OVA, as in figure 3A. (C) Silica was administered to *Il1r1*^{-/-} mice by i.t. instillation (n=4 mice, each group). Seven days later, the mice were exposed to aerosolized OVA, as in figure 3A. Alum was administered to *Casp1*^{-/-} (D), *Nlrp3*^{-/-} (E), *Tslpr*^{-/-} (F), *Il33*^{-/-} (G), *Irf3*^{-/-} *Irf7*^{-/-} (H) and STING mutant (*Tmem173*^{gt/gt}) mice (I) by i.t. instillation (n=4-8 mice, each group). Seven days after administration, the mice were exposed to aerosolized OVA, as in figure 3A. DATA represent at least two independent experiments (*p<0.05).

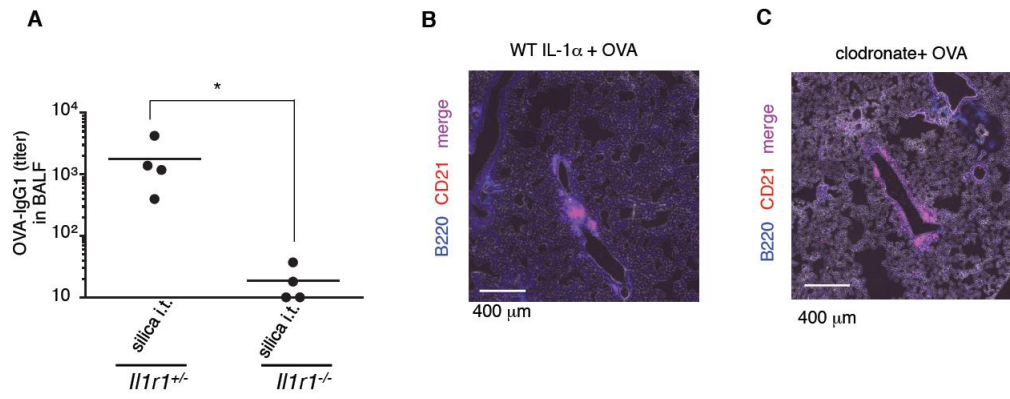


Figure S4 (related to Figure 4)

(A) Silica was administered to mice by i.t. instillation (n=4 mice, each group). Seven days after its administration, the mice were exposed to aerosolized OVA as in Figure 3A. Twenty-four hours after the last exposure, BALF was harvested and analyzed for OVA-specific IgG1 levels. (B) OVA or IL-1 α +OVA was administered to mice by i.t. instillation and the mice were exposed to aerosolized OVA as in Figure 4H and I. At 24 h after the last exposure, the lung sections were analyzed for iBALT formation with confocal microscopy. (C) Clodronate liposomes were administered to mice by i.t. instillation. Three days after their administration, the mice were exposed to aerosolized OVA as in Figure 4J and K. Lung sections were analyzed for iBALT formation. Bars, 400 μ m. The data represent at least two independent experiments (*p<0.05).

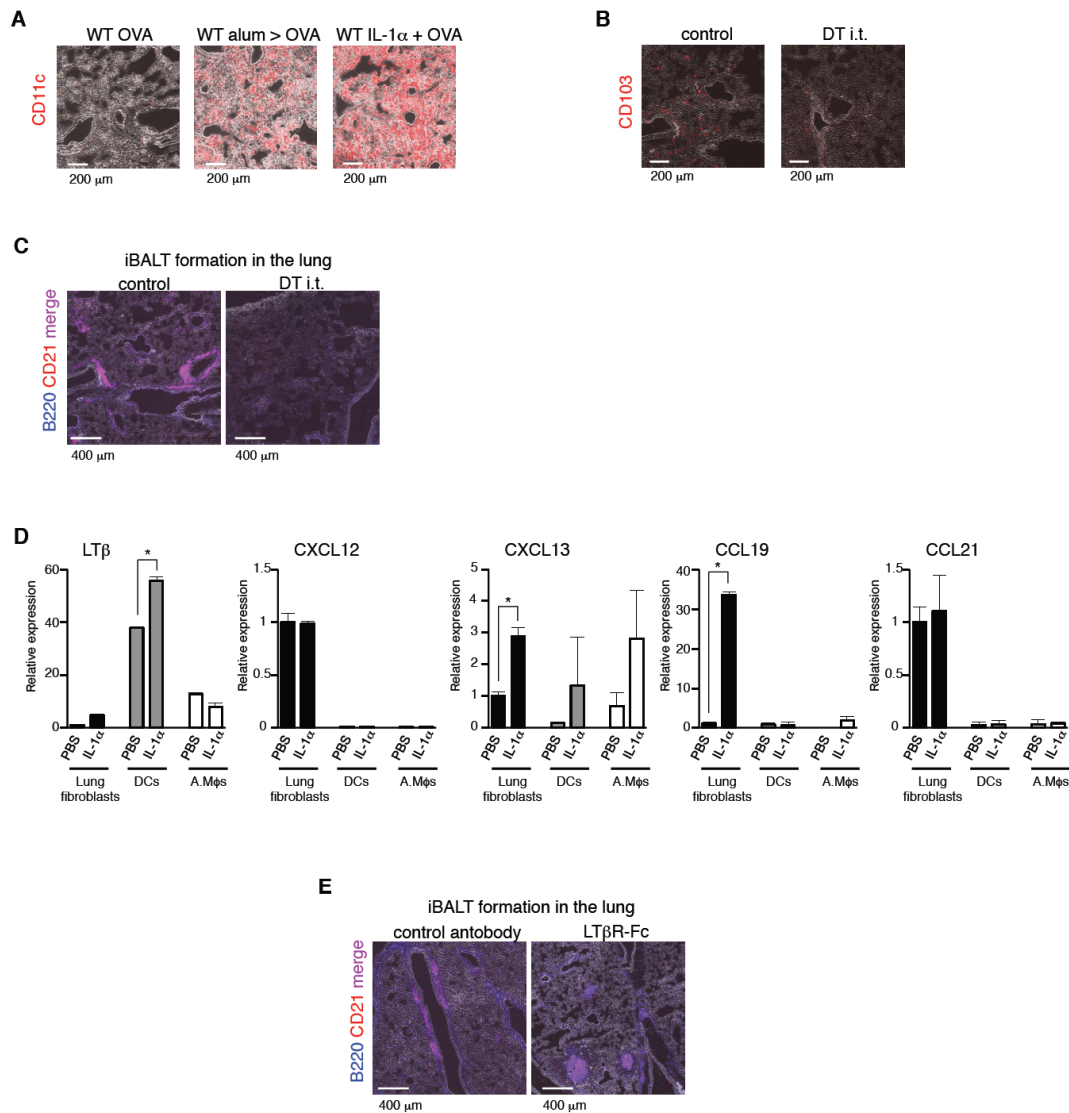


Figure S5 (related to Figure 5)

(A) Alum or OVA+IL-1 α was administered by i.t. instillation. At 3, 7 or 14 days after its administration, the mice were exposed to aerosolized OVA. Frozen sections of isolated lung specimens were stained for CD11c, and analyzed with confocal microscopy. Bars, 200 μ m. (B and C) CD11c-DTR mice were administered i.t. alum, and were exposed to aerosolized OVA 7 days later. DT was administered by i.t. instillation 1 day before the last OVA exposure as in figure 5B. Four days after the last OVA exposure, frozen lung sections were prepared, and stained with anti-CD103 antibody to detect lung DCs (B) or with anti-B220 and anti-CD21 antibodies to detect iBALT formation (C). Bars, 200 μ m in A and B, 400 μ m in C. (D) Lung fibroblasts, DCs, and alveolar macrophages (A.M ϕ s) were stimulated with IL-1 α for 6 h in vitro, and the relative expression of LT β and homeostatic chemokines were analyzed with quantitative RT-PCR. (E) Alum was administered by the i.t. instillation, and the mice were exposed to aerosolized OVA 7 days after its administration. LT β R-Fc was administered twice by the i.p. route as in figure 5C. Frozen lung sections were prepared, and stained with anti-B220 and anti-CD21 antibodies to detect iBALT formation. Bars, 400 μ m. DATA are shown as mean $\pm$ SD and represent at least two independent experiments (*p<0.05).

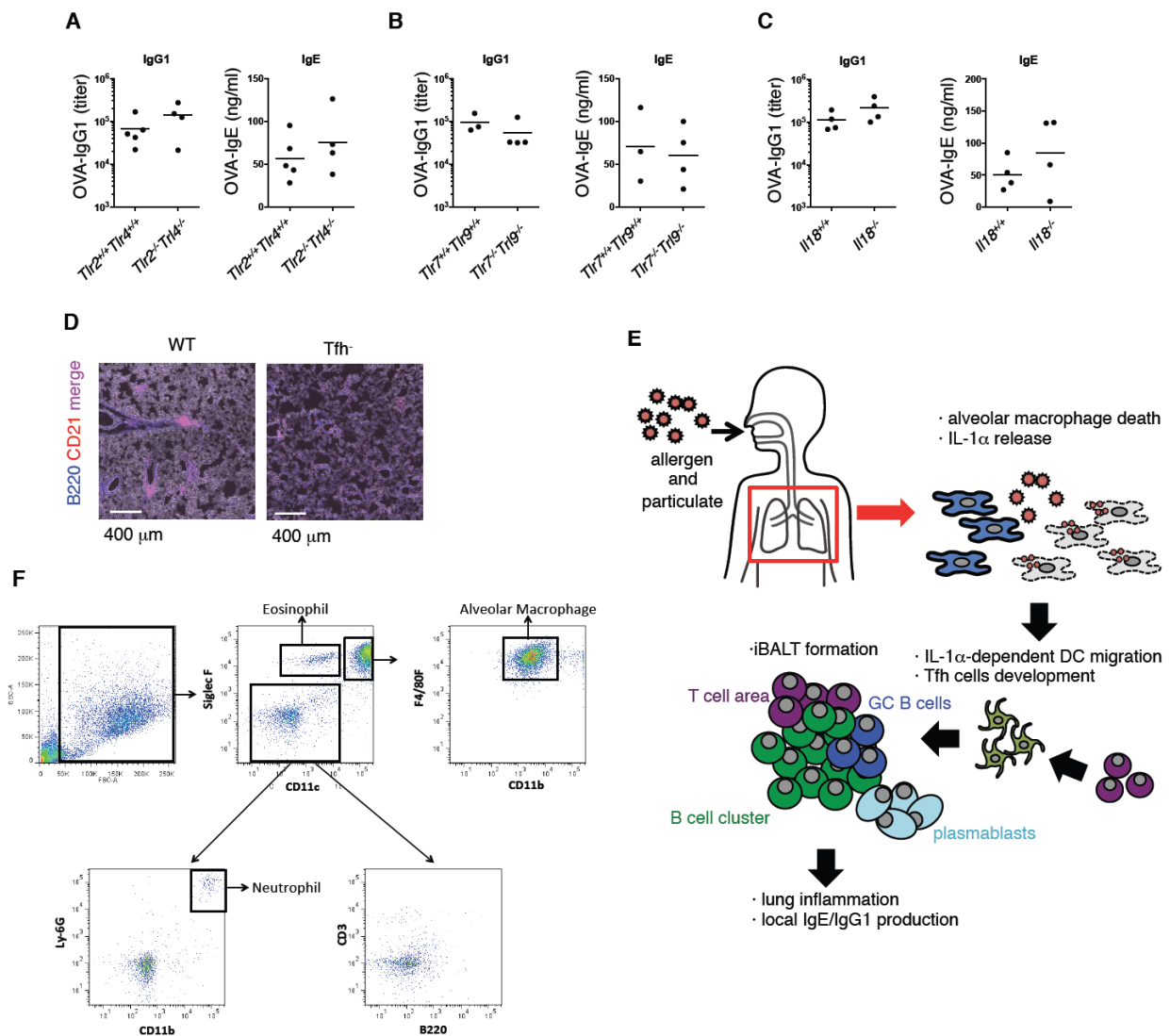


Figure S6 (relate to Figure 6, Discussion and Methods)

Alum was administered to *Tlr2*^{-/-}*Tlr4*^{-/-} (A), *Tlr7*^{-/-}*Tlr9*^{-/-} (B) and *Il18*^{-/-} mice (C) by i.t. instillation (n=3-4 mice, each group). Seven days after its administration, the mice were exposed to aerosolized OVA, as in Figure 3A. Twenty-four hours after the last exposure, the sera were collected and analyzed with OVA-specific IgG1 and IgE ELISAs. (D) *Cd4-cre Bcl6*^{fllox/fllox} mice (*Tfh*⁻) were administered i.t. alum, and were then exposed to aerosolized OVA as in Figure 6G and H. At 24 h after the last exposure, lung sections were analyzed for iBALT formation in lung sections with confocal microscopy. The data represent at least two independent experiments.

(E) The model of particulate-induced lung inflammation. Inhaled fine particulates engulfed by alveolar macrophages induce macrophage death. IL-1α is released as a DAMP and contributes to DC recruitment into the lungs. Activated DCs triggers iBALT formation through the development of Tfh cells. Mature iBALT functions as a tertiary lymphoid organ, which contains a germinal center and areas of T-cell, generates short-lived plasmablasts, and is involved in local and systemic IgE responses. (F) Flow-cytometric gating strategy for the analysis of BAL cells.

Supplemental Experimental Procedures

Animals

Il18^{-/-} mice were kindly donated by Dr. Nakanishi (Hyogo College of Medicine, Nishinomiya, Hyogo). *Il33*^{-/-} mice were obtained as described previously (Haenuki et al., 2012; Yasuda et al., 2012). *Tslpr*^{-/-} mice were kindly donated by Dr. Ziegler (Benaroya Research Institute, Seattle, USA) (Zhou et al., 2005). *Myd88*^{-/-}, *Tlr2*^{-/-}*Tlr4*^{-/-} and *Tlr7*^{-/-}*Tlr9*^{-/-} mice were obtained from Oriental BioService Inc (Kyoto, Japan). *Il1r1*^{-/-} mice were obtained as described previously (Hayashi et al., 2016). STING mutant mice (*Tmem173*^{gt/gt}) were obtained from Jackson Laboratories (Bar Harbor, ME, USA). *Asc*^{-/-}, *Nlrp3*^{-/-} and *Casp1*^{-/-} mice were obtained as described previously (Mariathasan et al., 2004; Mariathasan et al., 2006). *Irf3*^{-/-}*Irf7*^{-/-} mice were derived from *Irf3*^{-/-} (Tang et al., 2013) and *Irf7*^{-/-} mice provided by the RIKEN BRC (Ibaraki, Japan) through the National Bio-Resource Project of the Japanese Ministry of Education, Culture, Sports, Science and Technology (Honda et al., 2005). *Il1a*^{-/-} mice were obtained from the Center for Animal Disease Models, Tokyo University of Science (Chiba, Japan) (Horai et al., 1998). *Cd4-cre Bcl6*^{fllox/fllox} mice were kindly donated by Dr. Kurosaki (Osaka University, Osaka, Japan) (Ise et al., 2014; Onishi et al., 2015). All but the CD11c-DTR mice were bred in a C57BL/6 background. The CD11c-DTR mice (BALB/c background) kindly donated by Dr. Kunisawa (National Institutes of Biomedical Innovation, Health and Nutrition, Osaka) (Obata et al., 2013). To prepare the CD11c-DTR chimeric mice, BM cells were prepared from the femurs and tibias of the CD11c-DTR transgenic mice (12 weeks, old male) and used as the donor cells. Donor cells (1×10^7 cells) were intravenously injected into BALB/c mouse (6 weeks old, male) 6 h after their irradiation with 7 Gy X-irradiation with an MBR-1520R-3 device (Hitachi). The BM chimeric mice were used for experiments at least 8 weeks after transplantation. All the animal experiments were performed according to the Guidelines for the Care and Use of Animals approved by Osaka University and the National Institutes of Biomedical

Reagents

Alhydrogel (alum) was purchased from Invivogen (San Diego, CA, USA). Silica crystals (Min-u-sil 5 silica) were purchased from U.S. Silica (Berkeley Springs, WV, USA). NiO was purchased from Vacuum Metallurgical Co., Ltd (Chiba, Japan). Al₂O₃ (particle size: 40-50 nm) and TiO₂ were purchased from Wako Pure Chemical Industries (Osaka, Japan). Hydroxyapatite (particle size: 59 nm) was purchased from SofSera (Tokyo, Japan). OVA was purchased from Kanto Chemical Co. Inc. (Osaka, Japan). All particulates and OVA used for the in vivo experiments were prepared in endotoxin-free solution (< 1EU/ml [10 mg]). Endotoxin levels were determined with Toxicolor® (Seikagaku Corp., Tokyo, Japan). Poly-2-vinylpyridine N-oxide (PVNO) was purchased from Polysciences (Warrington, PA, USA). House-dust-mite allergen (HDM) was purchased from Cosmo Bio (Tokyo, Japan). GSK'872 was purchased from BioVision (Milpitas, CA, USA). Clodronate and empty liposomes (Clophosome-A anionic) were purchased from FormuMax Scientific (Palo Alto, CA, USA). Soluble LTβR-Fc for blocking LTβR signaling was kindly donated by Dr. Kunisawa (National Institutes of Biomedical Innovation, Health and Nutrition, Osaka).

