obtained similar results with intramuscular (i.m.) immunization of these mice, with respect to total IgG titer and IgG2a titer (fig. S1). In contrast to i.n. immunization, i.m. immunization failed to elicit BALF IgA in either wild-type or TLR7-deficient mice. There was an enhanced serum IgG1 response in TLR7-deficient mice relative to wild-type mice, but this response failed to protect the TLR7-deficient mice from lethal challenge with PR H1N1 (fig. S1, A and C). Thus, the inactivated WV vaccine requires TLR7-mediated, but not RIG-I–mediated, IPS-1 for its immunogenicity, which is consistent with findings for the live virus vaccination (*13*).

We also immunized ASC-deficient mice with WV (NC H1N1) because a role for the ASC inflammasome in the adaptive immune response to influenza virus infection is controversial (*14*, *19*). ASC-deficient mice did not show any defects in their adaptive immune responses to WV immunization relative to wild-type mice (Fig. 1, D and E). Notably, ASC was not involved in the immunogenicity of live virus immunization, except for serum IgG1 production, whereas $CD8^+$ T cell responses were not observed with WV immunization (fig. S2). Immunized wild-type and ASC-deficient mice were similarly protected against lethal PR H1N1 virus challenge relative to naïve wild-type mice (Fig. 1F). Therefore, ASC inflammasome activation was not essential for the induction of virus-specific B and CD4 T cell responses to WV.

The type I IFN receptor is important for the immunogenic response to inactivated WV vaccine but not to the live virus

Because both the inactivated WV and the live virus require TLR7 for their immunogenicity, we sought to identify the downstream effector molecule(s) involved. Type I IFNs, such as IFN-α and IFN-β, are known to have potent adjuvant activity (*20*, *21*), and a recent study showed that WV immunization substantially up-regulated the expression of IFN-inducible genes, such as *Cxcl10* (*22*). Therefore, as a systemic indicator of type I IFN responses, we examined the amounts of the cytokine CXCL10 in the sera of mice 24 hours after vaccination. The induction of CXCL10 was significantly reduced in TLR7-deficient mice relative to wild-type, IPS-1–deficient, and ASC-deficient mice (Fig. 2A). Similar results were obtained for messenger RNA (mRNA) analyses of IFN-β and CXCL10 in the lung (fig. S3A), suggesting that type I IFNs might be dominant effector molecules in this TLR7-dependent system. To test this hypothesis directly, we i.n. immunized mice deficient in the IFN-α and IFN-β receptor 2 (IFNAR2) with WV as in Fig. 1. IFNAR2-deficient mice failed to induce virus-specific antibodies (including BALF IgA and serum IgG) and $CD4^+$ T cell responses (Fig. 2B and fig. S3, B and C) relative to the heterozygous IFNAR2 knockout mice. As a result, mortality was increased and a significantly larger body weight loss was observed after lethal PR H1N1 challenge in the IFNAR2-deficient mice (Fig. 2C). Naïve wild-type and IFNAR2-deficient mice showed similar susceptibilities to PR H1N1 challenge, consistent with a previous study (*23*). Similar results were also observed after i.m. immunization (fig. S3, D and E). When naïve IFNAR2-deficient mice were immunized with live virus, there was no alteration of the virus-specific serum IgG and IFN-γ secretion by virus NP antigen-specific $CD4^+$ T cells ($NP_{260–283}$ specific to $I\text{-}A^b$) and $CD8^+$ T cells ($NP_{366–374}$ specific to $H\text{-}2D^b$) (fig. S3, F to I), consistent with a previous study (*23*). Together, these results suggest that TLR7, but not RLRs or NLRs, is required for immunogenicity of inactivated as well as live influenza virus vaccination. In addition, the type I IFN receptor–mediated signaling pathway was critical for the immunogenic response to WV but not to the live virus.

Live virus and inactivated WV vaccines induce type I IFNs through distinct DC types

DCs are a critical component of the innate immune system that recognize vaccines and mediate the adaptive immune response. There are different subtypes of DCs that can be divided loosely into conventional myeloid DCs (mDCs) and plasmacytoid DCs (pDCs), which express different receptors and secrete different cytokines on activation. To characterize in more detail the immune response triggered by the WV vaccine and live virus, we stimulated two types of bone marrow–derived DCs—Flt3

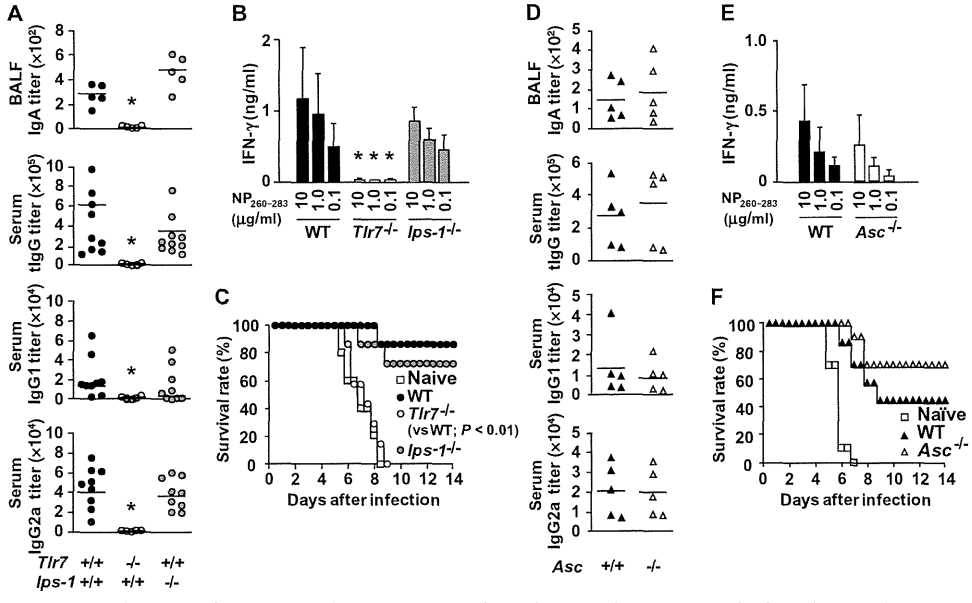

Fig. 1. TLR7-dependent, but not IPS-1–dependent or ASC-dependent, signaling is required for the induction of protective immune responses by inactivated WV. (**A** to **C**) Wild-type (WT), *Tlr7*-deficient, and *Ips-1*–deficient mice ($n = 9$ per group) were i.n. vaccinated with WV of NC (1.5 μg per mouse) twice, with a 2-week interval. One week after the second vaccination, we measured titers of antigen-specific mucosal (BALF) IgA and serum total IgG (tIgG), IgG1, and IgG2a (A) and IFN-γ production by $CD4^+$ T cells (B) by ELISA as described in Materials and Methods. *$P < 0.05$ versus WT mice. (C) Vaccinated mice ($n = 9$ per group) were challenged with $10 \times LD_{50}$ (median lethal dose) (2×10^4 PFU per mouse) of lethal influenza virus PR, and their survival was monitored. *$P < 0.05$ versus vaccinated WT mice. (**D** to **F**) WT and *Asc*-deficient mice ($n = 5$, each group) were similarly vaccinated, and their antigen-specific antibody responses (D), IFN-γ production by $CD4^+$ T cells (E), and survival (WT, $n = 7$; *Asc*-deficient, $n = 10$; naïve, $n = 10$) (F) were determined by ELISA as described in Materials and Methods. Each bar represents the mean (A and D) or mean ± SD (B and E). These results are representative of two independent experiments.

ligand–generated DCs (FL-DCs; which contain pDCs) and granulocyte-macrophage colony-stimulating factor (GM-CSF)–generated DCs (GM-DCs; which contain conventional DCs but no pDCs)—with the live virus or WV and then measured their IFN-β production by enzyme-linked immunosorbent assay (ELISA). The live virus strongly stimulated FL-DCs to produce IFN-β in a TLR7-dependent manner, whereas production by GM-DCs was in a TLR7-independent manner (Fig. 2D), consistent with our previous study (*13*). In contrast, WV-activated FL-DCs, but not GM-DCs, produced IFN-β, a process entirely dependent on TLR7 signaling (Fig. 2D), indicating that there is a clear distinction between the live virus and WV in terms of their abilities to activate DCs to secrete type I IFNs.

To confirm these in vitro observations in vivo, we next depleted pDCs in vivo to examine the role of these cells in the induction of adaptive immune responses to live virus and WV, as pDCs are known to play a key role in bridging the innate and adaptive immune responses (*24*). Wild-type mice were treated intravenously with an antibody to mPDCA-1 (*25*) and then immunized 24 hours later with the live virus or WV. Depletion of pDCs was confirmed in the spleen (fig. S4). After live virus vaccination, the concentrations of mRNAs derived from genes involved in induction of the adaptive immune response, specifically *Ifnb*, *Cxcl10*, *Il6*, and *Ccl2*, were clearly elevated in the lung irrespective of treatment with antibody to mPDCA-1 (Fig. 2E). In contrast, these transcriptional responses were severely impaired after WV vaccination in pDC-depleted mice relative to isotype control antibody–treated mice (Fig. 2E). Serum CXCL10 was also reduced in pDC-depleted mice treated with WV but not the live virus (Fig. 2F). Thus, both live virus and WV induced type I IFNs predominantly through pDCs in vivo; however, WV was dominantly recognized by pDCs, whereas live virus could also stimulate other cell types to activate innate immune responses.

Although it was previously reported that pDC activation is not essential for the induction of adaptive immune responses in live influenza virus infection (*26*, *27*), pDC depletion specifically rendered the inactivated WV nonimmunogenic, as measured by virus-specific IgG concentrations in serum (Fig. 3A). In these experiments, mice were treated with an antibody to mPDCA-1 twice at both the primary and the secondary vaccinations. To further examine the more detailed role of pDCs in the primary and/or secondary vaccinations, we treated mice with an antibody to mPDCA-1 at either the primary or the secondary (boost) vaccination. Virus-specific mucosal IgA, serum IgG (Fig. 3B), and $CD4^+$ T cell IFN-γ (Fig. 3C) were significantly impaired when pDCs were depleted in the primary, but not in the secondary, vaccination with the inactivated WV. Thus, pDC activation is essential for inducing B cell and $CD4^+$ T cell responses to the inactivated WV during primary, but not secondary, vaccination. By sharp contrast, pDC activation at priming was not required for inducing B cell and $CD4^+$ T cell responses with the live virus.

WV-loaded pDCs were sufficient to transfer immunogenicity to naïve mice, which requires intrinsic as well as extrinsic type I IFN signaling

To further examine the role of pDCs, we performed cell transfer experiments. FL-DCs from wild-type mice were separated into two populations, namely, a B220 (CD45R)–enriched population containing pDCs and a B220-depleted population containing virtually no pDCs (as indicated in Fig. 3D). The cell populations were pulsed with WV and injected intravenously into wild-type mice. The virus-specific IgG concentrations elicited by the B220-enriched FL-DCs were significantly higher than those elicited by the B220-depleted population (Fig. 3D). In addition, when we transferred B220-enriched FL-DCs derived from the IFNAR2-deficient mice (lacking type I IFN) into wild-type mice or vice versa, virus-specific IgG induction was significantly impaired in both cases (Fig. 3E). We also confirmed that IFNAR2-deficient FL-DCs secreted significantly less type I IFN relative to that of FL-DCs from the heterozygous IFNAR2 knockout mice (fig. S5A). When we tested TLR7 deficiency with

