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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

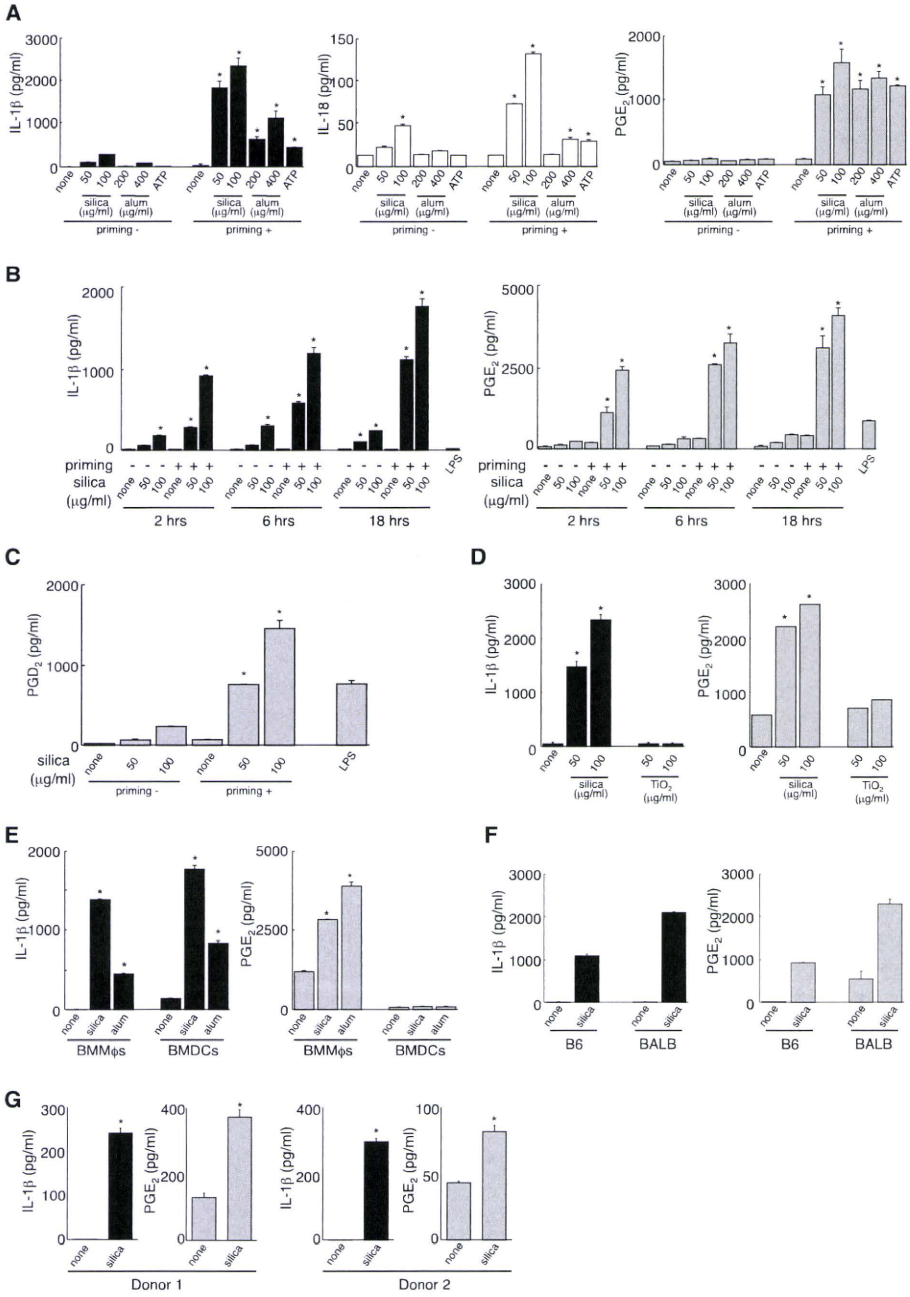


Figure 1. Inflammasome Activators Induce Macrophages to Produce PGE_2

(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 (*$p < 0.01$).