Cell preparation

The preparation of peritoneal macrophages and GM-CSF-derived BM macrophages has been described previously (Kuroda et al., 2011). Cultured alveolar macrophages were prepared with the method used to prepare Kupffer cells, described previously (Kitani et al., 2010). The lungs were isolated and suspended in 5 ml of RPMI1640 containing 200 µg/ml DNase I and 1.5 U/ml Liberase DL Research Grade (Roche Diagnostics GmbH, Indianapolis, IN, USA). The lungs were diced with scissors, incubated at 37°C for 1 h and homogenized by a gentle MACS™ Dissociator (program

B.01) (Miltenyi Biotech, Gladbach, Germany). The cell suspensions were washed twice with PBS and passed through 30 μm preseparation filters (Miltenyi Biotech). The cells, which contain both alveolar and interstitial macrophages, were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 5 $\mu\text{g/ml}$ insulin, but without antibiotics, and seeded into 175 cm^2 culture flasks (Corning, New York, NY, USA). The medium was replaced after 24 h, and then every 2 days. Fibroblastic cells grew and after 7 days in culture, macrophages (both alveolar and interstitial macrophages) proliferated on the cell sheet. Macrophages were easily detached with reciprocal shaking (170 strokes/min) for 2 h at 37 $^{\circ}\text{C}$. Floating cells were washed with PBS and suspended in RPMI 1640 supplemented with 10% FCS and antibiotics. Cultured macrophages were > 95% F4/80⁺ as determined with flow cytometry. A comparison of surface-marker expression on freshly isolated alveolar macrophages and cultured alveolar macrophages is described in Figure S1A. We prepared a single cell suspension of the lungs from five mice and the cells were divided into two 175 cm^2 culture flasks. We obtained more than 1×10^7 macrophages from two flasks. The adherent fibroblastic cells were positive for surfactant protein A, determined with immunohistochemistry, and were used as lung fibroblasts (Figure S1D). In some experiments, obtained F4/80⁺ cells were further separated into CD11c⁺ siglec F⁺ and CD11c^{low} siglec F^{low} cells with a BD InfluxTM cell sorter (BD Biosciences, Franklin Lake, NJ, USA), and used for in vitro culture.

The isolation of lamina propria macrophages has been previously described (Uematsu et al., 2008). Briefly, segments of the small intestine, from which Peyer's patches and epithelial cells were removed, were digested with collagenase D and DNase I. Single-cell suspensions were prepared with density gradient centrifugation, and stained with anti-CD11b (M170), anti-CD11c (HL3), anti-CD103 (M290) and anti-CX3CR1 antibodies. Small-intestinal lamina propria macrophages were sorted as CD11c^{int}, CD11b^{int}, CX3CR1⁺, CD103⁻ cells. This experiment was

performed once, but 50 mice were used to prepare the lamina propria macrophages.

In vitro culture

Freshly isolated or cultured alveolar macrophages were cultured at $1 \times 10^6/\text{ml}$ in 48- or 96-well plates (BD Biosciences). The cells were stimulated with 100 $\mu\text{g}/\text{ml}$ particulates (silica, alum, aluminum oxide or hydroxyapatite) for 6~8 h. LPS (1 $\mu\text{g}/\text{ml}$) stimulation was used as the positive control. In some experiments, an inhibitor (5 μM GSK'872 or 20 $\mu\text{g}/\text{ml}$ PVNO) was added to alveolar macrophages together with silica or alum. After stimulation, the cell-free supernatants were collected and used for ELISAs or a cytotoxicity assay. In some experiments, cell lysates were prepared with three freeze-thaw cycles and used for the IL-1 α ELISA (BioLegend, San Diego, CA, USA).

In vivo experiments

In the experiment shown in Figure 3D, HDM was used as the antigen instead of OVA. The mice were administered i.t. alum, and 7 days later, they were sensitized with HDM (10 μg) by the i.n. route on days 0, 2, 9, and 11. On day 12, the sera and BALF (bronchoalveolar lavage fluid) were collected.

Experiment shown in Figure 2C (PVNO treatment): We administered 50 μl of silica or silica mixed with PVNO (100 μg) to mice by i.t. instillation. Three days after i.t. instillation, the BALF was harvested and analyzed for the number of BAL cells and released DNA and IL-1 α in the BALF.

Experiment shown in Figures 2D-G: 50 μl of particulates, clodronate liposomes or empty liposomes was administered by i.t. instillation. BALF was collected 1, 3, 7 or 14 days after i.t. instillation of the particulate or liposomes. BALF was tested to measure DNA or cytokines. BAL

cells were counted and stained with anti-CD11c and anti-Siglec F antibodies. The number of alveolar macrophages was examined with flow cytometry. For the histological analysis in Figure 2H, I and J, lungs were isolated 14 days after i.t. instillation of alum, and used for H&E or iBALT staining.

Experiment shown in Figure 3B: Alum (1 mg in 200 μ l) was injected i.p. on day -7. OVA (10 μ g in 200 μ l) was injected i.p. on days 0 and 7 (alum $\rightarrow$ OVA $\rightarrow$ OVA). Injection of OVA alone (OVA $\rightarrow$ OVA) or alum mixed with OVA (alum+OVA $\rightarrow$ OVA) were used as the controls. On day 17, sera were collected and analyzed for OVA-specific antibodies with ELISA.

Experiment shown in Figure 4H and I: 50 μ l of saline, OVA (10 μ g) or OVA (10 μ g)+IL-1 α (10 ng) was administered by i.t. instillation on days 0. Mice were then exposed to OVA for six times (day 7, 9, 11, 18, 20, and 22). Sera were collected 24 h after the last exposure and tested for serum antibody responses. Lungs were isolated and used for iBALT staining (B220 and CD21).

Experiment shown in Figure 4J and K: 50 μ l of empty liposomes or clodronate liposomes were administered by i.t. instillation. Three days after i.t. instillation, the mice were exposed to aerosolized OVA on days 0, 2, and 4. After 7 days, the mice were exposed to OVA again three times every 2 days (on days 11, 13, and 15). Sera were collected 24 h after the last exposure and tested for serum antibody responses. Lungs were isolated and used for iBALT staining (B220 and CD21).

Experiment shown in Figure 5B: Chimeric CD11c-DTR mice were administered i.t. alum and exposed to aerosolized OVA, as in Figure 3A. On day 16, 50 ng of DT was administered by i.t. instillation, and the mice were then exposure to OVA once again (on day 17). Sera were collected 24 h after the last exposure and tested for serum antibody responses. Lungs were isolated and used for iBALT staining (B220 and CD21). A brief experimental design is shown in Figure 5B.

Experiment shown in Figure 5C: Mice were administered i.t. alum, and were exposed to

aerosol OVA as in Figure 3A. On day 0 and 11, 100µg of LTβR-Fc was administered by i.p. Sera were collected 24 h after the last exposure and tested for serum antibody responses. Lungs were isolated and used for iBALT staining (B220 and CD21). A brief experimental design is shown in Figure 5C.

To test the different routes of immunization (Figure 6C and D), we administered OVA (10 µg) adsorbed onto alum (100 µg) by intraperitoneal (i.p.) injection (200 µl). For intranasal (i.n.) injection, 30 µl of saline, OVA (50 µg) adsorbed onto alum (100 µg) was administered on days 0 and 7. The mice were sensitized with OVA (50 µg) by the i.n. route on days 14, 16, and 18. On day 19, sera and BALF were collected and analyzed for OVA-specific antibodies with an ELISA. A brief experimental design is shown in Figure 6C and D.

Experiment shown in Figure 6F, Mice were immunized with OVA (10 µg) adsorbed onto alum (100 µg) on day 0 by the i.p. (200 µl) or i.t. (50 µl) route. The mice were then exposed to OVA for six times (days 7, 9, 11, 18, 20, and 22). The mediastinal lymph nodes were isolated 24 h after the last exposure. The cells were cultured at 1×10^5 cells/well (200 µl) in 96-well plates (BD Biosciences). They were stimulated with or without OVA (100 µg/ml) for 5 days. The cell-free supernatants were collected and analyzed for cytokines with ELISAs.

Collection and analysis of BALF

BALF was collected by washing the lungs. Briefly, a plastic cannula (Terumo, Tokyo, Japan) was inserted into the incised trachea to flush the lungs twice with 550 µl of phosphate-buffered saline (PBS). The total volume recovered was about 1 ml. The BALF obtained was centrifuged at 300×g for 10 min to separate the cells and fluids. For the analysis of inflammatory cells in BALF in Figure 3C, the cells were stained with anti-CD11c, anti-CD11b, anti-Siglec F, anti-F4/80, anti-Ly6G, anti-CD3 and anti-B220 antibodies, and then analyzed by flow cytometry (LSRII; BD Biosciences).

The gating strategy is described in Figure S6F. All labeled antibodies were purchased from BD Biosciences or BioLegend.

Double-stranded DNA in BALF was measured with the Quant-iT™ Picogreen® ds DNA Assay Kit (Invitrogen, San Diego, CA, USA).

Histological analysis

To stain alveolar macrophages, the tissue samples were stained overnight at 4 °C with anti-F4/80 and anti-IL-1 α antibodies (BioLegend and Miltenyi Biotec). Images were analyzed with a FluoView (FV10i) confocal microscope (Olympus, Tokyo, Japan). To stain lung DCs, we used anti-CD11c and anti-CD103 antibodies to distinguish them from CD11c⁺ alveolar macrophages.

In some experiment, lungs were inflated with and fixed in 4% paraformaldehyde. To prepare paraffin sections, the tissues were gradually dehydrated and embedded in paraffin. The sections (5 μ m) were prepared and stained with H&E or PAS using the Periodic Acid-Schiff Kit (Sigma Aldrich, St. Louis, MO, USA). Representative images were acquired with a ScanScope® AT slide scanner (Leica, Germany).

ELISAs and cytotoxicity assay

Cytokines were measured using cytokine ELISA kits (R&D Systems, Minneapolis, MN, USA or BioLegend) according to the manufacturer's instructions. Serum OVA-IgE levels were determined with a DS Mouse IgE ELISA (OVA) kit (DS Pharma Biochemical, Osaka, Japan). For the OVA-specific IgG1 analysis, serial dilutions of sera were prepared in 96-well plates coated with 10 μ g/ml OVA or HDM. Horseradish-peroxidase-conjugated goat anti-mouse IgG1 antibody (Bethyl

Laboratories, Montgomery, TX, USA) was used as the secondary antibody. The reciprocal value of the serum dilution with an optical density at 450 nm (OD₄₅₀) of 0.5 was defined as the titer of the antigen-specific serum IgG1 or IgG2c. Cytotoxicity was measured by quantifying the release of lactate dehydrogenase (LDH) using the Cytotox 96® Non-Radioactive Cytotoxicity Assay from Promega (Madison, WI, USA).

Quantitative-PCR (qPCR) analysis

CD11c⁺-sorted GM-CSF-derived BM cells were used as DCs. Lung fibroblasts, DCs, and alveolar macrophages were stimulated with IL-1 α for 6 h. Total RNA was isolated with the SV Total RNA Isolation System (Promega), according to the manufacturer's instructions. RNA was reverse transcribed with ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan), according to the manufacturer's instructions. For qPCR, cDNA fragments were amplified with THUNDERBIRD Probe qPCR Mix (Toyobo), according to the manufacturer's instructions. The fluorescent TaqMan probe was detected with a StepOnePlus™ Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). The expression of *Cxcl12*, *Cxcl13*, *Ccl19*, *Ccl21b*, and *LTb* mRNAs was normalized to that of 18S rRNA. The experiments were repeated twice, with similar results.

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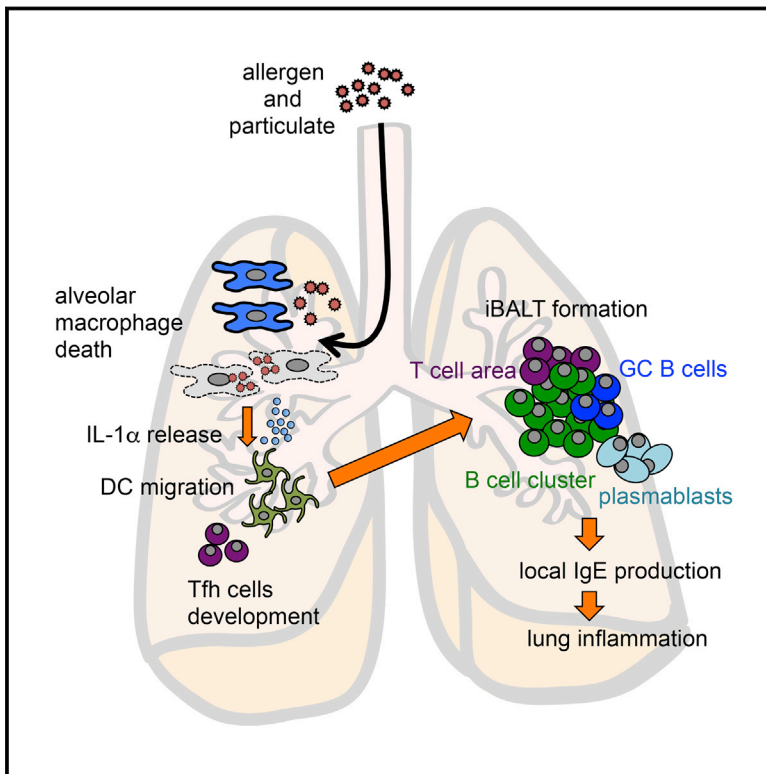
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Immunity

Inhaled Fine Particles Induce Alveolar Macrophage Death and Interleukin-1 α Release to Promote Inducible Bronchus-Associated Lymphoid Tissue Formation

Graphical Abstract



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In Brief

Inhaled fine particulates are known to induce allergic responses, but the exact cell types and immunological factors that initiate the lung-specific immune responses remain unclear. Kuroda et al. (2016) show that particulates trigger tertiary lymphoid organ formation in the lung as a consequence of alveolar macrophage death and IL-1 α release.

Highlights

- Particulates act as a long-lasting adjuvant to subsequent antigen inhalation
- Particulates induce alveolar macrophage death and IL-1 α release in the lung
- IL-1 α release in the lung is involved in iBALT formation
- Tfh cells induced by direct airway sensitization are required for iBALT formation



Inhaled Fine Particles Induce Alveolar Macrophage Death and Interleukin-1 α Release to Promote Inducible Bronchus-Associated Lymphoid Tissue Formation

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SUMMARY

Particulate pollution is thought to function as an adjuvant that can induce allergic responses. However, the exact cell types and immunological factors that initiate the lung-specific immune responses are unclear. We found that upon intratracheal instillation, particulates such as aluminum salts and silica killed alveolar macrophages (AMs), which then released interleukin-1 α (IL-1 α) and caused inducible bronchus-associated lymphoid tissue (iBALT) formation in the lung. IL-1 α release continued for up to 2 weeks after particulate exposure, and type-2 allergic immune responses were induced by the inhalation of antigen during IL-1 α release and iBALT formation, even long after particulate instillation. Recombinant IL-1 α was sufficient to induce iBALTs, which coincided with subsequent immunoglobulin E responses, and IL-1-receptor-deficient mice failed to induce iBALT formation. Therefore, the AM-IL-1 α -iBALT

axis might be a therapeutic target for particulate-induced allergic inflammation.

INTRODUCTION

The numbers of patients with allergic asthma or rhinitis have recently increased, especially in developed countries. The reason is unclear, but many studies have suggested that particulate pollution might exacerbate allergic responses (Ichinose et al., 2008; Løvik et al., 1997). Several nanometer- to micrometer-sized particulates, such as particulate matter 2.5 (PM_{2.5}), which is less than 2.5 μ m in diameter, can enter the respiratory tract and settle deep in the lungs, causing chronic pulmonary inflammation, including asthma (Granum and Løvik, 2002). In an epidemiological study, increased particulate matter in the air was significantly related to increased hospitalization for asthma (Schwartz et al., 1993). Particulates stimulate the immune system to generate inflammatory responses; aluminum salts (referred to as alum) and silica crystals particularly induce type 2 responses, characterized by eosinophil accumulation at the injection sites and elevated antigen-specific serum immunoglobulin E (IgE) levels

in vivo (Kool et al., 2011; Kuroda et al., 2011; Mancino et al., 1983; Marichal et al., 2011). Most particulates, including particulate pollutants, are thought to function as immune adjuvants enhancing allergen-specific type 2 responses. However, the basis for the adjuvant activity of these particulates and the underlying mechanisms by which they induce type 2 responses are still unclear.

Recent studies have demonstrated that innate immunity is required for the effective induction of adaptive immunity (Akira, 2011). In general, pathogen-derived factors, known as pathogen-associated molecular patterns, are recognized by innate immune cells via pattern-recognition receptors (PRRs). In addition, factors released from dying or stressed cells, damage-associated molecular patterns (DAMPs) (or “alarmins”), also contribute to inflammation, and are recognized by PRRs or cytokine receptors on innate immune cells. DAMPs can be critical for adjuvant activity and the induction of sterile inflammation (Bianchi, 2007; Desmet and Ishii, 2012; Rock et al., 2011; Willart and Lambrecht, 2009). Several studies have demonstrated that particulates stimulate innate cells to release DAMPs and that DAMP-dependent innate immune responses are required for the induction of adaptive immune responses (Kool et al., 2011; Marichal et al., 2011; McKee et al., 2013). Some small cytotoxic chemicals also function as adjuvants, inducing antigen-specific IgG1 and IgE production (Jacobson et al., 2013; Tonti et al., 2013). Therefore, particulate-induced DAMPs might participate in the induction and exacerbation of allergic responses.