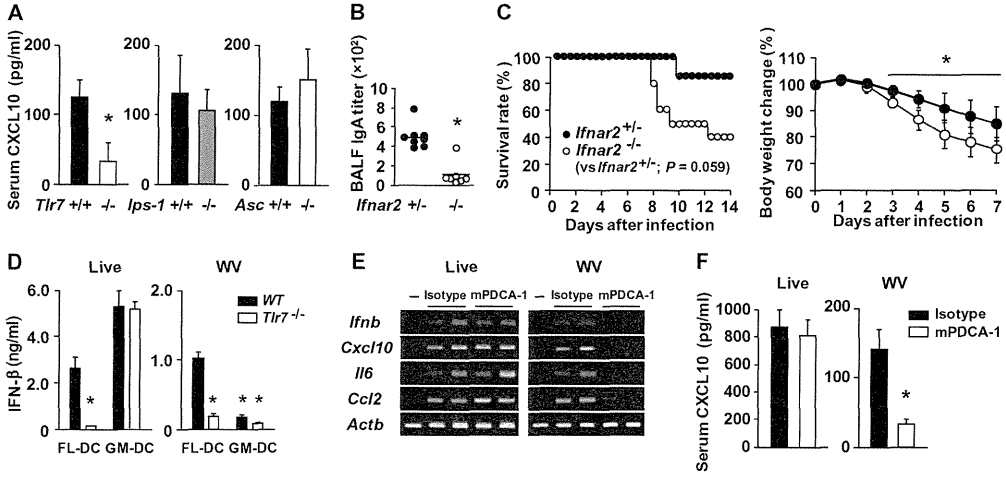

Fig. 2. Critical roles of type I IFN receptor–mediated signaling and pDC activation in inducing adaptive immune responses to the inactivated WV vaccine but not to the live virus. (**A**) *Tlr7*-deficient, *Ips-1*–deficient, *Asc*-deficient, and control mice ($n = 3$ per group) were i.n. vaccinated with WV (3.0 μg per mouse), and CXCL10 production in sera was measured by ELISA after 24 hours. *$P < 0.05$ versus control mice. (**B** and **C**) Control ($Ifnar2^{+/-}$) and type I IFN receptor–deficient ($Ifnar2^{-/-}$) mice ($n = 8$, each group) were i.n. vaccinated with WV (1.5 μg per mouse) as in Fig. 1, and BALF IgA (B) was measured by ELISA. *$P < 0.05$ versus control mice. The mice were then infected with lethal influenza PR at $10 \times LD_{50}$, and their survival and body weight (C) were monitored. *$P < 0.05$ versus control mice. (**D**) Type I IFN production by FL-DCs and GM-DCs from WT and *Tlr7*-deficient mice in response to the live virus [multiplicity of infection (MOI) = 10] and WV (5 μg/ml). IFN-β production was measured by ELISA 24 hours after the stimulation. *$P < 0.05$ versus WT FL-DC. (**E** and **F**) Innate immune responses to WV in pDC-depleted mice. Mice were treated with an antibody to mPDCA-1 24 hours before inoculation and then i.n. challenged with the live virus (1×10^5 PFU per mouse) or WV vaccine (5 μg per mouse). The expression of IFN-β, CXCL10, IL-6, and CCL2 mRNA in the lungs (E) and CXCL10 in sera (F) 24 hours after vaccination was measured by RT-PCR and ELISA. *$P < 0.05$ versus control mice. These results are representative of at least two independent experiments.

the same approach, we observed a substantial TLR7 signaling dependency in these FL-DC transfer experiments (fig. S5B). These results suggest that type I IFN–mediated signaling in pDCs, as well as in the recipient as yet unidentified cell type(s), is indispensable for eliciting the adaptive immune response to WV.

Split vaccine does not protect naïve mice, but immunogenicity can be improved with a pDC-activating adjuvant while it recalls memory T cells in human adult blood

Currently, the most widely used influenza vaccines in many countries comprise SV or SU, which mainly consist of purified protein antigens such as HA and neuraminidase. As mentioned earlier, vaccination of mice with SV led to significantly lower production of type I IFNs and related chemokines, such as CXCL10, at both the mRNA and the protein levels in the lung and serum, respectively (Fig. 4, A and B). SV also failed to activate DCs to produce type I IFNs in vitro (fig. S6A). These data suggest that the intrinsic TLR7 ligand (that is, viral genomic RNA) was lost during the SV production process. In support of this idea, removal of the RNA content from WV by ribonuclease treatment significantly decreased the TLR7-mediated type I IFN production by pDCs (fig. S6B). The reduced immunostimulatory activity of SV was associated with its diminished immunogenicity. When naïve mice were immunized with SV at the same dose, adjusted to the HA content (fig. S6C), as WV, the HA-specific IgG and CD4 T cell responses were significantly lower than those elicited by WV (Fig. 4, C and D). Together, these data strongly suggest that SV loses its built-in TLR7 adjuvant (viral genome RNA) during purification of WV, consistent with a recent study for the H5N1 virus (*28*).

Our results thus far raise the possibility of improving SV immunogenicity by adding a pDC-activating TLR ligand. Because pDCs express both TLR7 and TLR9, we examined whether addition of a TLR9 ligand to the "adjuvant-lost" split vaccine would replace the natural TLR7-mediated pDC activation. We used a second-generation TLR9 ligand of CpG DNA complexed with β-(1→3)-D-glucan, namely, schizophyllan (SPG) (*29*). This new TLR9 ligand is more potent and durable than naked CpG DNA, and it still retains the TLR9 ligand activity. Mice were i.n. immunized with WV, SV, or SV plus the SPG-CpG DNA conjugate (SV+SPG-CpG) and then evaluated for their adaptive immune responses. The SV+SPG-CpG induced robust type I IFN responses independently of TLR7 (Fig. 4, A and B). Correspondingly, the SV+SPG-CpG successfully enhanced HA-specific B cell and $CD4^+$ T cell responses to levels comparable to those from WV immunization of wild-type mice (Fig. 4, C and D). Immunization of TLR9-deficient mice provided further evidence that the responses induced by the SV+SPG-CpG were dependent on TLR9 but not on TLR7 (fig. S7A).

The protective efficacies of these three types of vaccines were also examined in mice. WV conferred protection against lethal PR H1N1 virus challenge in a TLR7-dependent and TLR9-independent manner, whereas the SV+SPG-CpG provided protection in a TLR9-dependent and TLR7-independent manner (Fig. 4E and fig. S7B). Notably, the original SV failed to provide protection against lethal PR H1N1 virus challenge in any of the groups of mice examined (Fig. 4E and fig. S7B). We also confirmed that the restored protective effect of the SPG-CpG adjuvant was mediated by type I IFN responses because IFNAR2-deficient mice failed to mount virus-specific B and T cell responses (fig. S7, C and D) and demonstrated no improved protection against infection (fig. S7E).

Although the usefulness of SV vaccination in the healthy adult human population has been recognized in many studies, our results are somewhat contradictory. Therefore, we tested the relevance of these observations in a human system. Human peripheral blood mononuclear cells (PBMCs) from healthy volunteers were stimulated with H1N1 live virus, inactivated H1N1 WV, and H1N1 SV for 24 hours, and then IFN-α and IFN-γ secretion was measured by ELISA. Consistent with the mice data, IFN-α was secreted with live virus and WV but not with SV stimulation (Fig. 4F). Depletion of pDCs with BDCA4 microbeads revealed that this IFN-α secretion was totally dependent on pDC in WV and partially in live virus stimulation (Fig. 4F). On the

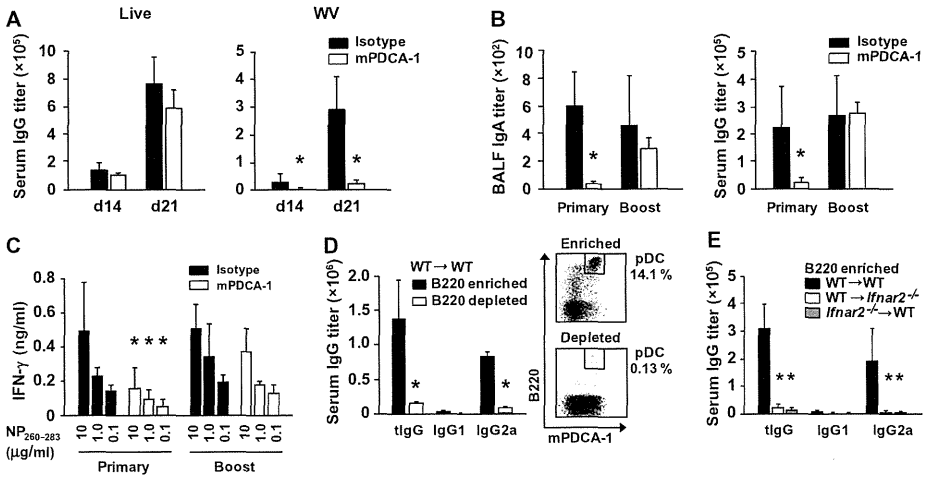

Fig. 3. Immunogenicity of the inactivated WV, but not of the live virus, depends on pDC activation at the primary vaccination. (**A**) The adaptive immune responses to WV (i.n.) were monitored in pDC-depleted mice ($n = 3$ per group). Mice were injected with an antibody to mPDCA-1 or isotype control antibody 24 hours before each vaccination, and serum IgG concentrations were measured by ELISA at the indicated times. *$P < 0.05$ versus control mice. (**B** and **C**) To determine whether pDCs are more important for priming or boosting after vaccination with inactivated WV, we treated one group of mice ($n = 3$) only with an antibody to mPDCA-1 before the primary vaccination (priming) and another group ($n = 3$) was treated only before the secondary vaccination (boosting). BALF IgA and serum IgG 1 week after the boost (B) and IFN-γ production from influenza-specific $CD4^+$ T cells (C) were measured by ELISA. *$P < 0.05$ versus control mice. (**D** and **E**) FL-DCs were separated into $B220^+$ and $B220^-$ DCs by MACS. Each DC was pulsed with WV in vitro, and $B220^+$ or $B220^-$ cells (5×10^5) were intravenously injected ($n = 3$ per group). Serum IgG was measured 2 weeks after the injection in WT mice. *$P < 0.05$ versus B220-enriched cells. (D) $B220^+$ cells (1×10^5) from WT and *Ifnar2*-deficient mice were intravenously transferred into untreated WT and *Ifnar2*-deficient mice ($n = 3$ per group), and serum IgG was measured by ELISA. *$P < 0.05$ versus WT to WT. (E) These results are representative of at least two independent experiments.

other hand, both WV and SV induced IFN-γ secretion comparably in the PBMC preparations even when the SV made from the swine-origin H1N1 A/California/04/2009 strain was used. Results obtained after $CD4^+$ and $CD8^+$ T cell depletion of the PBMC preparations revealed that virus-specific IFN-γ secretion was produced mainly by $CD4^+$ T cells in live virus and WV stimulation (Fig. 4G). These results suggest that SV could efficiently stimulate memory T cell responses without type I IFNs in a naturally (or seasonally) influenza virus–exposed human population.

DISCUSSION

Although TLR7 and certain NLRs have been shown previously to be involved in the induction of adaptive immune responses to influenza A virus infection (*13*, *14*), the current work represents a comprehensive study that directly compares the functions of TLRs, NLRs, and RLRs in the immunogenicity and efficacy of influenza inactivated WV vaccinations (Fig. 1 and fig. S1). We identified pDCs as an innate immune cell and type I IFNs as humoral factors that are essential for the immunogenicity of the inactivated WV vaccine (Figs. 2 and 3). Although our results demonstrate an essential role for pDCs in inactivated WV vaccination, other studies have identified a redundant role for pDCs in antiviral responses to live virus vaccination such as influenza virus (*26*, *27*). In addition, although TLR7 is expressed in a variety of cell types, including B cells and macrophages, our results strongly suggest an essential role for pDCs in mediating TLR7-induced innate and adaptive immune responses to inactivated influenza WV vaccination but not to live virus vaccination.

The critical role of pDCs in vaccine priming, but not in boosting, is apparent from results of the pDC depletion study, in which pDCs were removed from mice before vaccination with inactivated WV (Fig. 3, A to C). These findings parallel our in vitro data, in which the pDC-containing FL-DC preparation, but not the mDC-dominant GM-DC preparation, responded to inactivated WV to produce type I IFNs (Fig. 2D); however, these data do not necessarily exclude the involvement of other antigen-presenting cells for either priming or boosting. For example, pDCs pulsed with inactivated WV in vitro can prime naïve mice (Fig. 3D), but the type I IFNs produced by these pDCs were required not only for stimulating the pDCs themselves but also for priming as yet unidentified cell types of the recipient mouse (Fig. 3E). This suggests that cross talk exists between pDCs and the other as yet unidentified cells via type I IFN–mediated signaling. In addition, it will be of interest to examine whether other DCs, such as mDCs, are involved in this intercellular cross talk in such a way that pDCs can transfer the flu antigens to mDCs or that pDCs can present antigens directly to T cells and/or B cells. We note the distinct regulation of type I IFN induction by inactivated WV vaccination, which differed from IFN induction by live virus vaccination. Indeed, although type I IFNs were induced by both inactivated and live virus vaccinations, inactivated WV vaccines activated only TLR7 on pDCs,