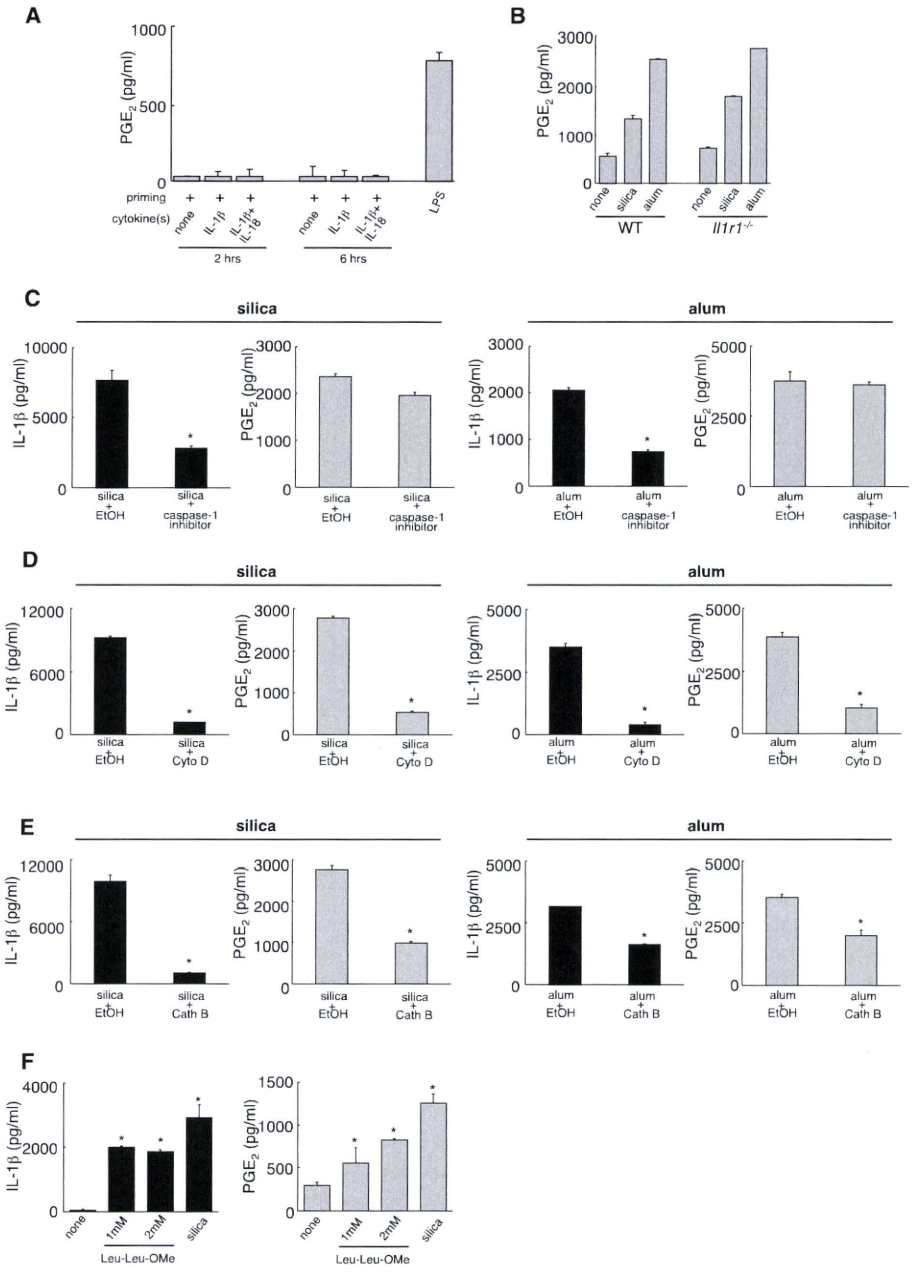


Figure 2. Silica- and Alum-Induced Lysosomal Damage Triggers PGE_2 Production in Macrophages via Caspase-1-Independent Mechanisms