Inducible bronchus-associated lymphoid tissues (iBALTs) are a tertiary lymphoid organ (TLO) that is not preprogrammed, but is induced in the lung by chronic inflammation, infections, or autoimmune diseases (Neyt et al., 2012; Rangel-Moreno et al., 2006). Lymphoid clusters are also frequently found in the lungs of patients with asthma and in a mouse model of asthma, and are currently regarded as iBALTs (Chvatchko et al., 1996; Elliot et al., 2004; Foo and Phipps, 2010). iBALTs are found in the lung parenchyma. It supports T and B cell proliferation and enhances immunoglobulin class switching in its germinal centers (GC) and also contributes to the survival of plasma cells in the lung (Foo and Phipps, 2010; Randall, 2010). iBALTs are reported to offer protective immunity against pathogens and induces virus-specific antibody production (GeurtsvanKessel et al., 2009; Moyron-Quiroz et al., 2004). However, iBALTs are sometimes considered detrimental, and is reported to correlate with disease severity in patients with rheumatoid arthritis (RA) and chronic obstructive pulmonary disease (COPD) (Neyt et al., 2012; Rangel-Moreno et al., 2006). Thus, iBALTs play important roles in the immune responses that are specifically occurring in the lung.

Some environmental particles such as sand dust are composed of mineral elements, including silicon and aluminum (Ichinose et al., 2008). Therefore, we established a particulate-induced lung inflammation model using the intratracheal (i.t.) instillation of particulate alum or silica. In this model, we found that the direct administration of particulates to the airway induced unique immune responses in the lung, involving interleukin-1 α (IL-1 α) release after particulate-induced alveolar macrophage death, and the subsequent formation of iBALT. Therefore, the release of DAMPs, triggered by exposure to particulates, and TLO formation might be important adjuvant effects in the induction of particulate-pollution-induced allergic inflammation.

RESULTS

Alveolar Macrophages Specifically Induce IL-1 α in Response to Particulates

When lungs are exposed to particulates such as alum or silica, the particulates are thought to be engulfed by phagocytes, such as alveolar macrophages, to be cleared from deep in the lung (Ling and van Eeden, 2009; Oberdörster et al., 2005). Therefore, we first examined the effects of particulates on alveolar macrophage function. We prepared in-vitro-differentiated alveolar macrophages (referred to as “cultured alveolar macrophages,” in Figure S1A), stimulated them with alum in vitro, and then measured the typical proinflammatory factors produced by macrophages. Alum stimulated alveolar macrophages to produce IL-1 α , but no other inflammatory factors were detected (Figure 1A). In a similar experiment using alveolar macrophages freshly isolated from mouse lungs, alum-stimulated cells produced IL-1 α (Figure 1B). Crystalline silica also stimulated cultured alveolar macrophages to produce IL-1 α . Both alum and silica induced cell death (Figure 1C). However neither aluminum oxide nor hydroxyapatite induced IL-1 α production or cell death.

A previous report shows that particulates, such as alum and monosodium urate crystals, induce IL-1 α release by bone marrow (BM)-derived dendritic cells (BMDCs) or BM macrophages that were primed with lipopolysaccharide (LPS) (Gross et al., 2012). However, we used alveolar macrophages without LPS priming. We also found that *Myd88*^{−/−} alveolar macrophages released IL-1 α after stimulation with alum (Figure 1D). A previous study shows that IL-1 α production is partly associated with the activation of apoptosis-associated speck-like protein containing CARD (ASC) in BMDCs and keratinocytes (Yazdi et al., 2010). However, particulates induced similar IL-1 α levels in wild-type (WT) and *Asc*^{−/−} alveolar macrophages (Figure 1E). These results indicate that both MyD88 (Toll-like receptor [TLR] signaling) and ASC (inflammasome) were not essential for the induction of IL-1 α in alveolar macrophages in response to particulates.

Cultured alveolar macrophages include two different populations: CD11c⁺ sialic-acid-binding immunoglobulin-type lectins (siglec)-F⁺ cells and CD11c^{low} Siglec-F^{low} cells. We observed higher amounts of IL-1 α from cells expressing CD11c⁺ and siglec-F⁺, typical surface markers of alveolar macrophages (Figure 1F). Moreover, neither peritoneal macrophages, granulocyte-macrophage colony-stimulating factor (GM-CSF)-derived BM-macrophages, nor lung fibroblasts produced IL-1 α in response to alum or silica, whereas these particles caused the same degree of cell death in all the cell types we tested (Figure 1G and Figure S1B, S1C–S1E). We also examined the release of IL-1 α by small intestinal lamina propria macrophages (Uematsu et al., 2008), however IL-1 α was not released by the lamina propria macrophages in response to alum (Figure 1H). Therefore, particulate-induced IL-1 α release was a unique characteristic of alveolar macrophages.

Because IL-1 α is released from dying cells and functions as a DAMP (Cohen et al., 2010; Rider et al., 2011), we next examined the intracellular IL-1 α in the lysates of alveolar macrophages after three freeze-thaw cycles. We detected intracellular IL-1 α in unstimulated alveolar macrophages and the amounts of

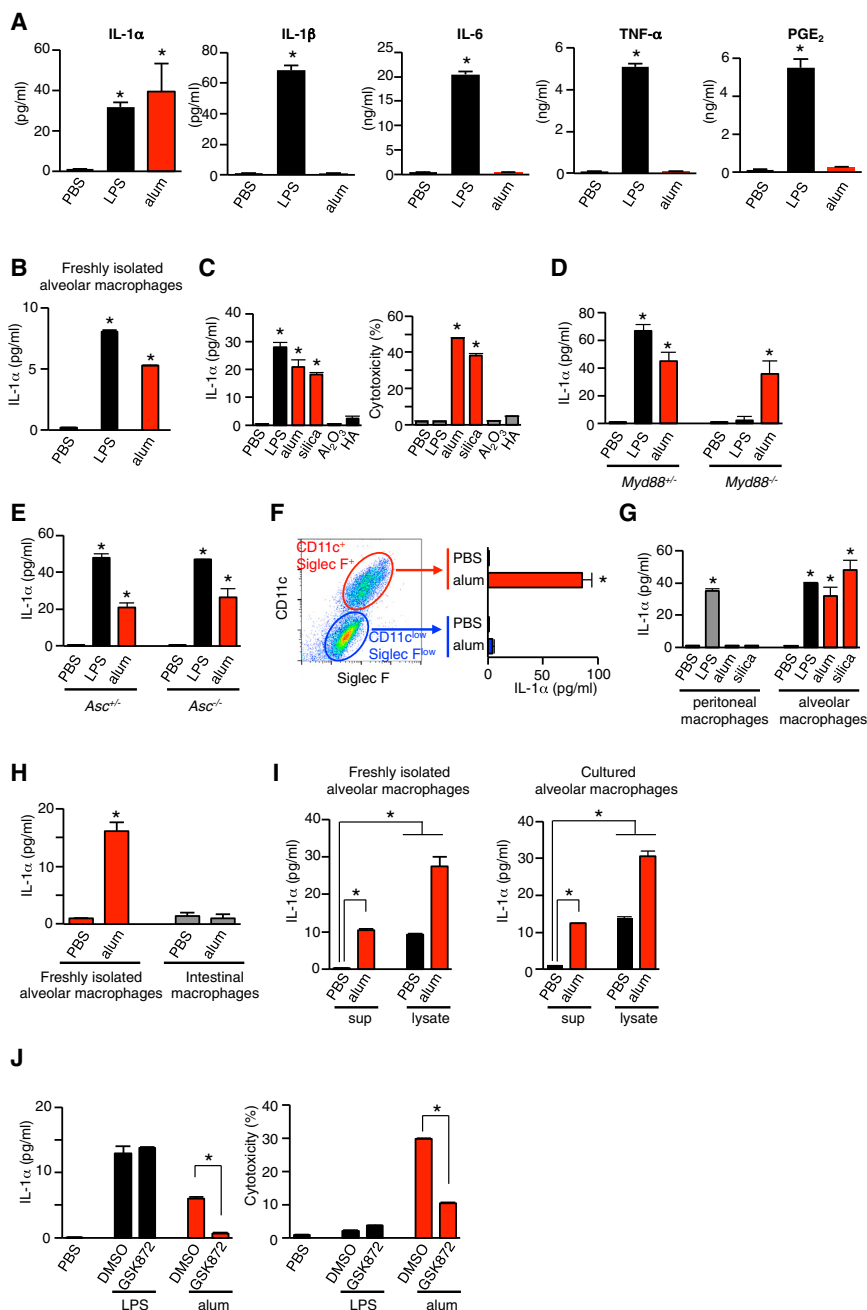


Figure 1. Alveolar Macrophages Release IL-1α in Response to Particulate Adjuvants

(A) Cultured alveolar macrophages were stimulated with LPS or alum for 6 hr. Supernatants were analyzed for cytokines with ELISAs.

(B and C) Freshly isolated (B) and cultured (C) alveolar macrophages were stimulated with LPS or particulates as indicated for 6 hr. Supernatants were used for cytokine ELISAs or to test for lactate dehydrogenase (LDH) release. Al₂O₃, aluminum oxide; HA, hydroxyapatite.

(D) Myd88- or (E) ASC-deficient cultured alveolar macrophages were stimulated with LPS or alum for 6 hr.

(F) Cultured alveolar macrophages were sorted into CD11c⁺ siglec-F⁺ and CD11c^{low} siglec-F^{low} cells, and then the sorted cells were stimulated with alum for 6 hr.

(G) Peritoneal or cultured alveolar macrophages were stimulated with LPS, alum, or silica for 6 hr.

(H) Freshly isolated alveolar and lamina propria macrophages were stimulated with alum for 8 hr.

(I) Freshly isolated and cultured alveolar macrophages were stimulated with alum for 6 hr. The culture supernatants and cell lysates were used for an IL-1α ELISA.

(J) Cultured alveolar macrophages were stimulated with alum in the presence of an RIPK3 inhibitor (GSK872). All macrophages were prepared from five to ten mice. Data are shown as mean ± SD and represent at least two independent experiments (*p < 0.05), except in Figure 1H, in which the experiment was performed once, but 50 mice were used for the preparation of the lamina propria macrophages. See also Figure S1.

Therefore, we suggest that alum-induced cell death was RIPK3-dependent but RIPK1-independent necroptosis (Moriwaki and Chan, 2016).

Particulates Induce IL-1α Release after Cell Death, Resulting in the Generation of B Cell Clusters

We next examined whether alveolar macrophages in the lung release IL-1α in vivo. An immunohistochemical analysis of lung tissues showed that some of the F4/80⁺ cells expressed IL-1α after the intratra-

cheal (i.e.) administration of alum (Figure 2A), whereas we could not observe IL-1α in other types of cells. Alveolar macrophages constitutively expressed intracellular IL-1α, but at very low levels (picograms/ml). In contrast, LPS-stimulated alveolar macrophages released IL-1α at picogram levels, but expressed 100-fold greater amounts of intracellular IL-1α (Figure S2A), and the intracellular IL-1α was easily detected with flow cytometry (Figure S2B). However, it was difficult to detect intracellular IL-1α in unstimulated or alum-stimulated alveolar macrophages with flow cytometry (Figure S2B). We detected a weak signal for intracellular IL-1α in some of the F4/80⁺ cells in bronchoalveolar lavage (BAL) cells from mice after the i.t. administration of alum (Figure S2C).

The mechanisms by which particulates induce the cell death of alveolar macrophages are unclear. We found that GSK872, a receptor-interacting serine/threonine protein kinase-3 (RIPK3) inhibitor that suppresses necroptosis, significantly blocked both IL-1α and cell death, indicating that alum-induced cell death was necroptotic (Figure 1J) (Silke et al., 2015). However, necrostatin did not suppress IL-1α release or cell death (Figure S1F).

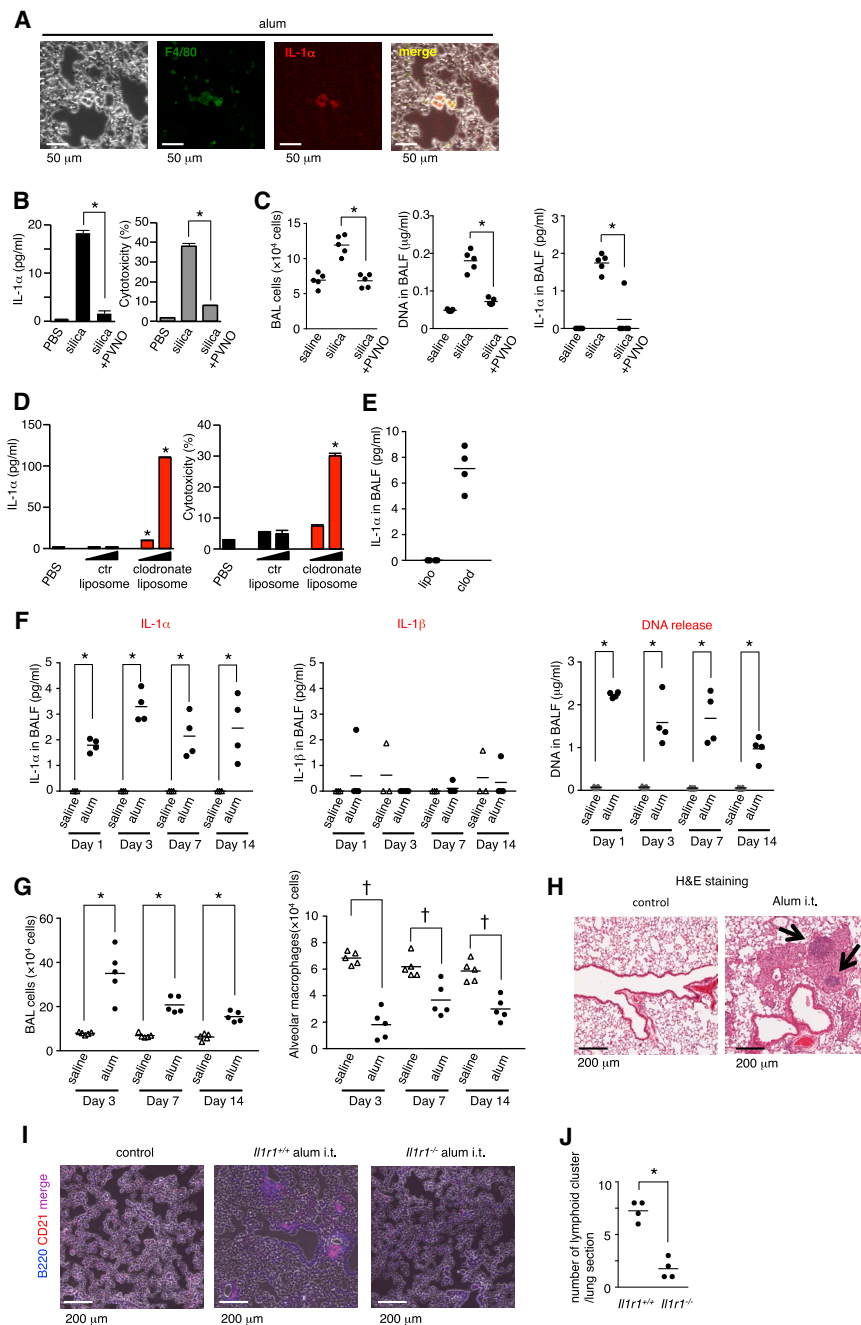


Figure 2. IL-1 α Is Released after Alveolar Macrophage Cell Death in the Lung

(A) Lungs ($n = 4$) were isolated 3 days after the i.t. instillation of alum. Frozen section of the isolated lung specimens were stained for F4/80 and IL-1 α , and analyzed with confocal microscopy. Scale bars represent 50 μ m.

(B) Cultured alveolar macrophages were stimulated with silica in the presence of PVNO. The supernatants were analyzed for cytokines with ELISAs or for LDH release.

(C) Silica was administered to mice ($n = 5$ mice, each group) by i.t. instillation with PVNO. BALF was harvested 3 days after particulate administration.

(D) Cultured alveolar macrophages were stimulated with empty or clodronate liposomes for 6 hr. (E) Clodronate (clod) or empty (lipo) liposomes were administered to mice ($n = 4$ mice, each group) by i.t. instillation. BALF was harvested 3 days after instillation and tested for IL-1 α with an ELISA.

(F) Alum was administered by i.t. instillation ($n = 4$ mice, each group). At 1, 3, 7, or 14 days after administration, BALF was harvested and analyzed for IL-1 α and IL-1 β with ELISAs or for DNA release.

(G) At 3, 7, or 14 days after i.t. alum administration, the total BAL cells and alveolar macrophages in the BALF were analyzed. ($n = 5$ mice, each group). (H) Histological analysis (H&E staining) of the lungs of mice ($n = 4$ mice, each group) 14 days after i.t. instillation of alum. Scale bars represent 200 μ m. Arrows indicate lymphoid clusters.