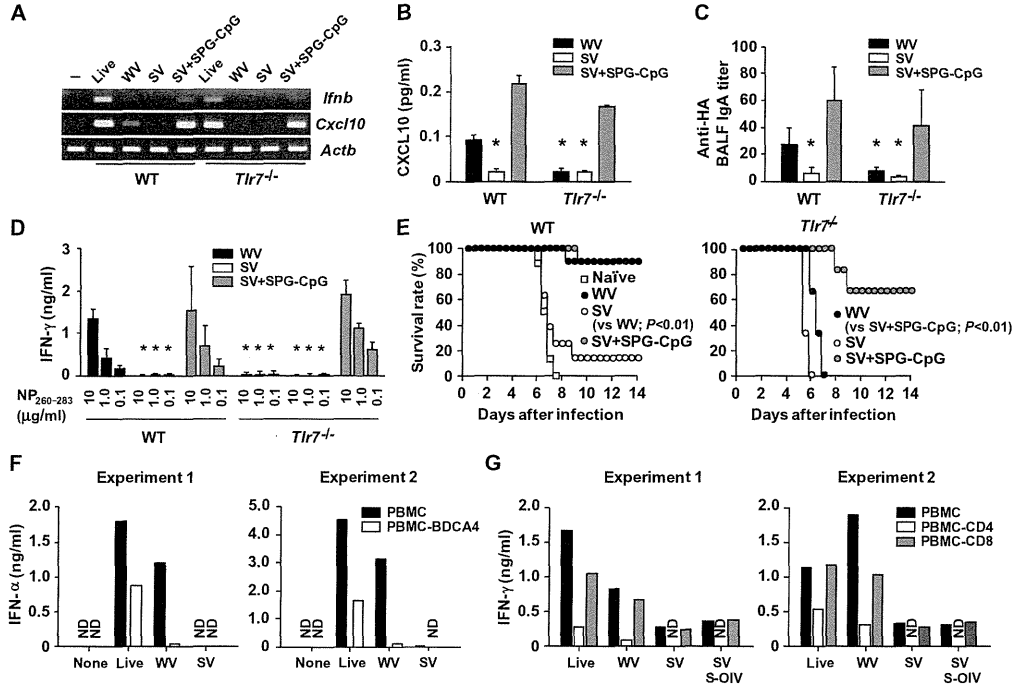

Fig. 4. Immunogenicity differences between WV and SV depend on type I IFN production via pDC activation in naïve hosts but not in primed hosts. (**A** and **B**) WT and *Tlr7*-deficient mice were i.n. vaccinated with three different vaccines as described in Materials and Methods. After 24 hours, IFN-β and CXCL10 expression in the lungs (A) and CXCL10 production in the sera (B) was measured by RT-PCR and ELISA. *$P < 0.05$ versus WT mice immunized with WV. (**C** and **D**) To compare the immunogenicities of the three vaccines, we i.n. vaccinated WT ($n = 9$) and *Tlr7*-deficient ($n = 6$) mice as described in Fig. 1 and determined antigen (HA)–specific BALF IgA, serum total IgG (C), and IFN-γ production by $CD4^+$ T cells (D) by ELISA. *$P < 0.05$ versus WT mice immunized with WV. (**E**) Vaccinated mice were challenged with the PR strain at $10 \times LD_{50}$, and their survival rates were determined as described in Materials and Methods. *$P < 0.05$ versus WT mice immunized with WV or control mice. These results are representative of at least two independent experiments. (**F** and **G**) IFN-α and IFN-γ production in response to inactivated influenza vaccines in human PBMCs. PBMCs from healthy volunteers were stimulated with live NC virus, WV, and SV. Total PBMCs and pDC-depleted PBMCs (PBMC-BDCA4) were stimulated with live NC virus (0.01 MOI), WV (1.0 μg/ml), and SV (0.5 μg/ml), and IFN-α production was measured by ELISA (F). Total PBMCs and CD4- or CD8-depleted PBMCs were stimulated with WV and SV of NC (10 and 5 μg/ml, respectively) and SV of swine-origin influenza virus (SV S-OIV) (5 μg/ml). IFN-γ production was measured by ELISA (G). These results are from 2 representatives of 10 volunteers.

whereas live virus activated both TLR7 on pDCs and other TLR7-independent pathways in the other cells, possibly mDCs (Fig. 2, D and E). Therefore, although TLR7, pDCs, and type I IFNs all were essential for inactivated WV vaccination, pDCs and type I IFNs were not essential for live virus vaccination.

The adaptor ASC is a critical component of NLRP3 inflammasome (*30*). In contrast to type I IFN responses, ASC-dependent inflammasome activation has been shown to play a critical role in the survival of the mice challenged with live influenza virus (*14*, *18*, *19*). However, the requirement for inflammasome activation to induce influenza-specific adaptive immune responses has been controversial (*14*, *19*). Our data indicate that ASC-dependent inflammasome activation is dispensable for inducing adaptive immune responses to WV and live virus, except for IgG1 production in live virus vaccination (Fig. 1 and fig. S2). Concurrent analysis comparing three innate immune signaling pathways, TLR, NLR, and RLR, enabled us to elucidate that the TLR-dependent pathway dominantly controlled the T helper 1–type protective immunity elicited by WV and live virus vaccination.

Although SV, which is now used as the first choice for influenza vaccination in many countries, was not protective in naïve mice, its decreased immunogenicity was fully restored by adding a new TLR9 ligand that stimulates pDCs to secrete type I IFNs (Fig. 4, A to E, and fig. S7). These data above further support the notion that pDC activation and their type I IFN production play a critical role in the induction of inactivated influenza vaccine immunogenicity in naïve hosts. These results might explain in part the well-known fact that the efficacy of adjuvant-less SV is lower in young children than in adults (*7*), in which SV is simply boosting the memory T and/or B cell responses. This is further supported by our results obtained using human PBMCs (Fig. 4, F and G), which suggest that most human adults have virus-specific $CD4^+$ T cells that produce IFN-γ in response not only to seasonal flu viruses but also to the novel swine H1N1 virus. Our results also indicate that memory T cells react to both internal proteins, such as those in SV, and a wide spectrum of influenza virus surface antigens, such as those on swine-origin H1N1 (*31*, *32*) and H5N1 (*33*). The age distribution of the affected population in swine-origin H1N1 and H5N1 infections, which was limited to the young, might reflect the importance of memory T cells established by recurrent exposure to seasonal influenza live viruses and vaccines (*34*, *35*)

LAIVs activate both influenza-specific IgA-secreting B cells and cytotoxic $CD8^+$ T cells (*36*), which provides certain advantages over inactivated vaccines including WV and SV. Although WV is now unavailable for seasonal influenza, it is cost-effective and can induce heterosubtypic protection not only against a challenge by H1N1 (Fig. 1C and fig. S1C) but also against H5N1 (*37*, *38*), as with LAIV (*39*). In addition, recent progress in manufacturing techniques could reduce the adverse event rate in i.m. WV immunization (*37*, *38*) to yield results that are quite different from those of past clinical trials (*3*, *40*). An i.n. WV immunization may produce a sufficient combination of efficacy, safety, and utility for both seasonal and pre-pandemic vaccines (*41*–*45*).

Together, analysis of the molecular and cellular mechanisms of different influenza vaccines provides useful information for improving vaccine immunogenicity and efficacy, as well as for choosing an appropriate form of influenza vaccine with a rational safety approach.

MATERIALS AND METHODS

Animals, cells, viruses, and reagents

The generation of *Tlr7*-, *Ips-1*–, *Ifnar2*-, and *Tlr9*-deficient mice, either on a 129/Ola × C57/BL6 or on a C57/BL6 background, has been described previously (*13*, *46*). ASC-deficient mice were a gift from V. M. Dixit (*47*).

All animal experiments were performed in accordance with the institutional guidelines for the Osaka University animal facility.

Purified influenza viruses, H1N1 (PR and NC), a recombinant HA protein of PR, and both inactivated WV and split vaccines of NC were prepared as previously described (*48*). Both types of vaccines were derived from the NC strain. Briefly, the viruses were purified from allantoic fluid by filtration (0.45 μm) followed by sedimentation through a linear sucrose gradient. For formalin-inactivated WV vaccines, purified viruses were treated with 0.1 to 0.2% formalin at 4°C for a week. For the ether-split vaccines (SV), the viruses were mixed with an equal volume of ether and then incubated for 30 min at room temperature with stirring. The mixture was centrifuged (3000 rpm, 15 min), and the aqueous phase was collected and evaporated. CpG DNA forming a triple helix with SPG, a natural polysaccharide composed of β-(1→3)-D-glucan, was used as the second-generation TLR9 ligand as previously described (*29*, *49*, *50*). DCs were prepared as described previously. Briefly, bone marrow cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 mM 2-mercaptoethanol, and human Flt3 ligand (100 ng/ml) (PeproTech) or murine GM-CSF (10 ng/ml) (PeproTech) for 7 to 9 days to use as FL-DCs and GM-DCs.

Influenza virus infection and vaccination

For influenza virus infection or vaccination, mice were anesthetized and administered i.n. with 30 μl of phosphate-buffered saline (PBS) (15 μl for each nares) containing serial amount of influenza NC viruses and vaccines. Mice were infected with 1×10^5 to 2×10^5 plaque-forming units (PFU) of virus per mouse or vaccinated with WV (1.5 to 3.0 μg per mouse) or SV (0.75 μg per mouse) with or without SPG-CpG (30 μg per mouse) twice at a 2-week interval. For the analysis of protection, mice were infected with the indicated doses of lethal PR strain.

Measurement of innate immune responses

Reverse transcription polymerase chain reaction (RT-PCR) was performed to measure mRNA expression levels of type I IFNs, cytokines, and chemokines using the RNA of the stimulated cells as previously described (*13*). Protein concentrations of IFN-α, IFN-β, and CXCL10 in the culture supernatants of the stimulated cells were measured using ELISA kits (IFN-α and IFN-β, PBL Biomedical Laboratories; CXCL10, R&D Systems).

Plasmacytoid DC depletion and cell transfer

Plasmacytoid DCs were depleted by intravenous injection of antibody to mPDCA-1 (500 μg) (Miltenyi Biotec) 24 hours before live virus infection or inactivated WV vaccination.

FL-DCs were separated into two populations, B220-enriched and B220-depleted population, by B220 antibody MACS microbeads (Miltenyi Biotec) according to the manufacturer's protocol to obtain B220-enriched FL-DC. Each cell population was incubated with WV (5 to 10 μg/ml) for 3 hours, and 1×10^5 to 5×10^5 cells per mouse

were injected intravenously into each type of mice. Immunological assays were performed 2 weeks after injection.

Confirmation of pDC depletion in spleen by flow cytometric analysis

After Fc blocking with an antibody to CD16/32, isolated spleen cells were stained with fluorescein isothiocyanate (FITC)–conjugated antibody to CD11c, phycoerythrin (PE)–conjugated antibody to CD45R/B220, and allophycocyanin-conjugated antibody to mPDCA-1 (Miltenyi Biotec) for 30 min at room temperature and washed with PBS containing 1% bovine serum albumin. Just before fluorescence-activated cell sorting (FACS) analysis using FACSCalibur and CellQuest software (BD Biosciences), 7-aminoactinomycin D (BD Biosciences) was added.

Measurement of antigen-specific T and B cell responses

After two i.n. vaccinations, B cell–mediated humoral responses were measured as immunoglobulin production by ELISA using goat antibody to mouse total IgG, IgG1, IgG2a, and IgA conjugated to horseradish peroxidase (Southern Biotech) as previously described (*1*). T cell–mediated cellular responses were monitored by measuring $NP_{260-283}$/I-A^b–specific or $NP_{366-374}$/H-$2D^b$–specific IFN-γ secretion of splenocytes and the frequency and cytotoxicity of H-$2D^b$–specific CD8 T cells as described previously (*13*).

Preparation of human PBMCs for cytokine analysis

PBMCs were obtained from 10 healthy adult volunteers (30 to 50 years old, 6 males and 4 females). All of the experiments using human PBMCs were approved by the Institutional Review Board of the Research Institute for Microbial Diseases, Osaka University. Cells were purified from heparinized blood by density centrifugation using Ficoll-Paque Plus (Amersham). Human pDCs, CD4, or CD8 T cells were depleted with BDCA4 and CD4 or CD8 antibody MACS microbeads (Miltenyi Biotec), respectively, according to the manufacturer's protocol. Plasmacytoid DC depletion was confirmed by FACS analysis staining with FITC-conjugated antibody to BDCA2 and PE-conjugated antibody to CD123 (Miltenyi Biotec). PBMCs or pDC-depleted PBMCs (1×10^6 to 2×10^6 cells) were stimulated with each influenza vaccine at the indicated concentration. Twenty-four hours later, IFN-α and IFN-γ (R&D Systems) were measured in supernatants by ELISA according to their manufacturers' protocol.

Statistical analysis

Statistical significance ($P < 0.05$) between groups was determined using the Student's *t* test. A survival curve was generated using Kaplan-Meier methodology, and the susceptibility of mice after infection was compared using the log-rank test.

SUPPLEMENTARY MATERIAL

www.sciencetranslationalmedicine.org/cgi/content/full/2/25/25ra24/DC1

Materials and Methods

Fig. S1. TLR7-dependent, but not IPS-1–dependent, signaling is required for the induction of protective immune responses with inactivated WV vaccine by i.m. immunization.