(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

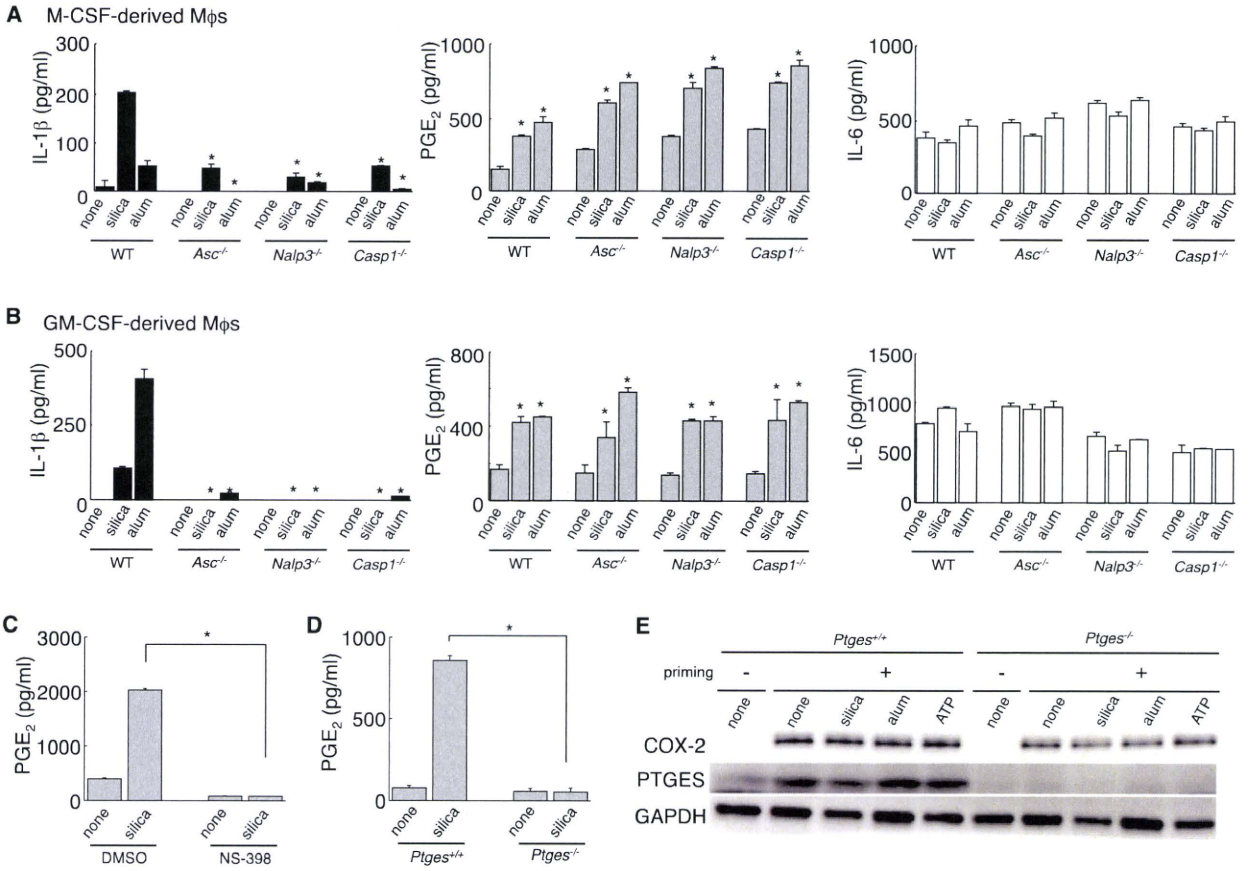


Figure 3. The Mechanisms of Particulate-Induced PGE_2 Production

(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

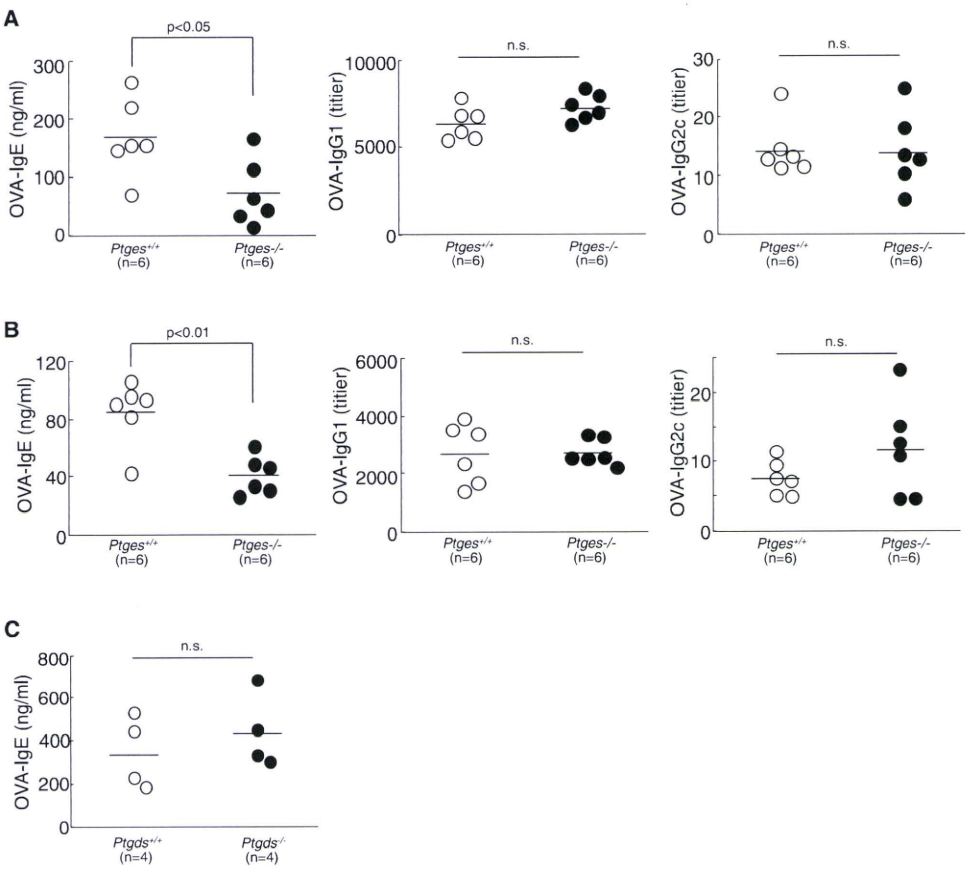