(I and J) Lungs were isolated from *Il1r1*^{+/+} and *Il1r1*^{-/-} mice ($n = 4$ mice, each group) 14 days after the i.t. instillation of alum. (I) Frozen section of the isolated lung specimens were stained for B220 and CD21 (scale bars represent 200 μ m), and (J) the number of lymphoid clusters in the lungs of *Il1r1*^{+/+} and *Il1r1*^{-/-} mice. All alveolar macrophages were prepared from five to ten mice. Data are shown as mean $\pm$ SD and represent at least two independent experiments (*, $p < 0.05$). See also Figure S2.

IL-1 α released by macrophages in response to particulates seemed to be linked to cell death (Figures 1C and 1J). It is previously reported that treating macrophages with poly-2-vinylpyridine-N-oxide (PVNO), a lysosome-stabilizing agent, suppresses their silica-induced cell death (Kuroda et al., 2011; Mancino et al., 1983). Indeed, treatment with PVNO rescued alveolar macrophages from cell death and reduced their IL-1 α release in response to silica in vitro (Figure 2B). Similarly, the host DNA, an indicator of cell death, and IL-1 α released in the BAL fluids (BALFs) and bronchoalveolar lavage (BAL) cell recruitment in the lung after the i.t. instillation of silica were also significantly reduced by PVNO treatment in vivo (Figure 2C). These results

indicate that particulate-induced IL-1 α release was mediated by cell death. Clodronate liposomes are frequently used for the specific depletion of phagocytosis. Indeed, clodronate liposomes induced cell death and IL-1 α release in alveolar macrophages (Figure 2D). In an in vivo analysis, we observed the reduced number of alveolar macrophages in the BALF after the i.t. instillation of clodronate liposomes (Figure S2D). Furthermore, IL-1 α was released into the BALF after treatment with clodronate liposomes (Figure 2E). These results indicate that the direct administration of particulates to the airways induced IL-1 α in the lung via alveolar macrophage death.

We found that IL-1 α and DNA were released into the BALF for 14 days after the i.t. instillation of alum (Figure 2F), whereas IL-1 β was not detected in the BALF at any time point. The numbers of alveolar macrophages were also still reduced 14 days after the i.t. instillation of alum, regardless of the increased number of

inflammatory cells in the lung (Figure 2G and Figure S2E). These results suggest that alum-induced IL-1 α persisted for 2 weeks, which might be mediated by ongoing alveolar macrophage death.

We next performed a histological analysis of the lung after i.t. alum instillation. Hematoxylin-and-eosin (H&E) staining revealed that, in addition to the infiltration of inflammatory cells, lymphoid clusters were induced 14 days after the i.t. instillation of alum (Figure 2H). These clusters were mainly composed of B cells, and were characterized by areas of B220⁺ CD21⁺ B cells (Figure 2I). The number of clusters was clearly reduced in IL-1-receptor-deficient (*Il1r1*^{-/-}) mice (Figures 2I and 2J). These results suggest that the prolonged IL-1 α release caused by particulate-induced cell death was involved in the formation of lymphoid clusters in the lung.

IL-1 α Release and iBALT Formation Play Roles in the Delayed and Sustained Adjuvant Effects

Particulate-induced lymphoid clusters seem to be iBALTs. Therefore, antigen-specific immune responses might be induced by the administration of antigen into the lung during the release of IL-1 α and the formation of iBALT. To test this, mice were initially administered i.t. alum and were exposed to aerosolized ovalbumin (OVA) 3, 7, and 14 days later. Despite the lag time, the exposure to aerosolized OVA after alum instillation induced high OVA-specific serum IgG1 and IgE concentrations and increased BAL cell recruitment (Figure 3A). OVA exposure alone had no or a weak effect on antibody responses and BAL cell recruitment. The antibody responses were detected in mice exposed to OVA even 14 days after the administration of alum, which correlated with the release of IL-1 α and the formation of iBALT (Figures 2F and 2I). These data suggest that immune responses were activated even when the antigen and adjuvant were administered separately, and that delayed exposure to the antigen long after particulate inhalation affected lung inflammation and the IgE response. We administered alum intraperitoneal (i.p.) on day -7, and then injected OVA i.p. twice, but no OVA-specific serum antibody responses were detected (Figure 3B). Therefore, the immune responses induced by the direct administration of particulates into the lung were regulated by different mechanisms than those induced by their i.p. injection. Crystalline silica and nickel oxide (NiO) nanoparticles also have strong adjuvant activity and induce type 2 immune responses (Kuroda et al., 2011). Mice were administered i.t. alum, silica, or NiO and then exposed to aerosolized OVA 7 days later. We observed significant increases in OVA-specific serum IgG1 and IgE concentrations and in the number of BAL cells (Figure S3A). A similar experiment using other noninflammatory particulates, aluminum oxide and hydroxyapatite, induced weak IgG1 and no IgE responses (Figure S3B). Therefore, not all particulates administered by i.t. instillation induced severe lung inflammation and IgE production. Elevated numbers of airway eosinophils and goblet cell hyperplasia (periodic acid-Schiff [PAS] staining) were also found in mice exposed to OVA 7 days after the i.t. instillation of alum (Figure 3C). We performed a similar experiment with house-dust-mite (HDM) allergen instead of OVA. Mice were administered i.t. alum, and after 7 days, were sensitized to HDM by the intranasal (i.n.) route. The i.t. injection of alum enhanced the HDM-specific antibody responses and the number of BAL cells (Figure 3D).

Il1r1^{-/-} mice displayed reduced OVA-specific IgG1 concentrations, and OVA-specific IgE was absent in the mice exposed to OVA 7 days after the i.t. instillation of alum (Figure 3E) or silica (Figure S3C). We performed similar experiments with IL-1 α -deficient mice, and OVA-specific IgG1 and IgE concentrations were significantly reduced in *Il1a*^{-/-} mice (Figure 3F). Many reports have shown that particulates stimulate DCs and macrophages to produce IL-1 β by activating the NLRP3 inflammasome (Eisenbarth et al., 2008; Hornung et al., 2008). However, NLRP3-, caspase-1-, and ASC-deficient mice exhibited significant amounts of serum IgG1 and IgE, similar to those in the WT mice (Figure 3G and Figures S3D and S3E). Thymic stromal lymphopoietin (TSLP) and IL-33, important molecules in allergic inflammation (Haenuli et al., 2012; Zhou et al., 2005), were not involved in particulate-induced IgE production (Figures S3F and S3G). Although interferon regulatory factor 3 (IRF3) and stimulator of interferon gene (STING) are involved in the adjuvanticity of alum (Marichal et al., 2011; McKee et al., 2013), neither IRF3 nor STING was involved in the antibody responses induced by i.t. instillation of alum (Figures S3H and S3I). These results indicate that the induction of IgE by direct airway sensitization with particulates involved mechanisms that differ from those reported previously.

IL-1 α in the Lung Acts as a Potent iBALT Inducer and Is Involved in Local Immune Responses

We examined the particulate-induced iBALT structure in detail. Exposure to OVA 7 days after the i.t. instillation of alum induced many lymphoid clusters characterized by B220⁺ and CD21⁺ cell aggregates (Figure 4A), as did the i.t. instillation of alum without OVA exposure (Figure 2I). However, more and larger iBALT structures, around 300 μ m in diameter, were induced with OVA exposure than those without OVA exposure. Some of these clusters contained GL-7⁺ GC B cell areas (Figure 4B). Small T cell areas were observed and CD138⁺ cells (plasmablasts) were also located close to B cell clusters (Figures 4C and 4D). Areas of CD138⁺ cells were also stained for labeled OVA (Figure 4C). These results indicate that particulate-induced iBALTs functioned as TLOs in the lung and induced antigen-specific responses. The number of iBALTs were significantly reduced in *Il1r1*^{-/-} mice exposed to OVA 7 days after the i.t. instillation of alum (Figures 4E and 4F). OVA exposure without i.t. alum instillation did not induce iBALT structures in the lung (Figures 4E and 4F). Given that iBALT structures are reported to affect the local humoral immunity (GeurtsvanKessel et al., 2009), we measured the OVA-specific antibody responses in the BALF. OVA exposure after alum or silica instillation induced significant concentrations of OVA-specific IgG1 and IgE in the BALF, and these responses were absent in *Il1r1*^{-/-} mice (Figure 4G and Figure S4A). The i.t. instillation of IL-1 α plus OVA induced strong OVA-specific IgE responses, so IL-1 released in the lung played an important role in IgE production (Figure 4H). The direct instillation of IL-1 α into the airway also induced iBALT structures in the lung (Figure 4I and Figure S4B). The i.t. instillation of clodronate liposomes induced the release of IL-1 α in the lung in vivo (Figure 2E). OVA-specific IgG1 and IgE responses were detected in mice exposed to OVA 3 days after the i.t. instillation of clodronate liposomes (Figure 4J), and we also observed iBALT structures in the lung (Figure 4K and Figure S4C). These

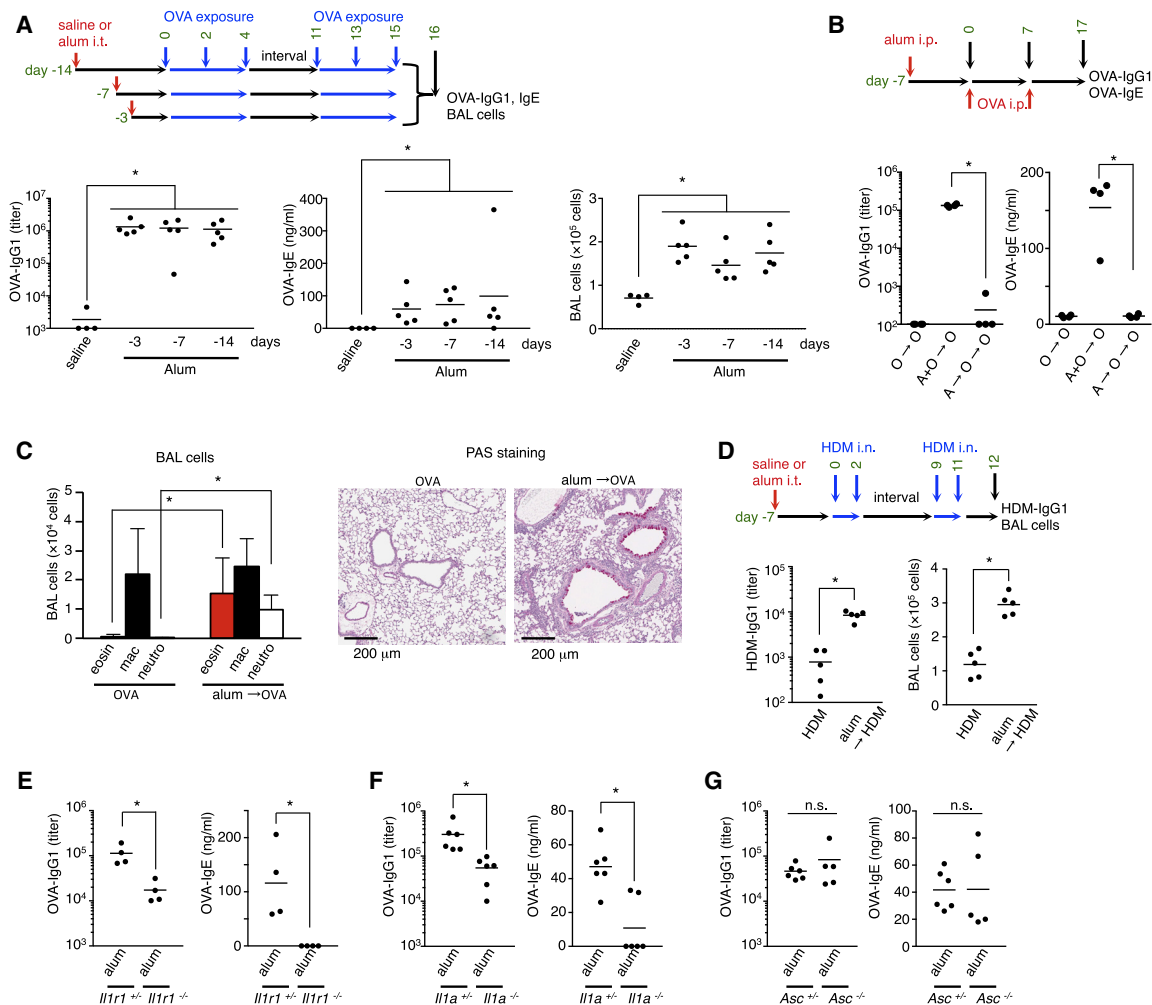


Figure 3. Delayed and Sustained Adjuvant Effect of Particulates Is Unique to the Lung

(A) Saline or alum was administered by i.t. instillation ($n = 4$ or 5 mice, each group). At 3, 7, or 14 days after its administration, the mice were exposed to aerosolized OVA. Sera and BALF were collected 24 hr after the last exposure.

(B) Alum was injected i.p. on day -7 and OVA was injected i.p. on days 0 and 7 (A $\rightarrow$ O $\rightarrow$ O) ($n = 4$ mice, each group). Injection of OVA alone (O $\rightarrow$ O) and alum mixed with OVA (A+O $\rightarrow$ O) were used as the controls.

(C) BAL cell and histological analyses (PAS staining) of the lungs from mice ($n = 4$ mice, each group) exposed to OVA 7 days after the i.t. instillation of alum. Scale bars represent 200 μ m.

(D) Saline or alum was administered by i.t. instillation ($n = 5$ mice, each group). Seven days after its administration, the mice were sensitized with HDM by i.n. administration.

(E–G) Alum was administered to *Il1r1*^{-/-} (E), *Il1a*^{-/-} (F), and *Asc*^{-/-} (G) mice ($n = 4$ – 6 mice, each group). Seven days later, the mice were exposed to aerosolized OVA as in (A). Data are shown as mean $\pm$ SD and represent at least two independent experiments (* $p < 0.05$). See also Figure S3.

results indicate that the IL-1 α released by the death of alveolar macrophages was essential for the particulate-induced formation of iBALT and both the systemic and local IgE responses in the lung.

Particulate-Induced IL-1 α Regulates iBALT Formation through DC Migration

DCs play an important role in the formation and maintenance of iBALT structures (GeurtsvanKessel et al., 2009; Halle et al., 2009). The recruitment of DCs was observed in the lung after the i.t. instillation of alum and exposure to OVA, but less DC migration into the lung was observed in *Il1r1*^{-/-} mice (Figure 5A).

The i.t. instillation of IL-1 α also caused a marked increase in DC recruitment into the lung (Figure S5A). Thus, particulate-induced IL-1 α was involved in the recruitment of DCs to the lung. Next, we depleted lung DCs (as CD11c⁺ cells) in vivo using CD11c-diphtheria toxin receptor (DTR) transgenic mice. Diphtheria toxin (DT) was administered by i.t. instillation 1 day before the last exposure to OVA. Four days later, we analyzed the antibody responses and iBALT formation. After the administration of DT, the number of DCs was reduced in the lung (Figure S5B). The lung-CD11c⁺-cell-depleted mice displayed reduced antigen-specific antibody responses and the reduced number of iBALT structures (Figure 5B and Figure S5C). These results indicate

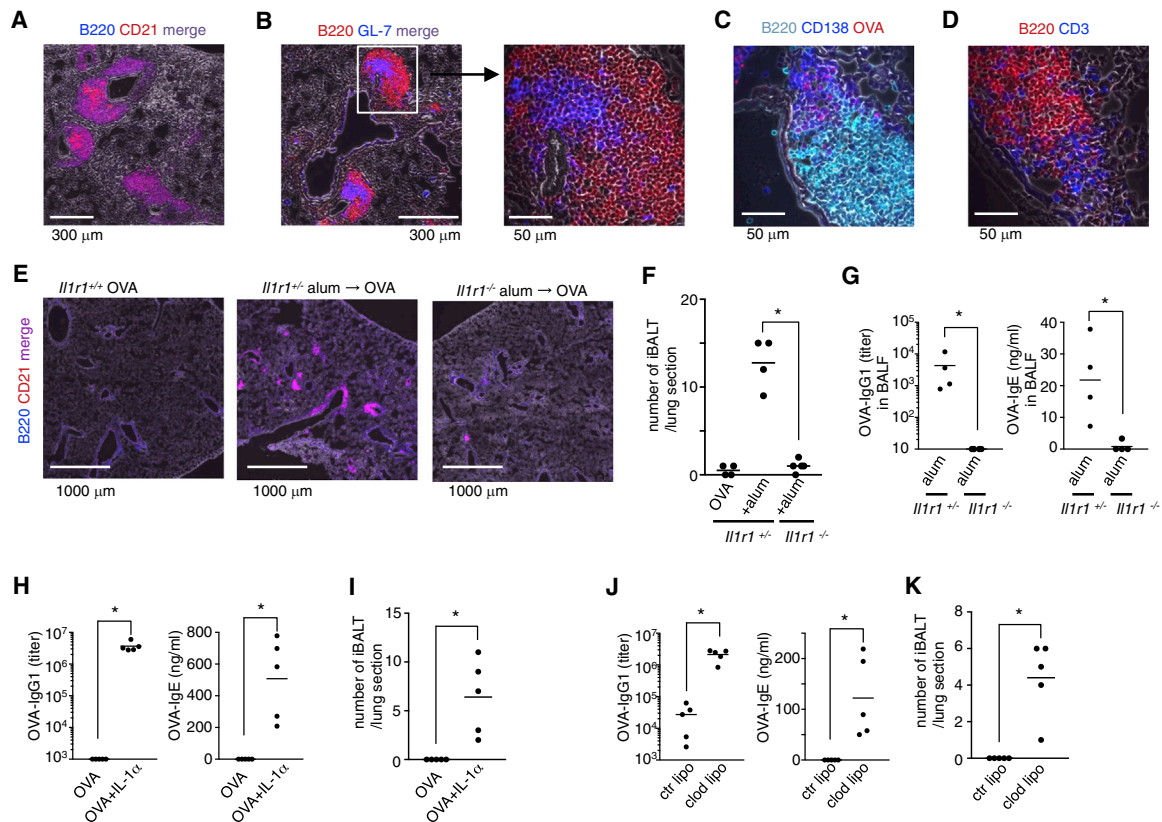


Figure 4. Exposure to Alum and Antigen Induces iBALTs in the Lung in an IL-1-Dependent Manner

(A–D) Frozen sections of lungs were isolated from mice ($n = 4$ mice, each group) exposed to OVA for 7 days after the i.t. instillation of alum. Frozen sections of specimens were stained for B220 and CD21 (A), B220 and GL7 (B), CD138, B220 and OVA (C), B220 and CD3 (D). All samples were analyzed with confocal microscopy. Scale bars represent 300 or 50 μ m, as indicated.