Fig. S2. ASC-dependent inflammasome activation was dispensable for adaptive immune response to influenza virus infection, except for systemic IgG1 production.

Fig. S3. Type I IFN receptor–mediated signaling was indispensable for adaptive immune response to WV but not to the live virus.

Fig. S4. Plasmacytoid DC depletion by mPDCA-1 antibody was confirmed in spleen.

Fig. S5. Type I IFN interaction between pDCs and other immune cells was required for WV vaccine immunogenicity.

Fig. S6. Different manner of type I IFN response to WV vaccine and split vaccine is dependent on the presence of the viral genome RNA.

Fig. S7. Indispensable role of type I IFN–mediated signaling in vaccination with split vaccine plus SPG-CpG.

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Immunity
Article

Silica Crystals and Aluminum Salts Regulate the Production of Prostaglandin in Macrophages via NALP3 Inflammasome-Independent Mechanisms

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SUMMARY

Particulates such as silica crystal (silica) and aluminum salts (alum) activate the inflammasome and induce the secretion of proinflammatory cytokines in macrophages. These particulates also induce the production of immunoglobulin E via a T helper 2 (Th2) cell-associated mechanism. However, the mechanism involved in the induction of type 2 immunity has not been elucidated. Here, we showed that silica and alum induced lipopolysaccharide-primed macrophages to produce the lipid mediator prostaglandin E_2 (PGE_2) and interleukin-1β (IL-1β). Macrophages deficient in the inflammasome components caspase 1, NALP3, and ASC revealed that PGE_2 production was independent of the NALP3 inflammasome. PGE_2 expression was markedly reduced in PGE synthase-deficient (*Ptges*$^{-/-}$) macrophages, and *Ptges*$^{-/-}$mice displayed reduced antigen-specific serum IgE concentrations after immunization with alum or silica. Our results indicate that silica and alum regulate the production of PGE_2 and that the induction of PGE_2 by particulates controls the immune response in vivo.

INTRODUCTION

Some particulates and crystals can stimulate the innate immune system to induce inflammatory responses. In particular, aluminum salts (referred to as alum) and silica crystals can induce type 2 inflammatory responses, which are characterized by the accumulation of eosinophils at the site of injection and the elevation of antigen-specific serum IgE and IgG1 amounts in vivo (Aimanianda et al., 2009; Marrack et al., 2009; Kumar et al., 2009). However, the basis for the adjuvanticity of these particulates and the mechanisms by which they elicit type 2 immunity remain poorly understood.

In the innate immune system, macrophages and dendritic cells (DCs) function as the first line of defense against foreign antigens. These cells can recognize pathogen-associated molecular patterns (PAMPs) through pattern-recognition receptors (PPRs) and can induce inflammatory responses (Akira et al., 2006). NOD-like receptors (NLRs) are intracellular PRRs (Ye and Ting, 2008). Among the known NLRs, NALP3 (also known as NLRP3, Cryopyrin, CIAS1, or PYPAF1) is one of the best characterized. Upon activation, NALP3 forms a multiprotein complex with apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and caspase-1. This complex, referred to as the NALP3 inflammasome, promotes the secretion of the proinflammatory cytokines interleukin-1β (IL-1β) and IL-18 by the action of caspase-1 (Franchi et al., 2009; Martinon et al., 2009; Schroder and Tschopp, 2010; Schroder et al., 2010). The activated NALP3 inflammasome contributes to antifungal host defense, antitumor immunity, and inflammation (Duewell et al., 2010; Ghiringhelli et al., 2009; Gross et al., 2009; Halle et al., 2008; Watanabe et al., 2008). In addition to being activated by PAMPs, the NALP3 inflammasome is also activated by ATP, various crystals (i.e., silica, asbestos and monosodium urate), and alum (Cassel et al., 2008; Dostert et al., 2008; Eisenbarth et al., 2008; Hornung et al., 2008). The NALP3 inflammasome has been shown to induce type 2 immune responses, and *Nalp3*$^{-/-}$ and *Asc*$^{-/-}$ mice display reduced type 2 immune responses to alum (Eisenbarth et al., 2008; Kool et al., 2008; Li et al., 2008). However, other reports have shown that the NALP3 inflammasome is dispensable for alum adjuvanticity (Franchi and Núñez, 2008; McKee et al., 2009). In addition, it has been reported that the NALP3 inflammasome is not required for antibody production in response to vaccination by the particulate adjuvant (Sharp et al., 2009). Thus, the role of the NALP3 inflammasome in the induction of type 2 immunity is still open to further investigation.

In addition to proinflammatory cytokines, lipid mediators such as prostaglandins (PGs) are also involved in the induction of

inflammatory responses (Narumiya, 2009). PGE_2, a well-characterized proinflammatory lipid mediator, is an arachidonic acid metabolite that is produced by various types of cells including antigen-presenting cells. Previous reports have shown that PGE_2 suppresses T helper 1 (Th1) cell-type responses by elevating intracellular cAMP concentrations in DCs, macrophages, and Th1 cells, thus inhibiting their ability to produce type 1 cytokines such as IL-12 and IFN-γ (Fabricius et al., 2010; Koga et al., 2009; Kuroda and Yamashita, 2003). In addition, PGE_2 can enhance IL-23 production by DCs and favors Th17 cell polarization and IL-17 production (Boniface et al., 2009; Yao et al., 2009). More recently, PGE_2 was shown to facilitate Th1 cell differentiation in the presence of IL-12 and high doses of the costimulatory CD28 antibody, via activation of the PI3-kinase pathway (Yao et al., 2009). Thus, PGE_2 has various functions in the regulation of immune responses. However, the involvement of PGE_2 in particulate-mediated adjuvanticity has not been investigated.

In this study, we showed that alum, silica, and ATP, which activate the NALP3 inflammasome, induced macrophages to produce IL-1β, IL-18 and PGE_2. Interestingly, PGE_2 production in macrophages was regulated by the spleen tyrosine kinase (Syk) and p38 MAP kinase pathway but did not depend on inflammasome activation. In addition, particulate-induced PGE_2 regulated antigen-specific serum IgE production in vivo. Our results suggest that the activation of the PGE_2 pathway by particulates may be an important signal for the induction of type 2 immune responses.

RESULTS

Silica, Alum, and ATP Induce PGE_2 Production in LPS-Primed Macrophages

Many reports have shown that alum, silica, and ATP stimulate macrophages to produce the caspase-1-dependent cytokines IL-1β and IL-18 by activating the NALP3 inflammasome (Cassel et al., 2008; Dostert et al., 2008; Eisenbarth et al., 2008; Hornung et al., 2008). These cytokines are thought to be important for regulation of immune responses. However, some studies have shown that caspase-1-dependent cytokines are dispensable for immune regulation (Franchi and Núñez, 2008; McKee et al., 2009; Sharp et al., 2009). Thus, we first ascertained whether inflammasome activators could induce the production of caspase-1-independent factor(s) in macrophages. To this end, we examined the production of several cytokines, chemokines and lipid mediators by macrophages in response to silica, alum and ATP. Silica, alum and ATP induced LPS-primed macrophages to produce IL-1β and IL-18, which is in agreement with previous reports (Cassel et al., 2008; Dostert et al., 2008; Eisenbarth et al., 2008; Hornung et al., 2008). These inflammasome activators also induced LPS-primed macrophages to produce PGE_2 (Figure 1A). A time-course analysis revealed that silica stimulation of LPS-primed macrophages for 2 hr was sufficient for detecting similar amounts of PGE_2 and IL-1β, in terms of pg/ml secreted (Figure 1B). In our experiments, we tested three different alum compounds: Imject alum (a mixture of aluminum hydroxide and magnesium hydroxide; Thermo Scientific), alhydrogel (aluminum hydroxide gel; Sigma-Aldrich) and LSL alum (aluminum hydroxide hydrate gel suspension; LSL). The experiments described in Figures 1A and 1B were performed with LSL alum as a stimulator. To test which alum compound was the most potent PGE_2 inducer in macrophages, we stimulated LPS-primed macrophages with the three different compounds. Each alum compound stimulated the production of PGE_2, but LSL alum (referred to hereafter as alum) induced the highest PGE_2 production in LPS-primed macrophages. Alhydrogel induced modest production of PGE_2 in LPS-primed macrophages, whereas Imject alum induced low amounts of PGE_2 (Figure S1A). It is important to note that LPS-primed macrophages did not produce any other well-characterized inflammatory cytokines or chemokines in response to silica (Figure S1B) or alum (data not shown).

Activated macrophages are known to produce PGD_2 (Mohri et al., 2003). Indeed, we found that LPS-primed macrophages produced PGD_2 in response to silica (Figure 1C). We also examined the effect of titanium dioxide (TiO_2), which does not cause severe inflammation on inhalational exposure and does not activate the NALP3 inflammasome (Cassel et al., 2008), on PGE_2 production by macrophages. As shown in Figure 1D, TiO_2 did not induce IL-1β or PGE_2 production in LPS-primed macrophages. Activation of the NALP3 inflammasome in DCs contributes to particulate-mediated adjuvanticity (Kool et al., 2008). Thus, we compared PGE_2 production by bone marrow (BM)-derived DCs and macrophages. BM-derived DCs produced IL-1β and PGE_2 in response to silica and alum, but the amounts of PGE_2 were markedly higher in macrophages than in DCs (Figure 1E). We have previously reported that macrophages from BALB/c mice produce higher amounts of PGE_2 than those from C57BL/6 mice (Kuroda et al., 2007; Kuroda and Yamashita, 2003). Macrophages from BALB/c mice produced ~2-fold greater amounts of IL-1β and PGE_2 in response to silica than macrophages from C57BL/6 mice (Figure 1F). Our observations are not restricted to mouse studies, given that human peripheral blood mononuclear cells (PBMCs) also produced PGE_2 and IL-1β in response to silica (Figure 1G). These results indicate that in addition to IL-1β and IL-18, macrophages also produce PG in response to silica, alum, and ATP.

Silica- and Alum-Induced PGE_2 Production Is Independent of the Activity of Caspase-1

IL-1β and IL-18 are known to induce PG production (Dinarello, 2002; Lee et al., 2004). However, simple exposure of LPS-primed macrophages to IL-1β and/or IL-18 did not induce the production of PGE_2 (Figure 2A). BM cells from WT and IL-1 receptor-deficient mice were cultured for 7 days with macrophage colony stimulating factor (M-CSF), and adherent macrophages were analyzed for silica- and alum-induced PGE_2 production. As shown in Figure 2B, WT and *Il1r1*$^{-/-}$ macrophages produced comparable amounts of PGE_2. In addition, treatment of silica- or alum-activated macrophages with a caspase-1 inhibitor significantly reduced their ability to produce IL-1β but did not affect their ability to produce PGE_2 (Figure 2C). Because silica and alum can induce cell death, we wanted to rule out the possibility that the enhanced production of PGE_2 in silica- and alum-activated macrophages was due to cell death. For this purpose, we measured cell death in silica- and alum-activated macrophages by quantifying the release of lactose dehydrogenase (LDH) into the culture media. Treatment of macrophages with a caspase-1 inhibitor reduced silica- and alum-induced

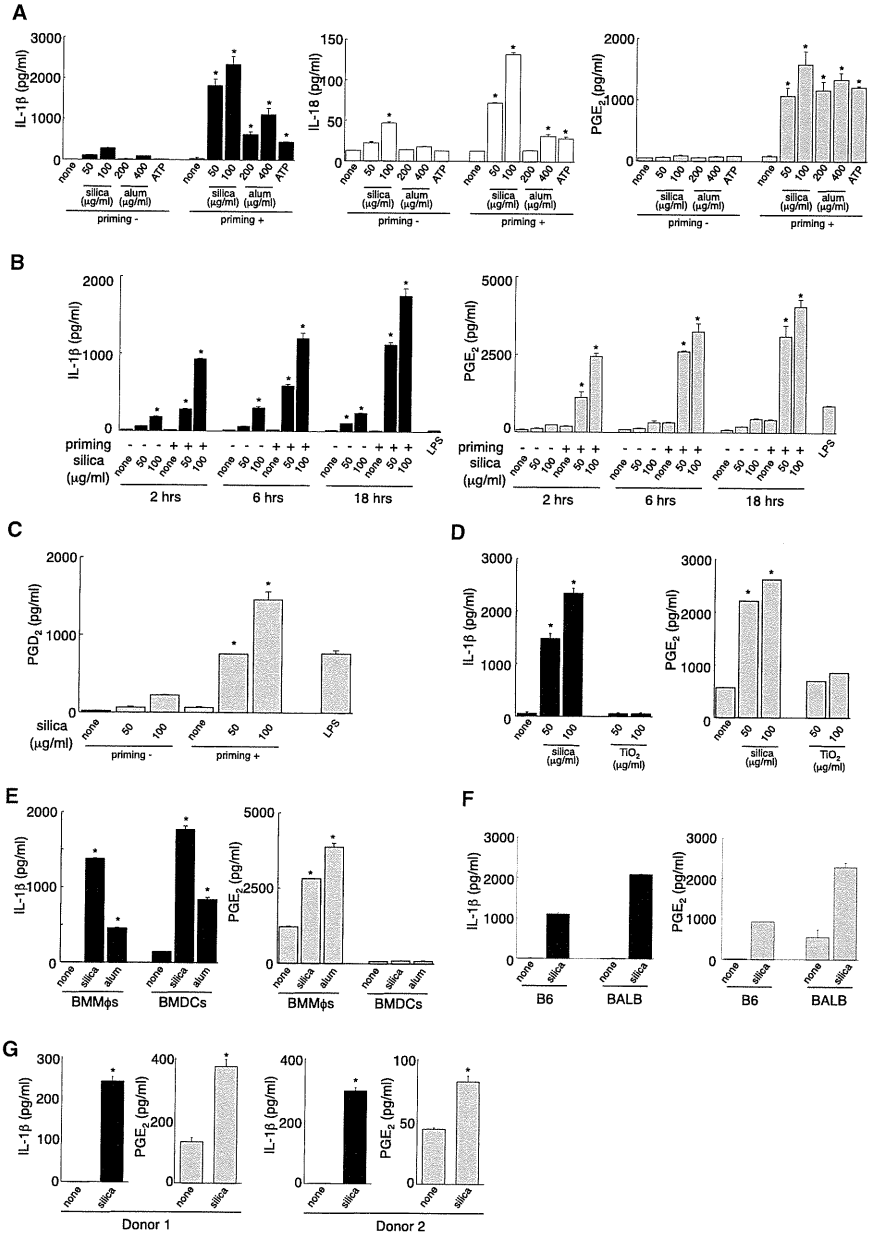

Figure 1. Inflammasome Activators Induce Macrophages to Produce PGE_2

(A) Peritoneal macrophages from BALB/c mice were primed for 3 hr with or without low-dose LPS (1 ng/ml) and then stimulated with silica, alum or ATP for 2 hr.

(B) Macrophages with or without LPS priming were stimulated with silica for 2, 6, or 18 hr. Macrophages stimulated with high-dose LPS (1 μg/ml) for 6 hr were used as controls.

(C) The culture supernatants of macrophages stimulated for 2 hr with silica were analyzed for PGD_2 production as indicated in (B).

(D) LPS-primed macrophages were stimulated with silica or TiO_2 for 2 hr.

(E) BM-derived macrophages and DCs were primed with LPS and then stimulated with 100 μg/ml silica or 400 μg/ml alum for 6 hr.

(F) Peritoneal macrophages from C57BL/6 (B6) and BALB/c (BALB) mice were primed with LPS and then stimulated with 100 μg/ml silica or 400 μg/ml alum for 2 hr.

(G) Human PBMCs were primed for 3 hr with LPS and then stimulated with silica for 3 hr. For all experiments, the amounts of IL-1β and PGE_2 in the culture supernatants were determined by ELISA. Data represent mean ± SE of three to five independent experiments (*$p < 0.01$).

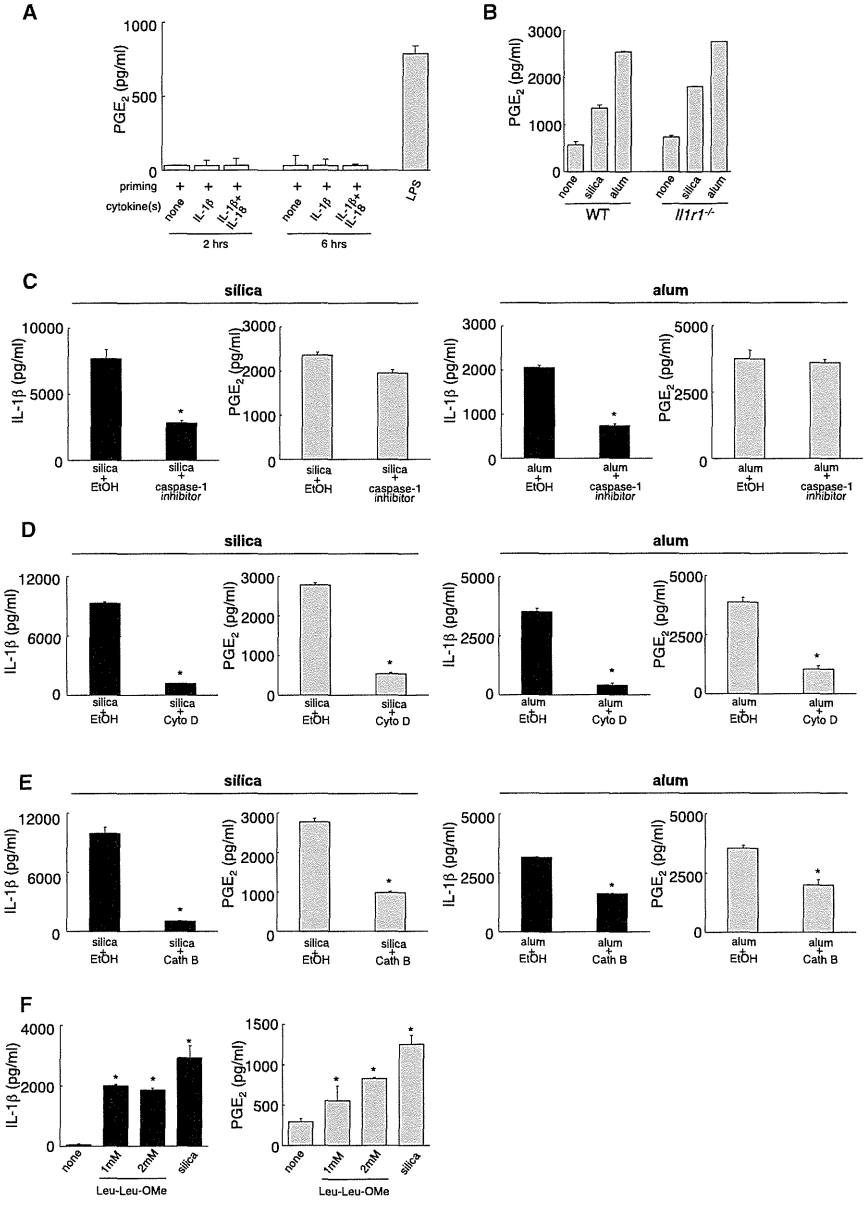

Figure 2. Silica- and Alum-Induced Lysosomal Damage Triggers PGE_2 Production in Macrophages via Caspase-1-Independent Mechanisms

(A) LPS-primed peritoneal macrophages were stimulated with IL-1β or IL-1β plus IL-18 for 2 or 6 hr. Macrophages stimulated with high-dose LPS were used as controls.

(B) M-CSF-derived BM macrophages from WT (C57BL/6) and *Il1r1*$^{-/-}$ mice were primed with low-dose LPS and then stimulated with 50 μg/ml silica or 200 μg/ml alum for 6 hr.

(C) LPS-primed macrophages were stimulated with 100 μg/ml silica or 400 μg/ml alum in the presence or absence of caspase-1 inhibitors for 2 hr.

(D and E) LPS-primed macrophages were stimulated with 100 μg/ml silica or 400 μg/ml alum in the presence or absence of cytochalasin D (D) or cathepsin B inhibitor (E) for 2 hr.

(F) LPS-primed macrophages were incubated with Leu-Leu-OMe for 2 hr. Data represent mean ± SE of three (A and C–E) or two (B and F) independent experiments (*$p < 0.01$).

cell death (Figure S2). This result was expected because caspase-1 is involved in promoting cell death. However, we have already shown that PGE_2 production in macrophages is independent of caspase-1 activity (Figure 2C and Figure S2). Therefore, these results indicate that caspase-1-dependent cytokine release and cell death are not responsible for silica- and alum-induced PGE_2 production in macrophages.

Silica and Alum Induce PGE_2 Production in Macrophages through Phagosomal Destabilization

Phagocytes engulf particulates such as silica or alum, which leads to lysosomal damage and rupture, followed by the release of lysosomal enzymes, such as cathepsin B, into the cytoplasm. Studies suggest that lysosomal damage can activate the NALP3 inflammasome and induce the production of IL-1β (Duewell et al., 2010; Hornung et al., 2008). To address whether the engulfment of particulates, lysosomal rupture, and release of lysosomal enzymes could induce PGE_2 production in macrophages, we cultured macrophages in the presence of cytochalasin D, which inhibits actin filament assembly and phagocytosis. Treatment of macrophages with cytochalasin D significantly reduced their ability to produce IL-1β and PGE_2 in response to silica and alum (Figure 2D).

The release of lysosomal enzymes such as cathepsin B is thought to trigger the activation of the NALP3 inflammasome. In addition, the cathepsin B inhibitor CA-074 has been shown to inhibit the NALP3 inflammasome (Duncan et al., 2009; Hornung et al., 2008; Sharp et al., 2009). We showed the production of IL-1β and PGE_2 by macrophages was partially inhibited by treating the cells with CA-074 (Figure 2E). Lysosomal damage has also been shown to trigger the activation of the NALP3 inflammasome. To investigate this process, we treated macrophages with leucyl-leucine methyl ester (Leu-Leu-OMe) to induce lysosomal damage (Hornung et al., 2008). Leu-Leu-OMe-treated macrophages produced large amounts of IL-1β (Figure 2F) as previously reported. Leu-Leu-OMe-treated macrophages also produced PGE_2. Altogether, these results suggest that, similar to the stimulatory effect of NALP3 inflammasome activation and IL-1β release, lysosomal damage and rupture triggers PGE_2 production in macrophages.

Silica- and Alum-Induced PGE_2 Production in Macrophages Is Independent of the NALP3 Inflammasome

We have shown that the NALP3 inflammasome activators silica, alum, and ATP induce LPS-primed macrophages to produce PGE_2. We have also demonstrated that PGE_2 production is independent of caspase-1 activity. To investigate whether the NALP3 inflammasome is involved in silica- and alum-induced PGE_2 production, we performed experiments similar to those described above using *Nalp3*$^{-/-}$, *Asc*$^{-/-}$, and *Casp1*$^{-/-}$ macrophages. M-CSF-derived BM macrophages from WT and inflammasome-deficient mice were used in this experiment. As shown in Figure 3A, macrophages deficient in NALP3, ASC, or caspase-1 failed to secrete IL-1β in response to silica and alum, which is consistent with previously published reports. However, macrophages from inflammasome-deficient mice produced slightly higher amounts of PGE_2 in response to silica and alum than cells from WT mice. IL-6 production was previously shown to be independent of the NALP3 inflammasome (Kumar et al., 2009; Yamamoto et al., 2004). Indeed, WT and inflammasome-deficient macrophages produced comparable amounts of IL-6. Similar results were obtained when we performed the same experiments with granulocyte-macrophage colony stimulating factor (GM-CSF)-derived macrophages (Figure 3B). These results indicate that silica and alum induce macrophages to produce PGE_2 through NALP3 inflammasome-independent mechanisms.

PGE_2 synthesis is regulated by cyclooxygenase (COX) and PGE synthase. In particular, COX-2 and PTGES (also known as mPGES-1) have been reported to regulate stimulation-dependent PGE_2 production in macrophages (Kuroda and Yamashita, 2003; Uematsu et al., 2002). As shown in Figure 3C, treatment with NS-398, which is a COX-2-specific inhibitor, significantly suppressed PGE_2 production in silica-activated macrophages. Similar to COX-2 inhibition, *Ptges*$^{-/-}$ macrophages did not produce detectable amounts of PGE_2 upon stimulation with silica (Figure 3D). However, neither COX-2 inhibition nor PTGES deficiency had an effect on silica-induced IL-1β production in macrophages, suggesting that IL-1β production and activation of the inflammasome are independent of PGE_2 production (Figure S3). Similar results were obtained when we performed the same experiments with alum-activated macrophages (data not shown). We then assessed the expression of COX-2 and PTGES in macrophages upon stimulation with silica and alum. As shown in Figure 3E, priming of macrophages with LPS induced the expression of the COX-2 and PTGES proteins. However, silica, alum, and ATP stimulation had no effect on COX-2 and PTGES expression in macrophages. This result indicates that silica- and alum-induced PGE_2 production in macrophages does not involve increased expression of COX-2 or PTGES. Collectively, these results indicate that silica- and alum-induced PGE_2 production in macrophages is mediated by the COX-2 and PTGES pathways.