(E and F) iBALT formation in *Il1r1*^{-/-} mice ($n = 4$ mice, each group). Immunohistochemical analysis of iBALT formation in the lungs (E) and the number of iBALT structures in lung sections (F) from WT and *Il1r1*^{-/-} mice. Scale bars represent 1,000 μ m.

(G) Antibody responses in BALF from mice ($n = 4$ mice, each group) exposed to OVA after i.t. alum instillation.

(H and I) OVA or IL-1 α +OVA was administered to mice ($n = 5$ mice, each group) by i.t. instillation and the mice were then exposed to aerosolized OVA. At 24 hr after last exposure, the sera and lungs were analyzed for antibody responses (H) and the number of iBALT structures in the lung sections (I).

(J and K) I.t. instillation of clodronate liposomes induces immune responses in the lung. Empty (ctr lipo) or clodronate (clod lipo) liposomes were administered to mice ($n = 5$ mice, each group) by i.t. instillation Three days after their administration, the mice were exposed to aerosolized OVA. Sera and lungs were analyzed for antibody responses (J) and the number of iBALT structures in the lung sections (K). Data represent at least two independent experiments (* $p < 0.05$). See also Figure S4.

that DCs were required for the particulate-induced formation of iBALT.

Lymphotoxin (LT) and several homeostatic chemokines, including CXCL12, CXCL13, CCL19, and CCL21, are important in controlling the formation of iBALT (Foo and Phipps, 2010; GeurtsvanKessel et al., 2009; Moyron-Quiroz et al., 2004; Randall, 2010; Rangel-Moreno et al., 2011). We observed that LT- β , but not other chemokines, was mainly expressed by DCs (Figure S5D). We next examined the role of LT- β in the formation of iBALT induced by the i.t. instillation of alum and exposure to OVA. The administration of the LT- β receptor-Fc fusion construct had no effect on the antibody responses (Figure 5C). Furthermore, the iBALT structures were slightly reduced, although they were still observed in the LT- β receptor-Fc-treated mice (Figure 5C and Figure S5E).

These data indicate that although DCs were required, unique factors, other than LT- β , participated in the particulate-induced formation of iBALT.

Exposure of Airways to Particulates Induces Unique Immune Responses in the Lung

Next, we examined whether the IL-1 α -iBALT axis explains the unique features of the lung allergic responses to inhaled antigens. It has been reported that i.p. or footpad immunization with OVA plus alum induces OVA-specific IgE responses independently of MyD88 (Schnare et al., 2001). However, a recent report demonstrates that the inhalation of OVA plus alum induces OVA-specific IgE responses that is totally dependent on MyD88 (Matsushita and Yoshimoto, 2014). Consistent with previous reports, the OVA-specific antibody concentrations were similar in WT and *Myd88*^{-/-} mice immunized i.p. with OVA plus alum (Figure 6A). In sharp contrast, OVA-specific antibody responses were significantly reduced in *Myd88*^{-/-} mice exposed to OVA 7 days after the i.t. instillation of alum (Figure 6B). This response was not mediated by TLRs or IL-18 (Figures S6A–S6C). The antibody responses in the serum (systemic) and BALF (local) seemed to be differentially regulated. Mice were

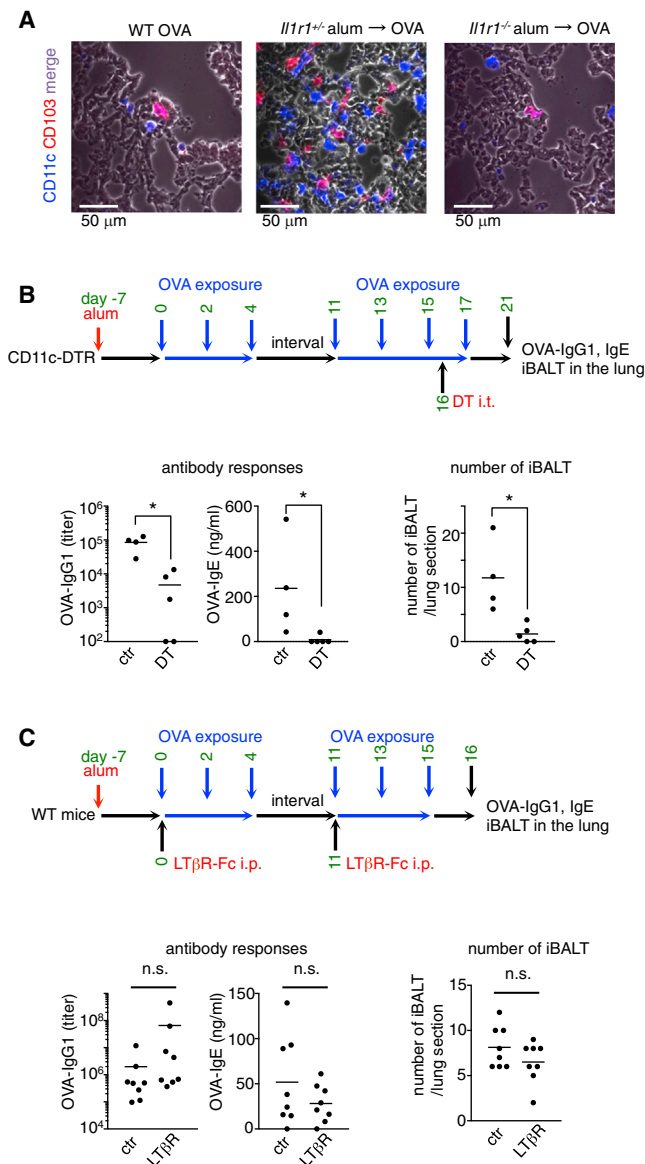


Figure 5. Particulate-Induced iBALT Formation Is Mediated by DC Recruitment

(A) Frozen sections of lungs isolated from WT and *Il1r1*^{-/-} mice ($n = 4$ mice, each group) exposed to OVA after i.t. alum instillation were stained for CD11c and CD103. Scale bars represent 50 μm, as indicated.
 (B) CD11c-DTR mice ($n = 4$ or 5 mice, each group) were administered i.t. alum, and were exposed to aerosolized OVA 7 days later. DT was administered by i.t. instillation 1 day before the last OVA exposure. Four days after the last OVA exposure, the sera and lungs were analyzed for antibody responses and the number of iBALT structures in the lung sections.
 (C) Alum was administered by i.t. instillation, and the mice were exposed to aerosolized OVA 7 days after its administration ($n = 8$ mice, each group). LTβR-Fc was administered by i.p. twice. Serum antibody responses and the numbers of iBALT structures in the lung sections were analyzed. Data represent at least two independent experiments (* $p < 0.05$). See also Figure S5.

immunized twice (days 0 and 7) with alum plus OVA by the i.p. or i.n. route, and then sensitized three times (days 14, 16, and 18) with OVA by the i.n. route. The serum (systemic) OVA-specific antibody responses were similar after i.p. and i.n. immunization

(Figure 6C). However, strong antibody responses in the BALF (local) were only observed in mice immunized by the i.n. route (Figure 6D). iBALTs especially affected the local immune responses (Figure 4G and Figure S4A). A histological analysis revealed that the lungs of mice immunized i.p. displayed very few, very small lymphoid clusters. In contrast, many large clusters were found in the lungs of mice immunized by the i.n. route (Figure 6E). We next examined the antigen-specific responses in regional lymph-node cells. Cells from the mediastinal lymph nodes secreted Th2 cytokines in response to OVA in mice that were immunized with i.p. alum plus OVA and then exposed to OVA (Figure 6F). However, the lymph-node cells of mice immunized with alum plus OVA by the i.t. route produced lower amounts of Th2 cytokines (Figure 6F). We observed no IFN-γ production in either case (data not shown). The infiltration of eosinophils and goblet cell hyperplasia might be moderate in mice directly sensitized to alum in the airway (Figure 3C) because smaller amounts of Th2 cytokines were produced by the lymph-node cells.

Recent studies have reported that IL-4 production by follicular helper T (T_{fh}) cells, but not conventional Th2 cells, is involved in the class-switch recombination to IgE (Harada et al., 2012; Vijayanand et al., 2012). Therefore, we examined the role of T_{fh} cells in particulate-induced lung inflammation, using *Cd4-cre Bcl6*^{flox/flox} mice as the T_{fh}-deficient mouse model (Onishi et al., 2015). The antibody responses were significantly reduced in the *Cd4-cre Bcl6*^{flox/flox} mice (Figure 6G). Moreover, the number of iBALT structures was also reduced by T_{fh}-deficiency (Figure 6H and Figure S6D). Thus, T_{fh} cells were required for both the IgE responses and iBALT formation.

Taken together, these results suggest that the direct exposure of the airway to particulates and antigens was more physiologically relevant than a model that was sensitized by i.p. and then exposed to antigen, and induced unique immune responses in the lung, which differed from those induced by i.p. sensitization.

DISCUSSION

Particulates, including particulate pollutants, probably increase the risk of allergic inflammation. Here, we have demonstrated that a DAMP, such as IL-1α, induced by particulates triggered lung immune responses through the generation of iBALT.

IL-1 is an important cytokine in murine allergic asthma models (Nakae et al., 2003; Schmitz et al., 2003). Here, we found that particulates such as alum and silica induce alveolar macrophage death and the release of IL-1α as a DAMP. We also observed IL-1α release in the lung after the specific depletion of phagocytes by the i.t. administration of clodronate liposomes. These observations suggest that macrophages were the major source of IL-1α in the lung after the administration of particulates through cell death. Indeed, *in vivo* analysis revealed that IL-1α tended to be released in the lung simultaneously with the release of host cell DNA, an indicator of cell death. Unlike IL-1β, pro-IL-1α is bioactive when released from dying cells, and IL-1α is an important factor in particulate-induced acute lung inflammation (Yazdi et al., 2010). A recent study also demonstrates that LPS-induced acute lung inflammation is mediated by the death of alveolar macrophages and the release of pro-IL-1α in the lung (Dagvadorj et al., 2015). Indeed, we observed that noninflammatory particulates (aluminum oxide and hydroxyapatite)

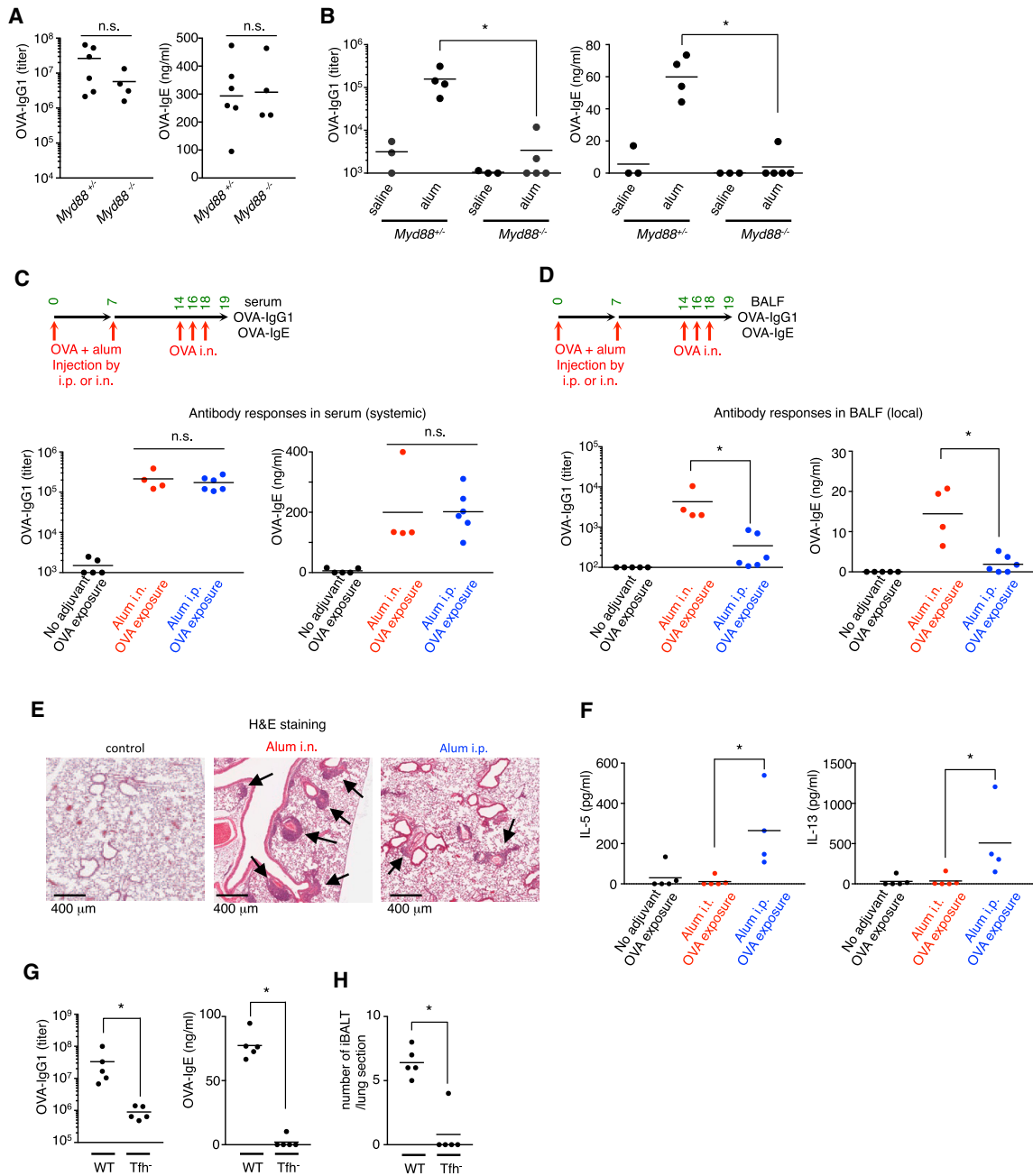


Figure 6. Direct Administration of Particulate Induces Unique Immune Responses in the Lung

(A) Alum+OVA was administered i.p. to *Myd88*^{-/-} mice, which were analyzed for serum OVA-specific IgE and IgG1 antibodies with ELISAs (n = 4–6 mice, each group).

(B) Saline or alum was administered to *Myd88*^{-/-} mice by i.t. instillation. Seven days after its administration, the mice were exposed to aerosolized OVA (n = 3–5 mice, each group), as in Figure 3A.

(C and D) Mice (n = 4–6 mice, each group) were immunized with alum+OVA twice (days 0 and 7) by the i.p. or i.n. route, and then sensitized three times with OVA by the i.n. route (days 14, 16, and 18). OVA-specific IgG1 and IgE levels in the serum (C) and BALF (D) were analyzed.

(E) Lungs were isolated from mice (n = 4) immunized with alum+OVA by the i.p. or i.n. route and then sensitized with OVA as in (C) and (D). The isolated lungs were analyzed with H&E staining. Arrows indicate lymphoid clusters.

(F) Mice (n = 4 or 5 mice, each group) were immunized with alum+OVA on day 0 by the i.p. or i.t. route, and then exposed to OVA for six times (days 7, 9, 11, 18, 20, and 22). Cells from mediastinal lymph nodes were cultured with OVA for 5 days. The culture supernatants were used for ELISA.

(G and H) *Cd4-cre Bcl6*^{flx/flx} mice Tfh⁻ were administered i.t. alum, and then exposed to aerosolized OVA as in Figure 3A (n = 5 mice, each group). The sera and lungs were analyzed for serum antibody responses (G) and the numbers of iBALT structures (H) in the lung sections. Data represent at least two independent experiments (*p < 0.05). See also Figure S6.

did not induce cell death and IgE responses. Thus, the cytotoxicity of particulates for alveolar macrophages and the consequently release of IL-1 α from these cells were considered to be critical in determining whether a particulate induces allergic inflammation. In addition, *in vivo* treatment with PVNO, which rescues the cell death induced by silica, reduced host DNA and IL-1 α levels after i.t. silica instillation. Therefore, controlling cell death or DAMP release could offer new options for the treatment of allergic inflammation. A recent study demonstrated that DCs and BM macrophages induce IL-1 α in response to particulates (Gross et al., 2012); however, they used LPS-primed cells. We observed that *Myd88*^{-/-} alveolar macrophages released IL-1 α in response to alum, indicating that LPS priming was not required for IL-1 α release from alveolar macrophages. Therefore, we suggest that IL-1 α release in response to particulates was a feature unique to alveolar macrophages and the specificity of tissue macrophages might reflect their tissue-specific immune responses. We are currently investigating which intracellular factors and events are associated with the release of IL-1 α and cell death by particulate-stimulated alveolar macrophages.

An important finding of this study is that the immune responses were activated even when the adjuvant (particulate) and antigen (OVA) were administered separately to the lungs. So far, adjuvants have been thought to enhance antigen-specific immune responses when administered concurrently with the antigens. However, antigen inhalation after the i.t. instillation of particulates enhanced the antigen-specific IgE responses. We have shown two possible mechanisms underlying the delayed and sustained adjuvant effects of particulates on the lung. The first is the continued release of IL-1 α . IL-1 α was released for at least 2 weeks after the i.t. instillation of alum, during alveolar macrophage cell death. Indeed, silica can be deposited in the lungs for 1 month *in vivo* (Yasuo Morimoto, personal communication). This release of IL-1 α was an extremely important event in the lung, bridging the innate and adaptive immune responses. The second important finding is that iBALT formation was a consequence of IL-1 α release. Therefore, antigen-specific IgE might be induced when the airway was exposed to antigens during the release of IL-1 α , with the subsequent generation of iBALT in the lung. The lung is not usually exposed to particulates and antigens concurrently, and we suggest that our separate exposure model is close to the natural course of allergen and particulate sensitization. Thus, once exposed to particulate pollution, the risk of allergen sensitization might persist for up to 2 weeks.

iBALTs are induced during chronic inflammation (Geurtsvan-Kessel et al., 2009; Halle et al., 2009; Moyron-Quiroz et al., 2004). In our model, iBALT formation was dependent on IL-1 signaling. IL-1 α played an important role in DC migration and chemokine expression. A recent report indicated that IL-17, produced by CD4⁺ T cells, is essential for the formation of iBALT (Rangel-Moreno et al., 2011), and IL-1 signaling is reported to induce IL-17-producing T cells (Chung et al., 2009; Sutton et al., 2009). The IL-1 α induced by particulates might be involved in the formation of iBALT through the induction of Th17, in addition to DC migration. Although we found that IL-1 α induced a strong antigen-specific IgE response, the detailed mechanism underlying the induction of IgE production is still unknown. A recent study reports that IL-1 directly stimulates CD4⁺ T cells to differentiate into Th2 cells, as well as Th17 cells (Ben-Sasson et al., 2009).

IL-1 α in the lungs might directly stimulate CD4⁺ T cells to induce IgE-producing B cells, as well as stimulating iBALT formation.

In this study, we found that the lymph-node responses in mice sensitized with i.t. treatments differed from those in i.p.-sensitized mice. The responses of lymph-node cells were weak in the mice sensitized by the i.t. administration of alum plus OVA. However, we found iBALT formation in the i.t.-sensitized mice and observed higher concentrations of antibody responses. Given that iBALT formation is reported to occur in lymph-node-deficient (lymphotoxin-deficient) mice (Moyron-Quiroz et al., 2004), these results suggest that sensitization directly via the airway mainly activated iBALTs, which functioned as lymph nodes. In addition, we observed the importance of Tfh for iBALT formation. It is demonstrated that IL-4 from Tfh cells is involved in class-switch recombination to IgE and IgG1, whereas conventional Th2 cells are associated with eosinophil activation (Harada et al., 2012). We found that the i.t. administration of particulates induced milder infiltration of eosinophils and milder goblet cell hyperplasia in the lung, corresponding to lower levels of Th2 cytokines released from the cells of the mediastinal lymph node. These findings suggest that direct sensitization of the airway preferentially activated Tfh cells rather than conventional Th2 cells. Indeed, the number of iBALT structures was significantly reduced in Tfh-deficient mice. The importance of Tfh cells in iBALT formation was reported by the transfer Tfh cells into T cell-deficient mice (Rangel-Moreno et al., 2011). Taken together, our findings suggest that Tfh cells were primed by DCs that have been induced to migrate into the lung by the release of IL-1 α and were then involved in iBALT formation and IgE production through Tfh-derived IL-4 production. Thus, both Tfh and CD11c⁺ cells were required for iBALT formation, and the Tfh-DC interaction might be important for the maintenance of the iBALT structure in the lung.

In conclusion, in investigating particulate-induced pulmonary inflammation, we found that lung-specific immune responses, including the release of IL-1 α and the formation of iBALT, were important in the induction of the local and systemic humoral responses (Figure S6E). To develop novel treatments for pulmonary inflammation, we must understand in detail the tissue-specific immune responses, the generation of iBALT, and the characteristics of the particulates themselves.

EXPERIMENTAL PROCEDURES

Animals

Female C57BL/6 mice were purchased from CLEA Japan Inc. (Osaka, Japan). Information about the gene-deficient and mutant mice is described in the [Supplemental Information](#). All the animal experiments were performed according to the Guidelines for the Care and Use of Animals approved by Osaka University and the National Institutes of Biomedical Innovation, Health and Nutrition.

Cell Preparation

Freshly isolated alveolar macrophages were prepared from BAL cells as adherent cells. Cultured alveolar macrophages were prepared from lung homogenate. Briefly, the lungs were isolated and diced with scissors, incubated with DNase I and Liberase at 37°C for 1 hr and homogenized by a gentle MACS™ Dissociator (Miltenyi Biotech, Gladbach, Germany). The cell suspensions were washed and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 5 μ g/ml insulin, but without antibiotics. After 7 days in culture, macrophages proliferated on the fibroblastic cell. Macrophages were easily detached with reciprocal

shaking (170 strokes/min) for 2 hr at 37°C. Floating cells were used for cultured alveolar macrophages. Cultured macrophages were > 95% F4/80⁺ as determined with flow cytometry. We described detailed method for preparation of cultured alveolar macrophages in the [Supplemental Information](#).

In Vivo Experiments

Particulates were administered by i.t. instillation. In brief, mice were anesthetized and hung on a board by their incisors. A 1 mL syringe fitted with a thin plastic needle was inserted into the trachea and 50 μ L of particulate solution was injected directly into the lungs.

We administered 50 μ L of particulates by i.t. instillation, according to the standard protocol used in this study. We used alum (100 μ g), silica (250 μ g), NiO (100 μ g), aluminum oxide (100 μ g), or hydroxyapatite (100 μ g), and saline was used as the negative control. Seven days after i.t. instillation, the mice were exposed to aerosolized OVA on days 0, 2, and 4 (three times every 2 days). After 7 days, the mice were exposed to OVA again three times every 2 days (on days 11, 13, and 15). To expose the mice to OVA, an aerosol of 1% OVA in H₂O was administered for 10 min. The aerosolized OVA was generated with a nebulizer (NE-C28, Omron Corp. Kyoto, Japan), and the mice were exposed in a chamber box (25 $\times$ 25 $\times$ 30 cm³). Twenty-four hours after the last exposure, their sera and BALF were collected. In some experiments, OVA exposure was started 3, 7, or 14 days after the i.t. instillation of alum. Experimental design is briefly shown in [Figure 3A](#).

All other in vivo experimental protocols are described in the [Supplemental Information](#).

Histological Analysis

To stain the lung tissues, we inflated the lungs with OCT Tissue-Tek (Sakura) diluted 1:4 in PBS, embedded in OCT and frozen in -80°C . Frozen sections (12 μ m) of the isolated lung specimens were prepared, fixed and permeabilized with the BD Cytfix/Cytoperm Plus Fixation/Permeabilization Kit (BD Biosciences), according to the manufacturer's instructions.

To detect iBALT, we stained lung specimens at 4°C with anti-B220, anti-CD21, anti-GL7, anti-CD138, and anti-CD3 antibodies (BioLegend or BD Biosciences), or OVA labeled with Alexa Fluor 555 (Molecular Probes, Thermo Fisher Scientific). We also measured the number of iBALT structures as the total number of B220⁺ and CD21⁺ clusters in five random area (1 mm²) in the lung.

Statistical Analysis

All the experiments were repeated at least twice and representative results are shown, except in [Figure 1H](#), which shows an experiment that was performed once, but 50 mice were used for the preparation of the lamina propria macrophages. The statistical analyses were performed with the Mann-Whitney U test in the GraphPad Prism software. Differences between groups were considered statistically significant at $p < 0.05$ and are indicated with an asterisk.

Other experimental procedures are described in the [Supplemental Information](#).

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.immuni.2016.11.010>.

AUTHOR CONTRIBUTIONS

E.K., K. Ozasa, B.T., K. Ohata, C.X.K., T. Kanuma, T. Kusakabe, S.K., N.T., S.U., and M. Hayashi performed experiments and data analysis. E.K., M. Horie, and Y.M. established in vivo model. S.K., K. Kabashima, S.F.Z., Y.I., W.I., T. Kurosaki, T.N., and J.K. provided mice and gave advice for in vivo experiment. E.K., T.A., K. Kobiyama, C.C., and K.J.I. established and helped experiment design. E.K. and K.J.I. performed and oversaw research and wrote the manuscript.

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Supplemental Information

Inhaled Fine Particles Induce Alveolar Macrophage

Death and Interleukin-1 α Release to Promote Inducible

Bronchus-Associated Lymphoid Tissue Formation

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Supplemental Figures

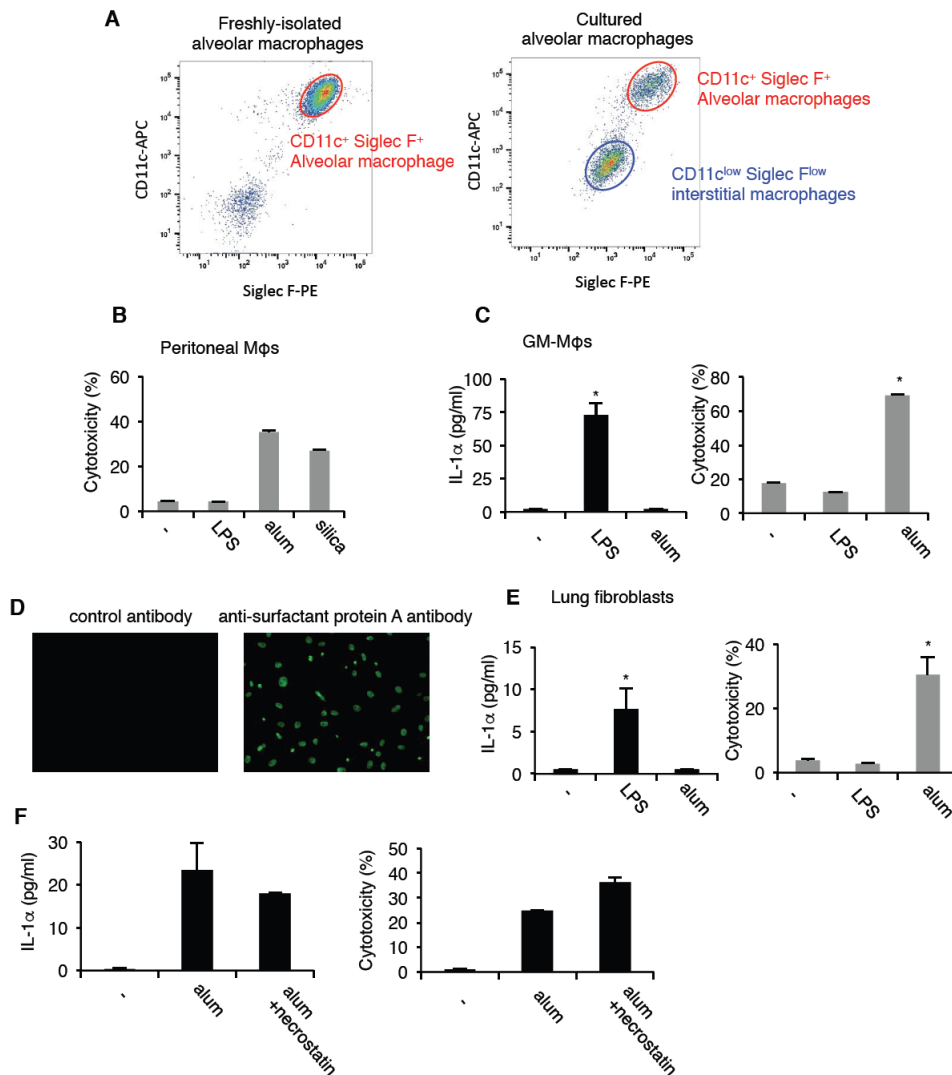


Figure S1 (related to Figure 1)

(A) Freshly isolated and cultured alveolar macrophages were obtained and stained with anti-CD11c and anti-Siglec-F antibodies. Cell-surface molecules were analyzed with flow cytometry (LSRII) (B and C) Thioglycolate-induced peritoneal macrophages and GM-CSF-derived macrophages were stimulated with LPS, alum or silica for 6 h. Supernatants were collected and analyzed for cytokines with ELISAs or for LDH release. (D) Lung fibroblasts were cultured on chamber slides (Mastunami, Osaka, Japan), fixed and permeabilized with the BD Cytotfix/Cytoperm™ Plus Fixation/Permeabilization Kit. Cells were stained overnight with anti-surfactant protein-A antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The cells were then stained with Alexa-Fluor-488-conjugated anti-rabbit IgG antibody (Cell Signaling Technology). (E) Lung fibroblasts were stimulated with LPS or alum for 6 h. Supernatants were collected and analyzed for cytokines with ELISAs or for LDH release. (F) Cultured alveolar macrophages were stimulated with alum in the presence or absence of necrostatin-1 (100 μM) for 6 hours. The culture supernatants were tested for IL-1α ELISA or for LDH release. DATA are shown as mean ± SD and represent at least two independent experiments (*p<0.05).

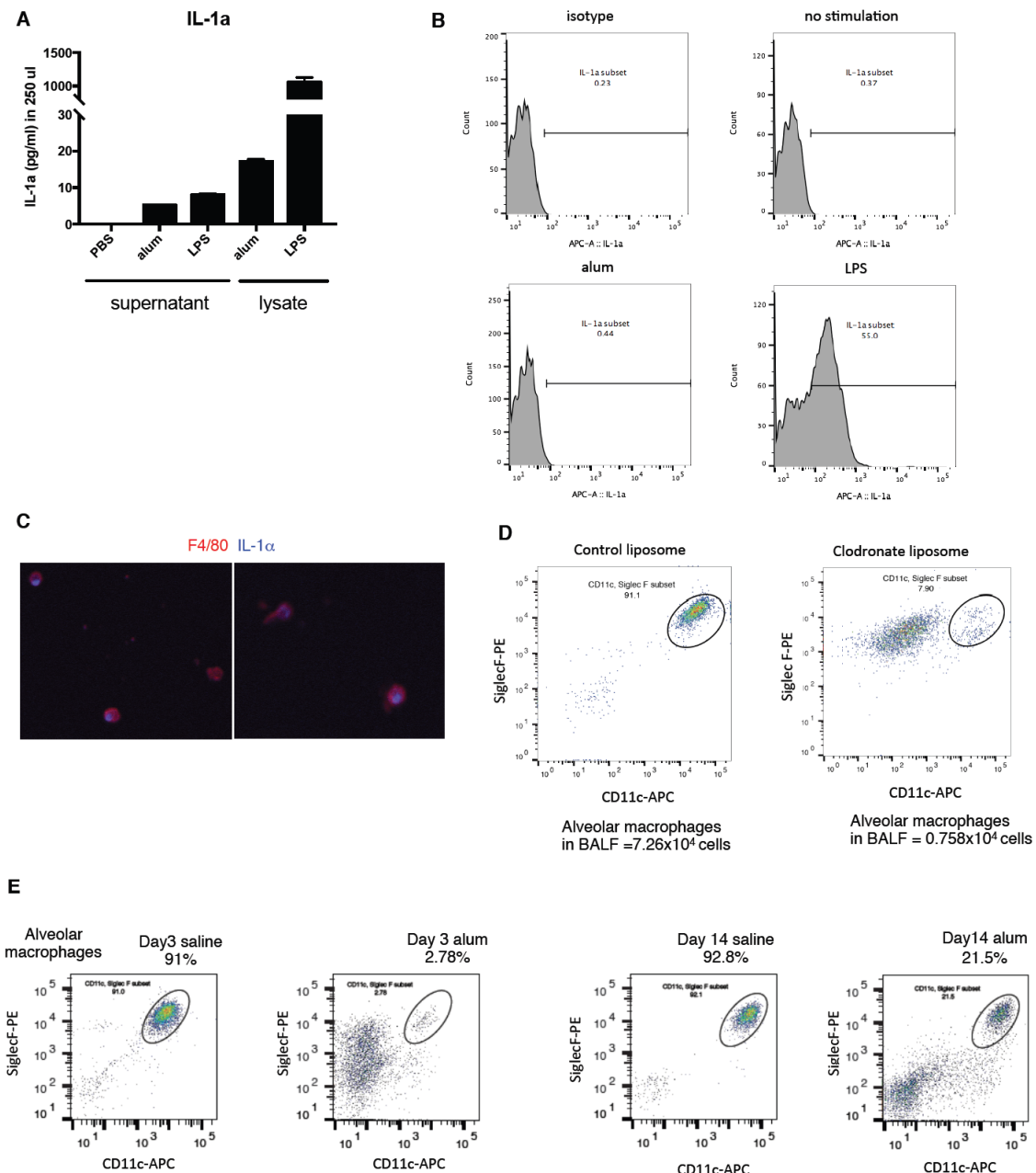


Figure S2 (related to Figure 2)

Analysis of intracellular IL-1 α in alveolar macrophages. (A) Cultured alveolar macrophages were stimulated with LPS or alum for 6 h. Supernatants were collected and analyzed for IL-1 α with an ELISA. Cell lysates were prepared with three freeze-thaw cycles and used for ELISAs. (B) Cultured alveolar macrophages were stimulated with or without alum or LPS for 6 h without brefeldin A. The cells were fixed and permeabilized with the BD Cytfix/Cytoperm™ Plus Fixation/Permeabilization Kit, stained with anti-F4/80 and anti-IL-1 α , and analyzed with flow cytometry. (C) Alum was administered by i.t. instillation. Three days later, BALF was harvested and BAL cells were cultured on chamber slides as in Figure S1D. Adherent cells were fixed, permeabilized and stained overnight with anti-IL-1 α and anti-F4/80 antibodies. Images were analyzed with confocal microscopy. (D) Clodronate liposomes (50 μ l) or control liposomes were administered to mice by i.t. instillation. BAL cells were isolated 3 days after i.t. instillation, and the alveolar macrophages (CD11c^{high} and siglec F^{high} cells) were analyzed with flow cytometry. (E) At 3 or 14 days after i.t. alum or saline administration, the BALF was harvested and alveolar macrophages (CD11c^{high} and siglec F^{high} cells) were analyzed. DATA represent at least two independent experiments.