Silica- and Alum-Induced Production of PGE_2 by Macrophages Regulates Immune Responses In Vivo

Given that *Ptges*$^{-/-}$ macrophages cannot produce PGE_2 while retaining inflammasome function and the ability to produce IL-1β in response to silica and alum (Figure 3 and Figure S3), we determined whether alum-induced PGE_2 production plays a role in regulating immune responses in vivo. We immunized *Ptges*$^{+/+}$ and *Ptges*$^{-/-}$ mice with alum plus OVA twice (day 0 and 7). Ten days after the last immunization, sera were collected and analyzed for amounts of OVA-specific IgE, IgG1, and IgG2c antibodies. In *Ptges*$^{+/+}$ mice, OVA-alum immunization stimulated the generation of OVA-specific IgE, IgG1, and IgG2c. In contrast, *Ptges*$^{-/-}$ mice displayed reduced amounts of OVA-specific IgE (Figure 4A). In contrast, the amounts of IgG1 and IgG2c in the sera taken from *Ptges*$^{+/+}$ and *Ptges*$^{-/-}$ mice were comparable (Figure 4A).

Because previous reports have shown that OVA-silica immunization induces OVA-specific antibody responses (Kumar et al., 2009), we assessed whether silica could trigger IgE responses. As shown in Figure 4B, OVA-silica immunization induced the generation of OVA-specific IgE, IgG1, and IgG2c antibodies. As observed with OVA-alum immunization, OVA-silica-immunized *Ptges*$^{-/-}$ mice displayed reduced amounts of OVA-specific IgE. In contrast, amounts of OVA-IgG1 and IgG2c antibodies were comparable between *Ptges*$^{+/+}$ and

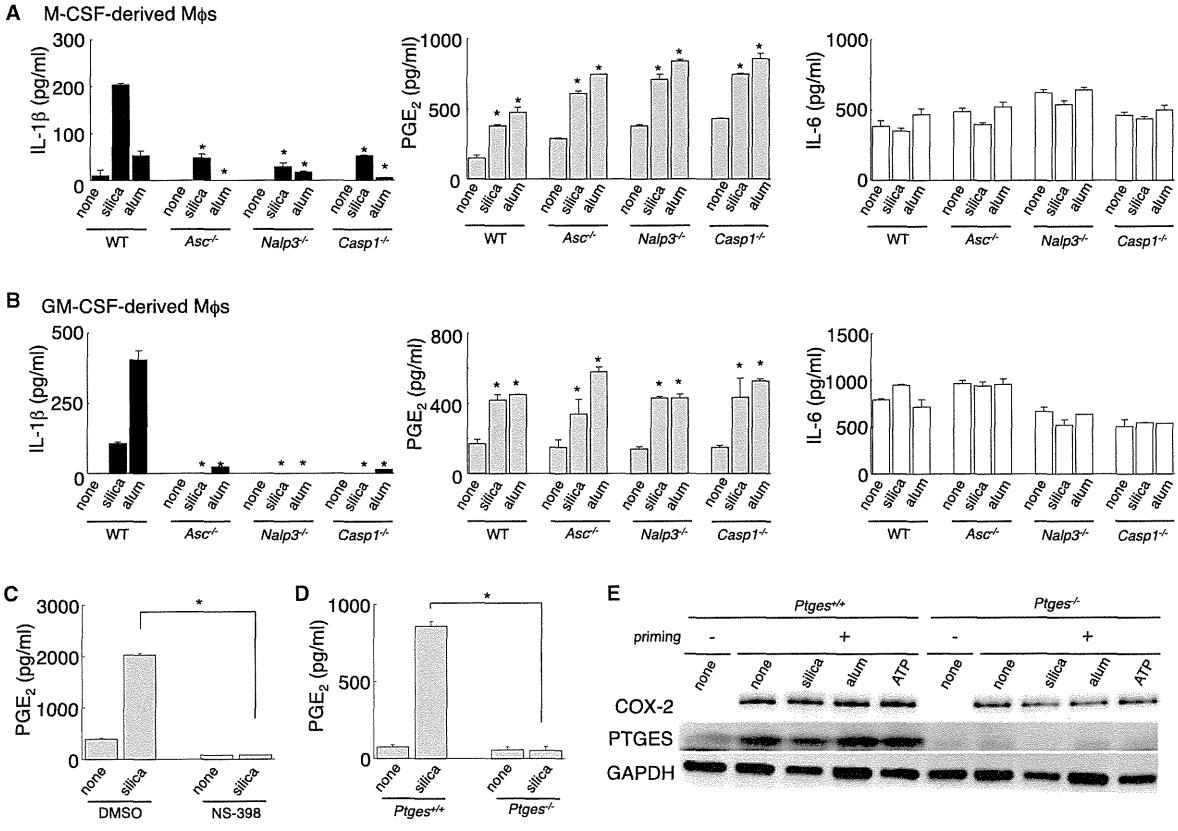

Figure 3. The Mechanisms of Particulate-Induced PGE_2 Production

(A and B) Silica- and alum-induced PGE_2 production is independent of the NALP3 inflammasome. M-CSF- (A) or GM-CSF- (B) derived BM macrophages from WT (C57BL/6), *Asc*$^{-/-}$, *Nalp3*$^{-/-}$, and *Casp1*$^{-/-}$ mice were primed with low-dose LPS and then stimulated with 100 μg/ml silica or 400 μg/ml alum for 6 hr.

(C–E) Silica-induced PGE_2 production is mediated by COX-2 and PTGES.

(C) LPS-primed BALB/c peritoneal macrophages were stimulated with 100 μg/ml silica in the presence or absence of the COX-2 inhibitor NS-398 for 2 hr.

(D) Peritoneal macrophages from *Ptges*$^{+/+}$ and *Ptges*$^{-/-}$ mice were primed with low-dose LPS and then stimulated with 100 μg/ml silica for 2 hr.

(E) *Ptges*$^{+/+}$ and *Ptges*$^{-/-}$ macrophages were primed with or without LPS and stimulated with 100 μg/ml silica, 400 μg/ml alum, or 1 mM ATP for 2 hr. Cell lysates were analyzed for COX-2, PTGES, and GAPDH (loading control) expression by western blotting. Data represent mean ± SE of two (A and B) or three (C and D) independent experiments (*$p < 0.01$).

Ptges$^{-/-}$ mice. We carried out similar in vivo experiments using *Nalp3*$^{-/-}$ and *Casp1*$^{-/-}$ mice, and we found that the amounts of OVA-IgE from WT and mutant mice were comparable (Figure S4A). We also assessed the effect of a Th1 cell adjuvant, *Propionibacterium* (*P.*) *acnes*, on antigen-specific IgE, IgG1, and IgG2c production in vivo. We found that *P. acnes* induced comparable amounts of OVA-IgG1 and IgG2c in *Ptges*$^{+/+}$ and *Ptges*$^{-/-}$ mice and did not induce OVA-IgE in either genotype (Figure S4B). Although *Ptges*$^{-/-}$ mice displayed reduced amounts of OVA-IgE after immunization, the total IgE concentrations were similar in unimmunized *Ptges*$^{+/+}$ and *Ptges*$^{-/-}$ mice (Figure S4C). This result indicates that *Ptges*$^{-/-}$ mice, unlike *Il4*$^{-/-}$ or *Stat6*$^{-/-}$ mice, are not Th2 cell type prone.

We also immunized hematopoietic PGD synthase-deficient (*Ptgds*$^{-/-}$) mice, in which macrophages cannot produce PGD_2, with OVA-alum and analyzed sera for amounts of OVA-specific IgE. As shown in Figure 4C, the amounts of OVA-specific IgE in the sera were similar in WT and *Ptgds*$^{-/-}$ mice. These results indicate that silica- and alum-induced PGE_2 production contributes to the generation of IgE antibodies in vivo. In fact, we found that PGE_2 promoted the production of IgE in spleen cells stimulated with LPS plus IL-4 or anti-CD40 plus IL-4 in vitro (Figure S4D).

Mice that received OVA alone did not exhibit an increase in OVA-specific serum IgE levels and, in fact, exhibited 50-fold lower amounts of OVA-specific IgG1 and IgG2c (Figure 5C and data not shown).

Particulate Nickel Oxide Induces Macrophages to Produce PGE_2, but Not IL-1β, and Enhances IgE production In Vivo

Nickel oxide (NiO) could induce macrophages to produce PGE_2, but not IL-1β or IL-18. NiO is a nanoparticle known to cause lung inflammation when inhaled (Nishi et al., 2009; Ogami et al., 2009). To determine whether NiO could activate macrophages, we

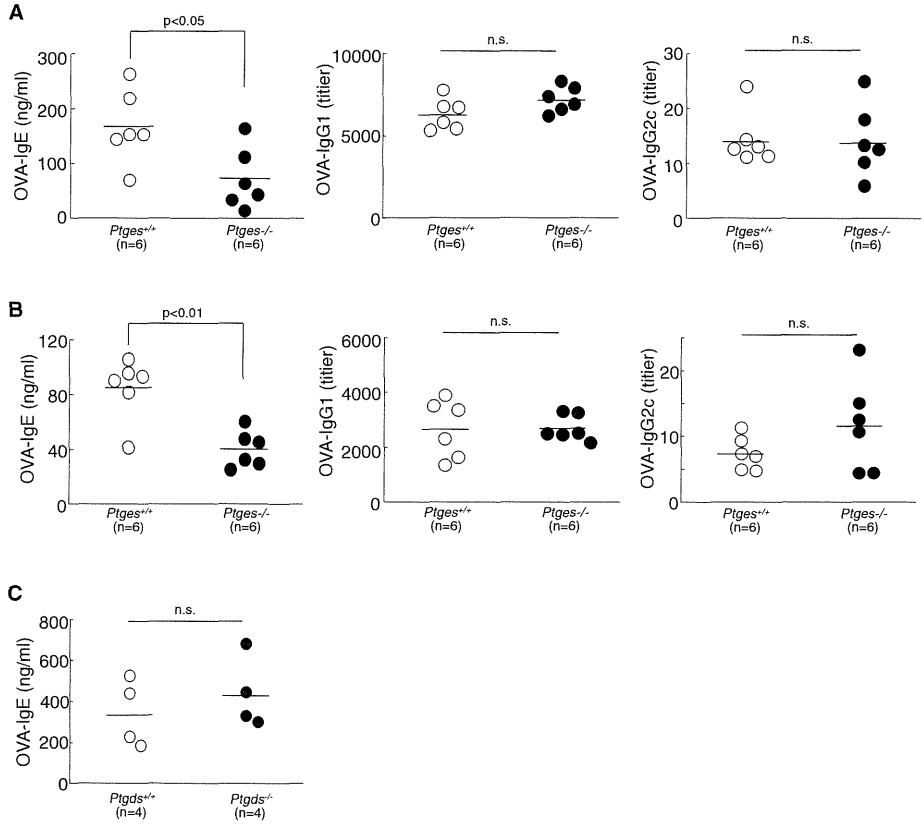

Figure 4. *Ptges*$^{-/-}$ Mice Display Reduced Antigen-Specific IgE Levels after Immunization with Silica or Alum

(A and B) *Ptges*$^{+/+}$ and *Ptges*$^{-/-}$ mice (n = 6) were immunized twice (day 0 and 7) with OVA plus alum (A) or OVA plus silica (B). Ten days after the last immunization, sera were collected and analyzed for OVA-specific IgE, IgG1 and IgG2c antibodies by ELISA.

(C) *Ptgds*$^{+/+}$ and *Ptgds*$^{-/-}$ mice (n = 4) were immunized twice (day 0 and 7) with OVA plus alum. Ten days after the last immunization, sera were collected and analyzed for OVA-specific IgE antibodies by ELISA.

carried out experiments similar to those in which we used silica and alum. We found that, as observed with TiO_2 (Figure 1D), NiO failed to induce IL-1β production in LPS-primed macrophages (Figure 5A). However, NiO-activated macrophages produced significant amounts of PGE_2 at amounts comparable to silica-activated macrophages. As expected, NiO-induced PGE_2 production in macrophages is independent of the NALP3 inflammasome (Figure 5B). In addition, mice immunized with OVA plus NiO exhibited a significant increase in OVA-specific serum IgE concentrations (Figure 5C). In contrast, TiO_2, which did not activate the NALP3 inflammasome or induce PGE_2 production in macrophages in vitro (Figures 1D and 5A), stimulated much weaker IgE responses than immunization with alum and NiO (Figure 5C). These results indicate that particulates that induce PGE_2 production, but not inflammasome activation in macrophages, positively regulate the generation of IgE antibodies in vivo.