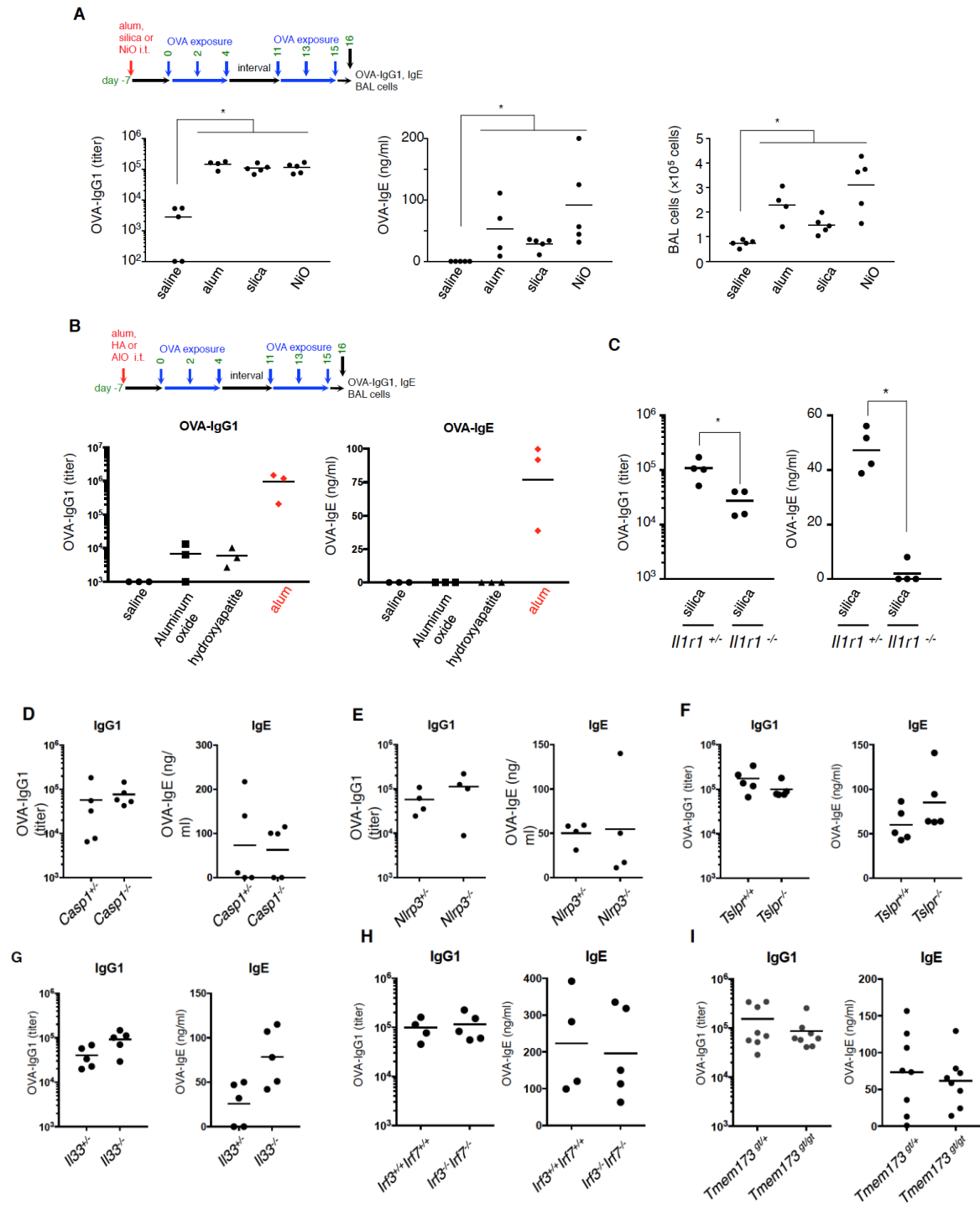


Figure S3 (related to Figure 3)

(A) Saline, alum (100 μ g), silica (250 μ g), or NiO (100 μ g) was administered to mice by i.t. instillation (n=5 mice, each group). Seven days later, the mice were exposed to aerosolized OVA, as in figure 3A. The sera were used for OVA-specific IgG1 and IgE ELISAs. (B) Saline, aluminum oxide (100 μ g) or hydroxyapatite (100 μ g) was administered to the mice by i.t. instillation (n=3 mice, each group), and the mice were then exposed to aerosolized OVA, as in figure 3A. (C) Silica was administered to *Il1r1*^{-/-} mice by i.t. instillation (n=4 mice, each group). Seven days later, the mice were exposed to aerosolized OVA, as in figure 3A. Alum was administered to *Caspr1*^{-/-} (D), *Nlrp3*^{-/-} (E), *Tslpr*^{-/-} (F), *Il33*^{-/-} (G), *Ir33*^{-/-} *Ir7*^{-/-} (H) and STING mutant (*Tmem173*^{gt/gt}) mice (I) by i.t. instillation (n=4-8 mice, each group). Seven days after administration, the mice were exposed to aerosolized OVA, as in figure 3A. DATA represent at least two independent experiments (*p<0.05).

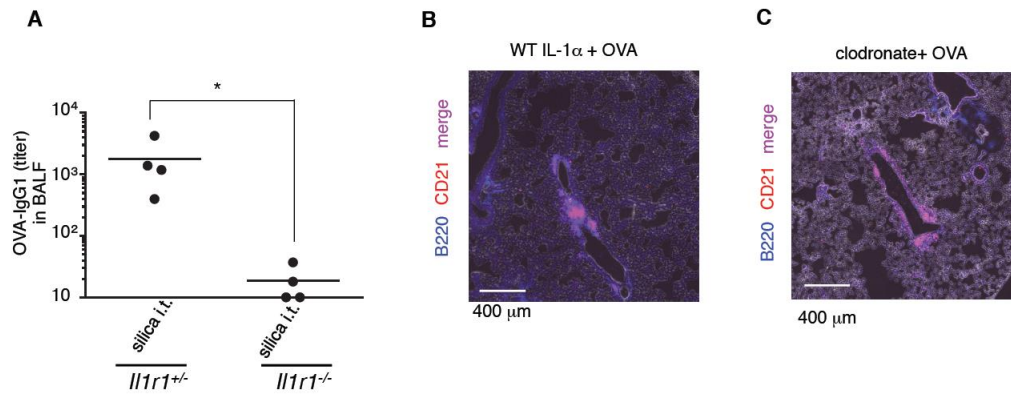


Figure S4 (related to Figure 4)

(A) Silica was administered to mice by i.t. instillation (n=4 mice, each group). Seven days after its administration, the mice were exposed to aerosolized OVA as in Figure 3A. Twenty-four hours after the last exposure, BALF was harvested and analyzed for OVA-specific IgG1 levels. (B) OVA or IL-1 α +OVA was administered to mice by i.t. instillation and the mice were exposed to aerosolized OVA as in Figure 4H and I. At 24 h after the last exposure, the lung sections were analyzed for iBALT formation with confocal microscopy. (C) Clodronate liposomes were administered to mice by i.t. instillation. Three days after their administration, the mice were exposed to aerosolized OVA as in Figure 4J and K. Lung sections were analyzed for iBALT formation. Bars, 400 μ m. The data represent at least two independent experiments (*p<0.05).

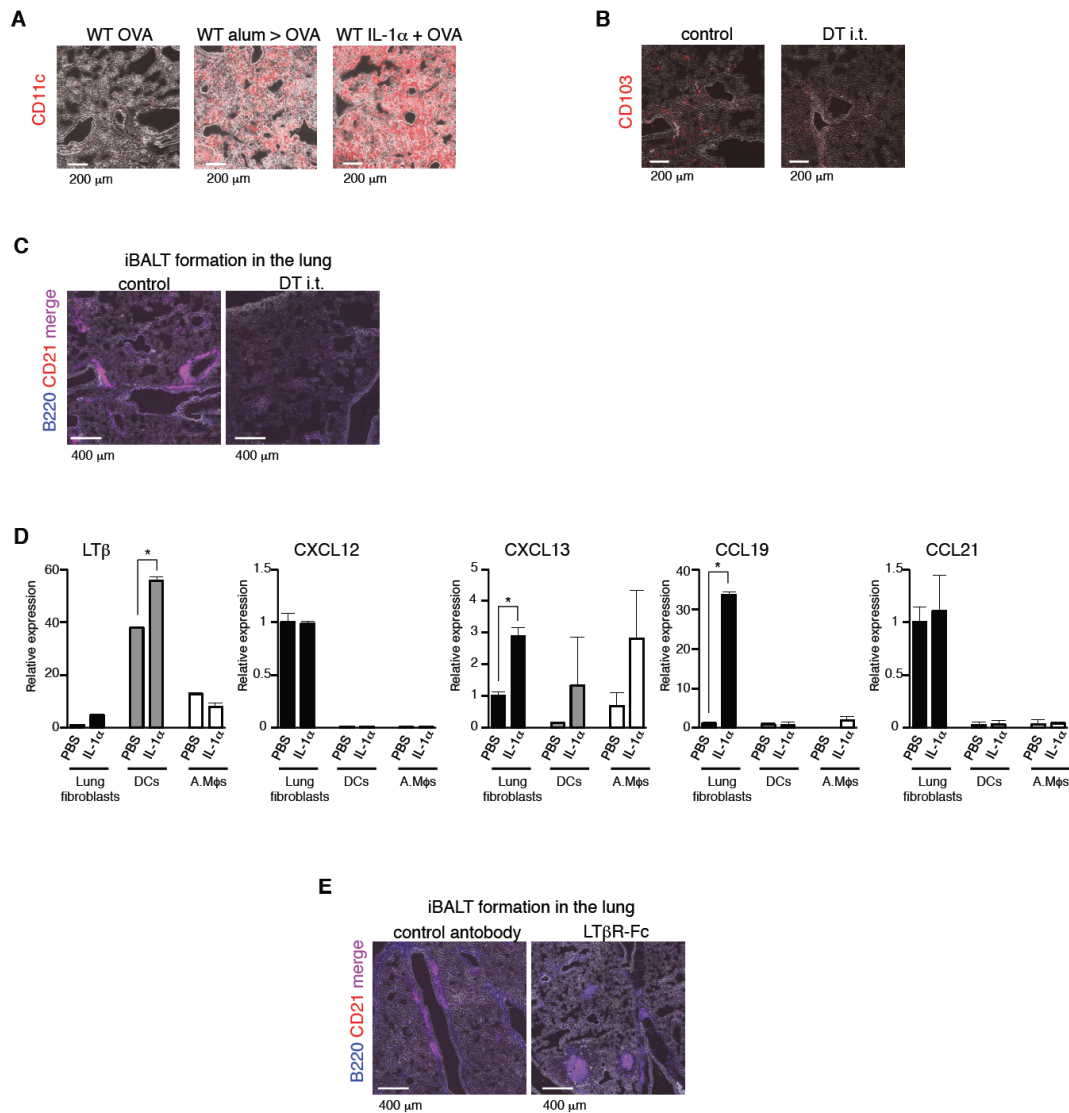


Figure S5 (related to Figure 5)

(A) Alum or OVA+IL-1 α was administered by i.t. instillation. At 3, 7 or 14 days after its administration, the mice were exposed to aerosolized OVA. Frozen sections of isolated lung specimens were stained for CD11c, and analyzed with confocal microscopy. Bars, 200 μ m. (B and C) CD11c-DTR mice were administered i.t. alum, and were exposed to aerosolized OVA 7 days later. DT was administered by i.t. instillation 1 day before the last OVA exposure as in figure 5B. Four days after the last OVA exposure, frozen lung sections were prepared, and stained with anti-CD103 antibody to detect lung DCs (B) or with anti-B220 and anti-CD21 antibodies to detect iBALT formation (C). Bars, 200 μ m in A and B, 400 μ m in C. (D) Lung fibroblasts, DCs, and alveolar macrophages (A.M ϕ s) were stimulated with IL-1 α for 6 h in vitro, and the relative expression of LT β and homeostatic chemokines were analyzed with quantitative RT-PCR. (E) Alum was administered by the i.t. instillation, and the mice were exposed to aerosolized OVA 7 days after its administration. LT β R-Fc was administered twice by the i.p. route as in figure 5C. Frozen lung sections were prepared, and stained with anti-B220 and anti-CD21 antibodies to detect iBALT formation. Bars, 400 μ m. DATA are shown as mean $\pm$ SD and represent at least two independent experiments (*p<0.05).

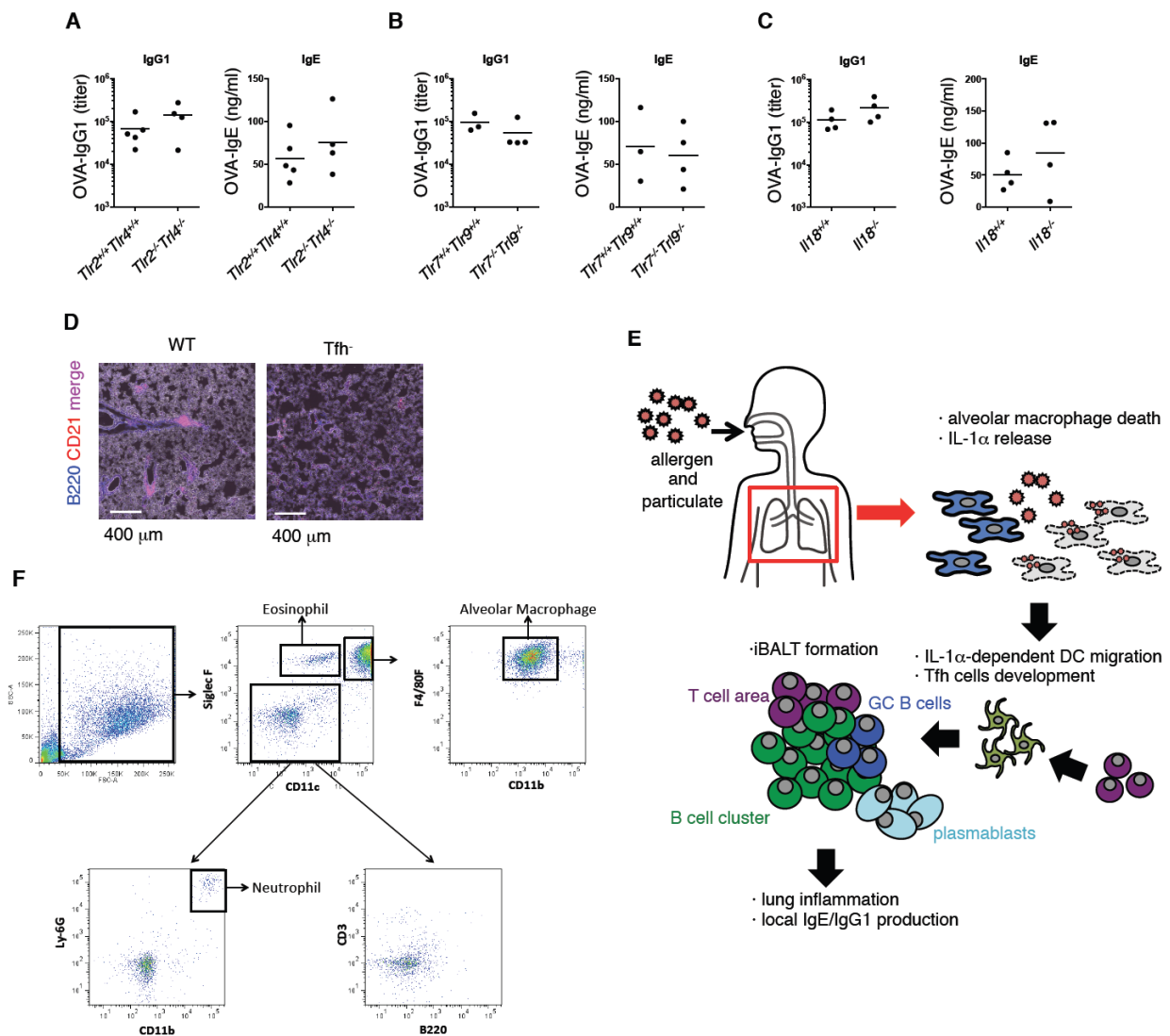


Figure S6 (relate to Figure 6, Discussion and Methods)

Alum was administered to *Tlr2*^{-/-}*Tlr4*^{-/-} (A), *Tlr7*^{-/-}*Tlr9*^{-/-} (B) and *Il18*^{-/-} mice (C) by i.t. instillation (n=3-4 mice, each group). Seven days after its administration, the mice were exposed to aerosolized OVA, as in Figure 3A. Twenty-four hours after the last exposure, the sera were collected and analyzed with OVA-specific IgG1 and IgE ELISAs. (D) *Cd4-cre Bcl6*^{fllox/fllox} mice (*Tfh*⁻) were administered i.t. alum, and were then exposed to aerosolized OVA as in Figure 6G and H. At 24 h after the last exposure, lung sections were analyzed for iBALT formation in lung sections with confocal microscopy. The data represent at least two independent experiments.