Silica-Dependent PGE_2 Production Is Regulated by the Syk and the p38 MAP Kinase Pathway

The mechanisms through which silica, alum, and ATP induce the production of PGE_2 in macrophages are unclear. Therefore, we sought to determine which signaling pathway was involved in the production of PGE_2. To this end, we stimulated LPS-treated macrophages with silica in the presence or absence of various signaling inhibitors, and we then determined which inhibitor(s) suppressed PGE_2 but had no effect on the production of IL-1β. As shown in Figure 6A, only wortmannin suppressed silica-induced production of IL-1β. In contrast, SB203580, U0126, and SP600125 suppressed silica-induced production of PGE_2. We also found that cyclosporin A, rapamycin, and wedelolactone partially suppressed silica-induced PGE_2 production. We conducted similar experiments by using ATP-activated macrophages. SB203580 and wortmannin enhanced ATP-induced IL-1β production, whereas the other inhibitors reduced IL-1β production. With the exception of rapamycin, all the inhibitors reduced PGE_2 production in ATP-activated macrophages (Figure 6B). It is worth noting that SB203580 had no effect on the amounts of COX-2 and PTGES expressed by macrophages (Figure S5A). Taken together, these data suggest that the p38 MAP kinase inhibitor SB203580 preferentially suppresses the production of PGE_2 in both silica- and ATP-activated macrophages. We also sought to ascertain whether silica and ATP

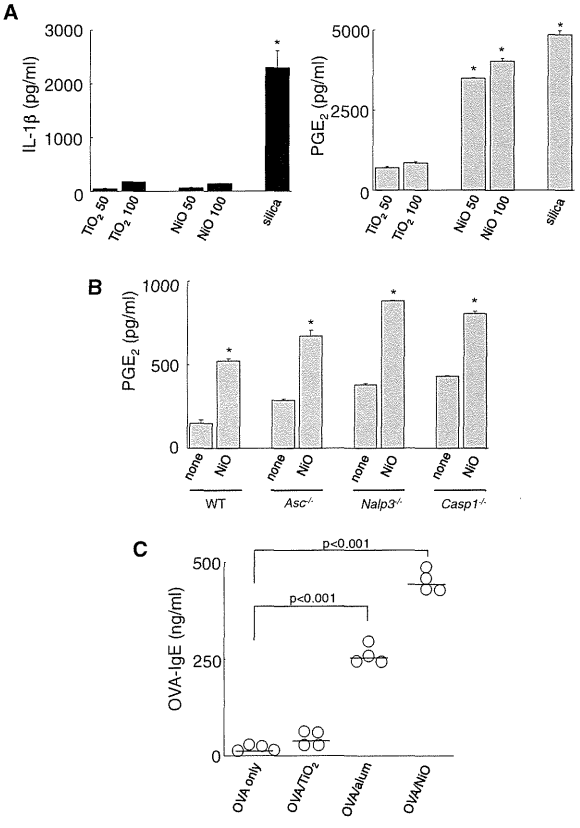

Figure 5. NiO Functions as a Th2 Adjuvant

(A) LPS-primed BALB/c peritoneal macrophages were stimulated with 100 μg/ml silica, 50 or 100 μg/ml NiO, or 50 or 100 μg/ml TiO_2 for 2 hr.

(B) BM-derived macrophages from WT (C57BL/6), *Asc*$^{-/-}$, *Nalp3*$^{-/-}$ or *Casp1*$^{-/-}$ mice were primed with low-dose LPS and then stimulated with 100 μg/ml NiO for 6 hr. Data represent mean ± SE of two (B) or three (A) independent experiments (*$p < 0.01$).

(C) C57BL/6 mice (n = 4) were immunized twice (day 0 and 7) with OVA alone, OVA plus TiO_2, OVA plus alum, or OVA plus NiO. Ten days after the last immunization, sera were collected and analyzed for OVA-specific IgE antibodies by ELISA.

could activate p38 MAP kinase in macrophages and found that stimulation of LPS-primed macrophages with silica and ATP induced p38 MAP kinase phosphorylation and activation (Figure 6C). Several reports have shown that ATP stimulates p38 MAP kinase, which then activates cytosolic phospholipase A_2 ($cPLA_2$) (Gijón et al., 2000; Ulmann et al., 2010). As shown in Figure 6D, treatment with a $cPLA_2$ inhibitor preferentially suppressed PGE_2 production, but not IL-1β production (data not shown), in both ATP- and silica-activated macrophages.

Next, we examined the mechanisms involved in silica-mediated activation of p38 MAP kinase. We have shown that lysosomal rupture by Leu-Leu-OMe activated macrophages to produce PGE_2 (Figure 2E). However, SB203580 significantly suppressed the production of PGE_2 but not IL-1β in Leu-Leu-OMe-treated macrophages (Figure 6E). In addition, treatment of macrophages with poly-2-vinylpyridine-N-oxide (PVNO), which is a lysosomal stabilizing agent (Allison et al., 1966; Von Behren et al., 1983), also suppressed silica-induced PGE_2 production, which was similar to SB203580 treatment (Figure 6F). Treatment with PVNO had no effect on ATP-induced PGE_2 production, given that ATP is not involved in lysosome damage (Hornung et al., 2008). These results indicate that lysosomal damage is involved in PGE_2 production via the activation of p38 MAP kinase in silica-activated macrophages.

Several reports indicate that Syk plays an important role in antifungal responses by activating the NALP3 inflammasome (Gross et al., 2009). In addition, Syk has been shown to be involved in malarial hemozoin-mediated and monosodium urate crystal-mediated inflammasome activation (Ng et al., 2008; Shio et al., 2009). A Syk inhibitor markedly suppressed the production of PGE_2 in both silica- and Leu-Leu-OMe-treated macrophages (Figure 6G). In contrast, Syk inhibition partially suppressed ATP-induced PGE_2 production, suggesting that the signaling pathway involved in PGE_2 production is different between particulate- and ATP-activated macrophages. Syk was partially associated with IL-1β production in silica-stimulated macrophages (Figure 6G). In addition, knockdown of Syk by siRNA in macrophages significantly reduced PGE_2 and IL-1β production compared to cells transfected with control siRNA (Figures S5B and S5C). Syk might act upstream of p38 MAP kinase because Syk inhibition suppressed phosphorylation of p38 MAP kinase in silica-stimulated macrophages (Figure 6H). Taken together, these results suggest that lysosomal damage triggers Syk activation, and then activated Syk upregulates $cPLA_2$ activity via the phosphorylation of p38 MAP kinase.

DISCUSSION

The NALP3 inflammasome has been reported to be activated by alum and involved in alum adjuvanticity and IgE production (Eisenbarth et al., 2008; Kool et al., 2008; Li et al., 2008). However, whether the NALP3 inflammasome is required for alum adjuvanticity is controversial. Here, we found that silica, alum, and ATP, which normally activate the NALP3 inflammasome, stimulate macrophages to produce PGE_2 through mechanisms that do not involve the NALP3 inflammasome. We also found that PGE_2 production by macrophages regulates the generation of antigen-specific IgE antibody in vivo.

The cells of the innate immune system can sense cellular danger and stress via the NALP3 inflammasome. Particulates, such as silica and alum, function as danger signals to activate the NALP3 inflammasome. We found that lysosomal damage and rupture and the subsequent leakage of lysosomal enzymes into the cytoplasm promoted the production of PGE_2 by macrophages. These results demonstrate that the danger signal caused by particulate-induced phagosomal destabilization activates the NALP3 inflammasome and induces macrophages to produce PGE_2. However, *Nalp3*$^{-/-}$, *Asc*$^{-/-}$, and *Casp1*$^{-/-}$ macrophages produced PGE_2 in amounts similar to WT macrophages, suggesting that the lysosomal damage caused by silica and alum activates at least two different pathways, the NALP3 inflammasome pathway and the PGE_2-inducing pathway. We also examined the pathway involved in PGE_2 induction and found that lysosomal damage triggered the production of PGE_2 via the

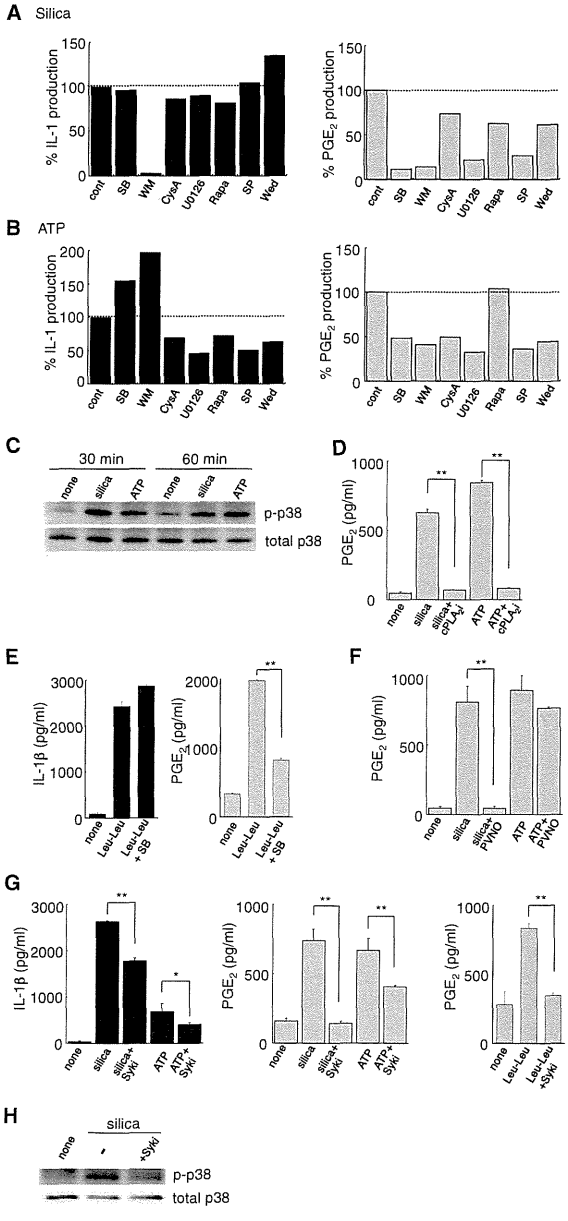

Figure 6. Silica-Induced PGE_2 Production in Macrophages Is Regulated by the Syk/p38 MAP Kinase Pathway

(A and B) LPS-primed BALB/c peritoneal macrophages were stimulated with 100 μg/ml silica (A) or 1 mM ATP (B) for 2 hr in the presence or absence of signaling inhibitors as described in the Experimental Procedures. The results are expressed as the percentage (%) of IL-1β and PGE_2 produced, and the amounts of IL-1β and PGE_2 produced by macrophages stimulated with silica or ATP in the absence of inhibitors were used as the 100% controls.

(C) Macrophages were stimulated with 100 μg/ml silica or 1 mM ATP for the indicated time. Cell lysates were subjected to western blot analysis with anti-phospho-p38 MAP kinase and total p38 MAP kinase antibodies.

(D) LPS-primed macrophages were stimulated with silica or ATP for 2 hr in the presence or absence of 10 μM $cPLA_2$ inhibitor (cPLAi).

(E) LPS-primed macrophages were incubated with 1 mM Leu-Leu-OMe for 2 hr in the presence or absence of p38 MAP kinase inhibitor.

(F) Macrophages were incubated with or without 20 μg/ml PVNO for 5 hr. Then cells were primed with LPS. Primed macrophages were stimulated with silica or ATP for 2 hr.

(G) LPS-primed macrophages were stimulated with 100 μg/ml silica, 1 mM ATP, or 1 mM Leu-Leu-OMe for 2 hr in the presence or absence of 1 μM Syk inhibitor (Syki).

(H) Macrophages were stimulated with 100 μg/ml silica for 30 min in the presence or absence of 1 μM Syk inhibitor. Cell lysates from stimulated macrophages were subjected to western blot analysis. Data represent mean ± SE of two (C and H) or three (A, B, and D–G) independent experiments (*$p < 0.05$, **$p < 0.01$).

activation of Syk and the p38 MAP kinase. Syk is known to play an important role in adaptive immune receptor signaling (Mócsai et al., 2010), and it is involved in malarial hemozoin-mediated and monosodium urate crystal-mediated inflammasome activation (Ng et al., 2008; Shio et al., 2009). Monosodium urate crystals have been reported to induce prostaglandin synthesis in phagocytic cells (Gordon et al., 1985), and we also found that monosodium urate crystals stimulates macrophages to produce PGE_2 and IL-1β in our experimental system (data not shown). In addition, $cPLA_2$ activation and prostaglandin production are regulated by the activation of Syk (Suram et al., 2006). We also found that curdlan, which activates Dectin-1 and Syk, stimulated macrophages to produce higher amounts of PGE_2 in a Syk- and p38 MAP kinase-dependent manner (data not shown). Thus, Syk activation triggered by lysosomal damage promotes the production of PGE_2 in macrophages.