(E) The model of particulate-induced lung inflammation. Inhaled fine particulates engulfed by alveolar macrophages induce macrophage death. IL-1 α is released as a DAMP and contributes to DC recruitment into the lungs. Activated DCs triggers iBALT formation through the development of Tfh cells. Mature iBALT functions as a tertiary lymphoid organ, which contains a germinal center and areas of T-cell, generates short-lived plasmablasts, and is involved in local and systemic IgE responses. (F) Flow-cytometric gating strategy for the analysis of BAL cells.

Supplemental Experimental Procedures

Animals

Il18^{-/-} mice were kindly donated by Dr. Nakanishi (Hyogo College of Medicine, Nishinomiya, Hyogo). *Il33*^{-/-} mice were obtained as described previously (Haenuki et al., 2012; Yasuda et al., 2012). *Tslpr*^{-/-} mice were kindly donated by Dr. Ziegler (Benaroya Research Institute, Seattle, USA) (Zhou et al., 2005). *Myd88*^{-/-}, *Tlr2*^{-/-}*Tlr4*^{-/-} and *Tlr7*^{-/-}*Tlr9*^{-/-} mice were obtained from Oriental BioService Inc (Kyoto, Japan). *Il1r1*^{-/-} mice were obtained as described previously (Hayashi et al., 2016). STING mutant mice (*Tmem173*^{gt/gt}) were obtained from Jackson Laboratories (Bar Harbor, ME, USA). *Asc*^{-/-}, *Nlrp3*^{-/-} and *Casp1*^{-/-} mice were obtained as described previously (Mariathasan et al., 2004; Mariathasan et al., 2006). *Irf3*^{-/-}*Irf7*^{-/-} mice were derived from *Irf3*^{-/-} (Tang et al., 2013) and *Irf7*^{-/-} mice provided by the RIKEN BRC (Ibaraki, Japan) through the National Bio-Resource Project of the Japanese Ministry of Education, Culture, Sports, Science and Technology (Honda et al., 2005). *Il1a*^{-/-} mice were obtained from the Center for Animal Disease Models, Tokyo University of Science (Chiba, Japan) (Horai et al., 1998). *Cd4-cre Bcl6*^{fllox/fllox} mice were kindly donated by Dr. Kurosaki (Osaka University, Osaka, Japan) (Ise et al., 2014; Onishi et al., 2015). All but the CD11c-DTR mice were bred in a C57BL/6 background. The CD11c-DTR mice (BALB/c background) kindly donated by Dr. Kunisawa (National Institutes of Biomedical Innovation, Health and Nutrition, Osaka) (Obata et al., 2013). To prepare the CD11c-DTR chimeric mice, BM cells were prepared from the femurs and tibias of the CD11c-DTR transgenic mice (12 weeks, old male) and used as the donor cells. Donor cells (1×10^7 cells) were intravenously injected into BALB/c mouse (6 weeks old, male) 6 h after their irradiation with 7 Gy X-irradiation with an MBR-1520R-3 device (Hitachi). The BM chimeric mice were used for experiments at least 8 weeks after transplantation. All the animal experiments were performed according to the Guidelines for the Care and Use of Animals approved by Osaka University and the National Institutes of Biomedical

Reagents

Alhydrogel (alum) was purchased from Invivogen (San Diego, CA, USA). Silica crystals (Min-u-sil 5 silica) were purchased from U.S. Silica (Berkeley Springs, WV, USA). NiO was purchased from Vacuum Metallurgical Co., Ltd (Chiba, Japan). Al₂O₃ (particle size: 40-50 nm) and TiO₂ were purchased from Wako Pure Chemical Industries (Osaka, Japan). Hydroxyapatite (particle size: 59 nm) was purchased from SofSera (Tokyo, Japan). OVA was purchased from Kanto Chemical Co. Inc. (Osaka, Japan). All particulates and OVA used for the in vivo experiments were prepared in endotoxin-free solution (< 1EU/ml [10 mg]). Endotoxin levels were determined with Toxicolor® (Seikagaku Corp., Tokyo, Japan). Poly-2-vinylpyridine N-oxide (PVNO) was purchased from Polysciences (Warrington, PA, USA). House-dust-mite allergen (HDM) was purchased from Cosmo Bio (Tokyo, Japan). GSK'872 was purchased from BioVision (Milpitas, CA, USA). Clodronate and empty liposomes (Clophosome-A anionic) were purchased from FormuMax Scientific (Palo Alto, CA, USA). Soluble LTβR-Fc for blocking LTβR signaling was kindly donated by Dr. Kunisawa (National Institutes of Biomedical Innovation, Health and Nutrition, Osaka).

Cell preparation

The preparation of peritoneal macrophages and GM-CSF-derived BM macrophages has been described previously (Kuroda et al., 2011). Cultured alveolar macrophages were prepared with the method used to prepare Kupffer cells, described previously (Kitani et al., 2010). The lungs were isolated and suspended in 5 ml of RPMI1640 containing 200 µg/ml DNase I and 1.5 U/ml Liberase DL Research Grade (Roche Diagnostics GmbH, Indianapolis, IN, USA). The lungs were diced with scissors, incubated at 37°C for 1 h and homogenized by a gentle MACS™ Dissociator (program

B.01) (Miltenyi Biotech, Gladbach, Germany). The cell suspensions were washed twice with PBS and passed through 30 μm preseparation filters (Miltenyi Biotech). The cells, which contain both alveolar and interstitial macrophages, were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 5 $\mu\text{g/ml}$ insulin, but without antibiotics, and seeded into 175 cm^2 culture flasks (Corning, New York, NY, USA). The medium was replaced after 24 h, and then every 2 days. Fibroblastic cells grew and after 7 days in culture, macrophages (both alveolar and interstitial macrophages) proliferated on the cell sheet. Macrophages were easily detached with reciprocal shaking (170 strokes/min) for 2 h at 37 $^{\circ}\text{C}$. Floating cells were washed with PBS and suspended in RPMI 1640 supplemented with 10% FCS and antibiotics. Cultured macrophages were > 95% F4/80⁺ as determined with flow cytometry. A comparison of surface-marker expression on freshly isolated alveolar macrophages and cultured alveolar macrophages is described in Figure S1A. We prepared a single cell suspension of the lungs from five mice and the cells were divided into two 175 cm^2 culture flasks. We obtained more than 1×10^7 macrophages from two flasks. The adherent fibroblastic cells were positive for surfactant protein A, determined with immunohistochemistry, and were used as lung fibroblasts (Figure S1D). In some experiments, obtained F4/80⁺ cells were further separated into CD11c⁺ siglec F⁺ and CD11c^{low} siglec F^{low} cells with a BD InfluxTM cell sorter (BD Biosciences, Franklin Lake, NJ, USA), and used for in vitro culture.

The isolation of lamina propria macrophages has been previously described (Uematsu et al., 2008). Briefly, segments of the small intestine, from which Peyer's patches and epithelial cells were removed, were digested with collagenase D and DNase I. Single-cell suspensions were prepared with density gradient centrifugation, and stained with anti-CD11b (M170), anti-CD11c (HL3), anti-CD103 (M290) and anti-CX3CR1 antibodies. Small-intestinal lamina propria macrophages were sorted as CD11c^{int}, CD11b^{int}, CX3CR1⁺, CD103⁻ cells. This experiment was

performed once, but 50 mice were used to prepare the lamina propria macrophages.

In vitro culture

Freshly isolated or cultured alveolar macrophages were cultured at $1 \times 10^6/\text{ml}$ in 48- or 96-well plates (BD Biosciences). The cells were stimulated with 100 $\mu\text{g}/\text{ml}$ particulates (silica, alum, aluminum oxide or hydroxyapatite) for 6~8 h. LPS (1 $\mu\text{g}/\text{ml}$) stimulation was used as the positive control. In some experiments, an inhibitor (5 μM GSK'872 or 20 $\mu\text{g}/\text{ml}$ PVNO) was added to alveolar macrophages together with silica or alum. After stimulation, the cell-free supernatants were collected and used for ELISAs or a cytotoxicity assay. In some experiments, cell lysates were prepared with three freeze-thaw cycles and used for the IL-1 α ELISA (BioLegend, San Diego, CA, USA).

In vivo experiments

In the experiment shown in Figure 3D, HDM was used as the antigen instead of OVA. The mice were administered i.t. alum, and 7 days later, they were sensitized with HDM (10 μg) by the i.n. route on days 0, 2, 9, and 11. On day 12, the sera and BALF (bronchoalveolar lavage fluid) were collected.

Experiment shown in Figure 2C (PVNO treatment): We administered 50 μl of silica or silica mixed with PVNO (100 μg) to mice by i.t. instillation. Three days after i.t. instillation, the BALF was harvested and analyzed for the number of BAL cells and released DNA and IL-1 α in the BALF.

Experiment shown in Figures 2D-G: 50 μl of particulates, clodronate liposomes or empty liposomes was administered by i.t. instillation. BALF was collected 1, 3, 7 or 14 days after i.t. instillation of the particulate or liposomes. BALF was tested to measure DNA or cytokines. BAL

cells were counted and stained with anti-CD11c and anti-Siglec F antibodies. The number of alveolar macrophages was examined with flow cytometry. For the histological analysis in Figure 2H, I and J, lungs were isolated 14 days after i.t. instillation of alum, and used for H&E or iBALT staining.

Experiment shown in Figure 3B: Alum (1 mg in 200 μ l) was injected i.p. on day -7. OVA (10 μ g in 200 μ l) was injected i.p. on days 0 and 7 (alum $\rightarrow$ OVA $\rightarrow$ OVA). Injection of OVA alone (OVA $\rightarrow$ OVA) or alum mixed with OVA (alum+OVA $\rightarrow$ OVA) were used as the controls. On day 17, sera were collected and analyzed for OVA-specific antibodies with ELISA.

Experiment shown in Figure 4H and I: 50 μ l of saline, OVA (10 μ g) or OVA (10 μ g)+IL-1 α (10 ng) was administered by i.t. instillation on days 0. Mice were then exposed to OVA for six times (day 7, 9, 11, 18, 20, and 22). Sera were collected 24 h after the last exposure and tested for serum antibody responses. Lungs were isolated and used for iBALT staining (B220 and CD21).

Experiment shown in Figure 4J and K: 50 μ l of empty liposomes or clodronate liposomes were administered by i.t. instillation. Three days after i.t. instillation, the mice were exposed to aerosolized OVA on days 0, 2, and 4. After 7 days, the mice were exposed to OVA again three times every 2 days (on days 11, 13, and 15). Sera were collected 24 h after the last exposure and tested for serum antibody responses. Lungs were isolated and used for iBALT staining (B220 and CD21).

Experiment shown in Figure 5B: Chimeric CD11c-DTR mice were administered i.t. alum and exposed to aerosolized OVA, as in Figure 3A. On day 16, 50 ng of DT was administered by i.t. instillation, and the mice were then exposure to OVA once again (on day 17). Sera were collected 24 h after the last exposure and tested for serum antibody responses. Lungs were isolated and used for iBALT staining (B220 and CD21). A brief experimental design is shown in Figure 5B.

Experiment shown in Figure 5C: Mice were administered i.t. alum, and were exposed to

aerosol OVA as in Figure 3A. On day 0 and 11, 100µg of LTβR-Fc was administered by i.p. Sera were collected 24 h after the last exposure and tested for serum antibody responses. Lungs were isolated and used for iBALT staining (B220 and CD21). A brief experimental design is shown in Figure 5C.

To test the different routes of immunization (Figure 6C and D), we administered OVA (10 µg) adsorbed onto alum (100 µg) by intraperitoneal (i.p.) injection (200 µl). For intranasal (i.n.) injection, 30 µl of saline, OVA (50 µg) adsorbed onto alum (100 µg) was administered on days 0 and 7. The mice were sensitized with OVA (50 µg) by the i.n. route on days 14, 16, and 18. On day 19, sera and BALF were collected and analyzed for OVA-specific antibodies with an ELISA. A brief experimental design is shown in Figure 6C and D.

Experiment shown in Figure 6F, Mice were immunized with OVA (10 µg) adsorbed onto alum (100 µg) on day 0 by the i.p. (200 µl) or i.t. (50 µl) route. The mice were then exposed to OVA for six times (days 7, 9, 11, 18, 20, and 22). The mediastinal lymph nodes were isolated 24 h after the last exposure. The cells were cultured at 1×10^5 cells/well (200 µl) in 96-well plates (BD Biosciences). They were stimulated with or without OVA (100 µg/ml) for 5 days. The cell-free supernatants were collected and analyzed for cytokines with ELISAs.

Collection and analysis of BALF

BALF was collected by washing the lungs. Briefly, a plastic cannula (Terumo, Tokyo, Japan) was inserted into the incised trachea to flush the lungs twice with 550 µl of phosphate-buffered saline (PBS). The total volume recovered was about 1 ml. The BALF obtained was centrifuged at 300×g for 10 min to separate the cells and fluids. For the analysis of inflammatory cells in BALF in Figure 3C, the cells were stained with anti-CD11c, anti-CD11b, anti-Siglec F, anti-F4/80, anti-Ly6G, anti-CD3 and anti-B220 antibodies, and then analyzed by flow cytometry (LSRII; BD Biosciences).

The gating strategy is described in Figure S6F. All labeled antibodies were purchased from BD Biosciences or BioLegend.

Double-stranded DNA in BALF was measured with the Quant-iT™ Picogreen® ds DNA Assay Kit (Invitrogen, San Diego, CA, USA).

Histological analysis

To stain alveolar macrophages, the tissue samples were stained overnight at 4 °C with anti-F4/80 and anti-IL-1 α antibodies (BioLegend and Miltenyi Biotech). Images were analyzed with a FluoView (FV10i) confocal microscope (Olympus, Tokyo, Japan). To stain lung DCs, we used anti-CD11c and anti-CD103 antibodies to distinguish them from CD11c⁺ alveolar macrophages.

In some experiment, lungs were inflated with and fixed in 4% paraformaldehyde. To prepare paraffin sections, the tissues were gradually dehydrated and embedded in paraffin. The sections (5 μ m) were prepared and stained with H&E or PAS using the Periodic Acid-Schiff Kit (Sigma Aldrich, St. Louis, MO, USA). Representative images were acquired with a ScanScope® AT slide scanner (Leica, Germany).

ELISAs and cytotoxicity assay

Cytokines were measured using cytokine ELISA kits (R&D Systems, Minneapolis, MN, USA or BioLegend) according to the manufacturer's instructions. Serum OVA-IgE levels were determined with a DS Mouse IgE ELISA (OVA) kit (DS Pharma Biochemical, Osaka, Japan). For the OVA-specific IgG1 analysis, serial dilutions of sera were prepared in 96-well plates coated with 10 μ g/ml OVA or HDM. Horseradish-peroxidase-conjugated goat anti-mouse IgG1 antibody (Bethyl

Laboratories, Montgomery, TX, USA) was used as the secondary antibody. The reciprocal value of the serum dilution with an optical density at 450 nm (OD₄₅₀) of 0.5 was defined as the titer of the antigen-specific serum IgG1 or IgG2c. Cytotoxicity was measured by quantifying the release of lactate dehydrogenase (LDH) using the Cytotox 96® Non-Radioactive Cytotoxicity Assay from Promega (Madison, WI, USA).

Quantitative-PCR (qPCR) analysis

CD11c⁺-sorted GM-CSF-derived BM cells were used as DCs. Lung fibroblasts, DCs, and alveolar macrophages were stimulated with IL-1 α for 6 h. Total RNA was isolated with the SV Total RNA Isolation System (Promega), according to the manufacturer's instructions. RNA was reverse transcribed with ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan), according to the manufacturer's instructions. For qPCR, cDNA fragments were amplified with THUNDERBIRD Probe qPCR Mix (Toyobo), according to the manufacturer's instructions. The fluorescent TaqMan probe was detected with a StepOnePlus™ Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). The expression of *Cxcl12*, *Cxcl13*, *Ccl19*, *Ccl21b*, and *LTb* mRNAs was normalized to that of 18S rRNA. The experiments were repeated twice, with similar results.

Supplemental References

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