We demonstrated that the p38 MAP kinase pathway is critical for PGE_2 (but not IL-1β) production in silica- and ATP-stimulated macrophages. Several reports have shown that p38 MAP kinase activates $cPLA_2$ and induces arachidonic acid release and PGE_2 secretion in a similar manner as Syk (Gijón et al., 2000; Ulmann et al., 2010). In addition, Syk has been reported to be required for p38 MAP kinase activation under the stress conditions (He et al., 2002). We showed that Syk inhibition suppressed phosphorylation of the p38 MAP kinase. These reports and our findings suggest that Syk activates $cPLA_2$ and PGE_2 production via the p38 MAP kinase pathway in particulate-activated macrophages.

We also found that ATP stimulated macrophages to induce PGE_2 production. However, the required signaling pathway might be different between particulate- and ATP-activated macrophages. Inhibition of the p38 MAP kinase and $cPLA_2$ significantly suppressed PGE_2 production in both silica- and ATP-activated macrophages. In contrast, Syk inhibition partially suppressed ATP-induced PGE_2 production. We have shown that lysosomal damage triggers Syk activation, and ATP has been reported to not be involved in lysosome damage (Hornung et al., 2008). However, ATP stimulation regulates Syk in osteoclasts (Hazama et al., 2009). Hazama et al. and our findings suggest that ATP-activated Syk is partially involved in PGE_2 production in macrophages independent of lysosomal damage. The detailed mechanism involved in lysosomal damage-triggered activation of Syk and p38 MAP kinase remains unclear. In addition, the involvement of cathepsin B in PGE_2 production should be clarified. As such, we are currently investigating which

intracellular factor(s) or event(s) are associated with activation of Syk and p38 MAP kinase in particulate-activated macrophages.

We have herein shown that *Nalp3*$^{-/-}$ and *Casp1*$^{-/-}$ mice produce similar amounts of OVA-IgE to WT mice. These results suggest that NALP3 inflammasome-dependent cytokines, such as IL-1β and IL-18, are not required for particulate-induced IgE responses. Our data in this study demonstrate that inflammasome activation and PGE_2 production are not required for the induction of antigen-specific IgG1 and IgG2c production. We found that treatment of macrophages with PVNO suppressed the production of PGE_2; however, a previous study has reported that treatment with PVNO prevent the adjuvant effect of silica on both IgE and IgG1 antibody production in vivo (Mancino et al., 1983). Taken together, these reports and our findings suggest that factor(s) induced by phagosomal destabilization other than IL-1β, IL-18, and PGE_2 are also associated with alum and silica adjuvanticity. Furthermore, these results also suggest that the properties of individual particulates are very important for the control of acquired immune responses when particulates are used as adjuvants.

The effect of PGE_2 on Th1 and Th2 cell responses is a complex issue. Previous studies have reported that PGE_2 induces type 2 immunity by suppressing the production of cytokines from Th1 cells, macrophages, and DCs (Koga et al., 2009; Kuroda and Yamashita, 2003; Fabricius et al., 2010). However, PGE_2 facilitates the differentiation of Th1 cells in the presence of IL-12 and high doses of CD28 antibody through the activation of the PI3-kinase pathway (Yao et al., 2009). Why does PGE_2 function as an activator of Th1 and Th2 cells? We speculate that the cytokine milieu, containing IL-4 and IL-12, among many other cytokines, influences the effects of PGE_2 on the immune systems. PGE_2 facilitates Th1 cell responses in the presence of IL-12 (Yao et al., 2009). In contrast, herein we show that silica and alum induce macrophages to produce only caspase-1-dependent cytokines and PGE_2, but not other inflammatory cytokines, including IL-12. Similar to the findings of others (Fedyk and Phipps, 1996; Roper et al., 1995), our findings also showed that PGE_2 cooperates with IL-4 to promote IgE production in spleen cells in vitro. Our results suggest that PGE_2 facilitates the generation of either Th1 or Th2 cell responses, depending on the balance of IL-12 and IL-4 amounts.

Our in vivo and in vitro experiments suggest that PGE_2 functions as an activator of IgE production in B cells. Consistent with these results, previous reports have shown that PGE_2 facilitates IgE production in LPS plus IL-4-stimulated B cells (Fedyk and Phipps, 1996; Roper et al., 1995). However, contrary to our findings, Garrone et al. showed that PGE_2 suppresses IgE production in anti-CD40 plus IL-4-stimulated B cells (Garrone et al., 1994). This discrepancy between their results and ours might be due to the different types of cells used. We carried out our experiments with unsorted spleen cells, but Garrone et al. used purified B cells (Garrone et al., 1994). Our findings suggest that non-B cells affected by PGE_2 might stimulate B cells to induce IgE.

In conclusion, we have found that silica and alum stimulate macrophages to produce PGE_2 through a pathway that is dependent on Syk and p38 MAP kinase. PGE_2 generated by this mechanism regulates type 2 immune responses in vivo. Our results suggest that manipulating particulate-induced cytokines and PGE_2 production could open new possibilities for the treatment of allergic inflammation, infectious diseases, and cancer.

EXPERIMENTAL PROCEDURES

Animals

Female C57BL/6 and BALB/c mice were purchased from Charles River Laboratories Japan (Yokohama, Japan). *Ptges*$^{-/-}$ mice were generated by S. Uematsu and S. Akira at Osaka University (Uematsu et al., 2002) and were backcrossed to the C57BL/6 background for five generations; their WT littermates were used as controls. *Ptgds*$^{-/-}$ mice (BALB/c background) were established by Y. Urade at the Osaka Bioscience Institute (Mohri et al., 2006). *Asc*$^{-/-}$, *Nalp3*$^{-/-}$ and *Casp1*$^{-/-}$ mice on C57BL/6 background were described previously (Mariathasan et al., 2004, 2006). *IL1r1*$^{-/-}$ mice were obtained from Jackson Laboratories. All the animal experiments were carried out in accordance to the guidelines for the care and use of animals approved by the University of Occupational and Environmental Health.

Reagents

We used three different types of alum compounds in this study. LSL alum was purchased from Cosmo Bio Co. Ltd. (Tokyo, Japan); alhydrogel was purchased from Sigma Aldrich (St. Louis, MO); and Imject alum was purchased from Pierce (Rockford, IL). All the cytokines except IL-18 were purchased from PeproTech (Rocky Hill, NJ). Recombinant mouse IL-18 and the mouse IL-18 ELISA kit were purchased from MBL (Nagoya, Japan). The cytokine and chemokine ELISA kits were purchased from PeproTech. The PGE_2 and PGD_2-MOX EIA kits were purchased from Cayman Chemical (Ann Arbor, MI). The silica crystals (Min-u-sil 5 silica) were purchased from U.S. Silica (Berkeley Springs, WV). NiO was purchased from Vacuum Metallurgical (Chiba, Japan). TiO_2 and ATP were purchased from Wako Chemical (Osaka, Japan). The PI3 kinase inhibitor wortmannin was purchased from Sigma Aldrich. The caspase-1 inhibitor I (YVAD-CHO), cathepsin B inhibitor (CA-074 Me), p38 MAP kinase inhibitor (SB203580), cyclosporine A, MEK1/2 (Erk) inhibitor (U0126), rapamycin, JNK inhibitor (SP600125), NF-κB inhibitor (wedelolactone), and Syk inhibitor were purchased from Calbiochem (Merck; Darmstadt, Germany). Cytochalasin D was purchased from Enzo Life Science (Plymouth Meeting, PA). Cytosolic PLA_2 inhibitor (Arachidonyl Trifluoromethyl Ketone) was purchased from Cayman Chemical. Leu-Leu-OMe was purchased from Chem-Impex International (Wood Dale, IL). Poly-2-vinylpyridine N-oxide (PVNO) was purchased from Polysciences (Warrington, PA). The following antibodies were used for western blot analysis: anti-GAPDH (Fitzgerald Industries International, Concord, MA); anti-COX-2 and anti-PTGES (mPGES-1) (Cayman Chemical); and anti-p38 MAP kinase and anti-phospho-p38 MAP kinase (Thr180/Tyr182) (Cell Signaling, Danvers, MA). The cells were cultured in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% FBS (BioWhittaker, Walkersville, MD, USA), 2 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin (all from Life Technologies, Rockville, MD, USA).

Cell Preparation

Peritoneal macrophages were generated by injecting mice i.p. with 2 ml 4% thioglycolate broth (Eiken, Tokyo, Japan) and harvested as described previously (Kuroda and Yamashita, 2003). BM-derived macrophages were prepared by culturing BM cells in the presence of 10 ng/ml of M-CSF or GM-CSF as described previously (Kuroda et al., 2007; Kuroda et al., 2009). The purity of the BM-derived macrophages was more than 95% F4/80 and Mac-1 positive as determined by flow cytometric analysis. BM-derived DCs were prepared by culturing BM cells in the presence of 10 ng/ml of GM-CSF for 5 days. $CD11c^+$ cells were then enriched from the culture with the MACS (Miltenyi Biotec, Bergisch Gladbach, Germany). The enriched $CD11c^+$ BM-derived DCs were 80% CD11c pure, as determined by flow cytometry. Human PBMC were purified by density gradient using Lymphoprep (Axis-Sheld Poc AS, Oslo, Norway).

In Vitro Stimulation

In all the experiments, the cells were cultured at a density of 5×10^5/ml/well in 24-well plates (Falcom 3047; BD Biosciences, Franklin Lake, NJ), unless

otherwise specified. Macrophages and BM-derived DCs were primed with or without 1 ng/ml LPS (low dose) for 3 hr and then stimulated with 50 or 100 μg/ml silica, 200 or 400 μg/ml alum, 1 mM ATP, 50 or 100 μg/ml TiO_2, or 50 or 100 μg/ml NiO for 2, 6, or 18 hr. In other experiments, 10 ng/ml of IL-1 or IL-18 or 1 or 2 mM of Leu-Leu-OMe was used instead of silica or alum. In some experiments, inhibitors were added to LPS-primed macrophages together with silica, alum, or ATP. The working concentrations of the inhibitors used were as follows: 10 μM caspase-1 inhibitor I, 10 μM CA-074 Me, 2 μM cytochalasin D, 1 μM NS-398, 10 μM SB203580, 100 nM wortmannin, 100 nM cyclosporin A, 10 μM U0126, 100 nM rapamycin, 10 μM SP600125, 20 μM wedelolactone, 10 μM $cPLA_2$ inhibitor, and 1 μM Syk inhibitor. After stimulation, cell-free supernatants were collected and used for ELISA. In some experiments, macrophages stimulated with 1 μg/ml LPS (high dose) for 6 hr were used as controls. For the PVNO treatment, macrophages were incubated with or without PVNO for 5 hr. Cells were washed and then primed with low-dose LPS. LPS-primed macrophages were stimulated with silica. For the western blot analysis, LPS-primed macrophages were stimulated with silica, alum, or ATP for 30 and 60 min. The cells were then lysed with RIPA lysis buffer and used for western blot analysis as described previously (Kuroda et al., 2009). Human PBMCs were cultured at a density of 1×10^6/ml/well in 24-well plates and primed with 0.1 ng/ml LPS for 3 hr. Primed PBMC were then stimulated with 50 μg/ml silica for 3 hr.

Immunization

The mice were immunized twice (day 0 and 7) i.p. with 100 μg OVA plus 2 mg alum, 100 μg OVA plus 0.5 mg silica or 100 μg OVA plus 0.5 mg NiO in 200 μl of PBS. Ten days after the last immunization, sera were collected and analyzed for the OVA-specific IgE, IgG1, and IgG2c antibodies by ELISA.

ELISA

The amounts of cytokines, chemokines, and PGs were measured with either cytokine/chemokine ELISA kits or PGE_2/PGD_2 EIA kit in accordance with the manufacturer's instructions. The amounts of OVA-IgE in the sera were determined with the DS mouse IgE ELISA (OVA) kit (DS Pharma Biochemical, Osaka, Japan). For the analysis of OVA-IgG1 and IgG2c, serial dilutions of sera were prepared in 96-well plates coated with 10 μg/ml OVA. HRP-conjugated goat anti-mouse IgG1 or IgG2c (Bethyl Laboratories, Montgomery, TX) were used as secondary antibodies. The reciprocal value of serum dilution being absorbance (OD 405 nm) at 0.5 was defined as the titer of antigen-specific serum IgG1 and IgG2c.

Statistical analysis

All the experiments were repeated between two to five times and representative results are shown. The statistical analyses were performed with the Student's t test. p values of < 0.05 were considered statistically significant and marked with an asterisk.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.immuni.2011.03.019.

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