Figure 4. *Ptges*$^{-/-}$ Mice Display Reduced Antigen-Specific IgE Levels after Immunization with Silica or Alum

(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

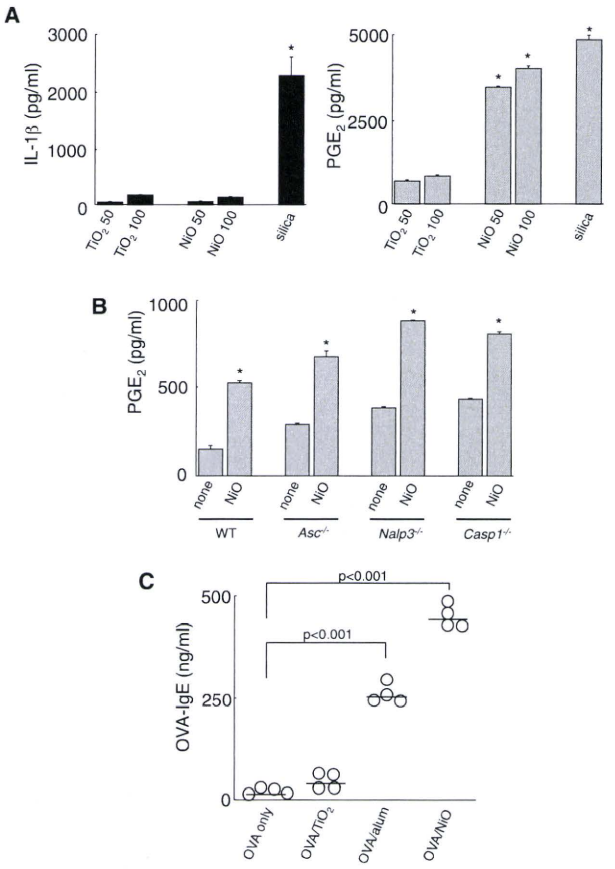


Figure 5. NiO Functions as a Th2 Adjuvant

(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.

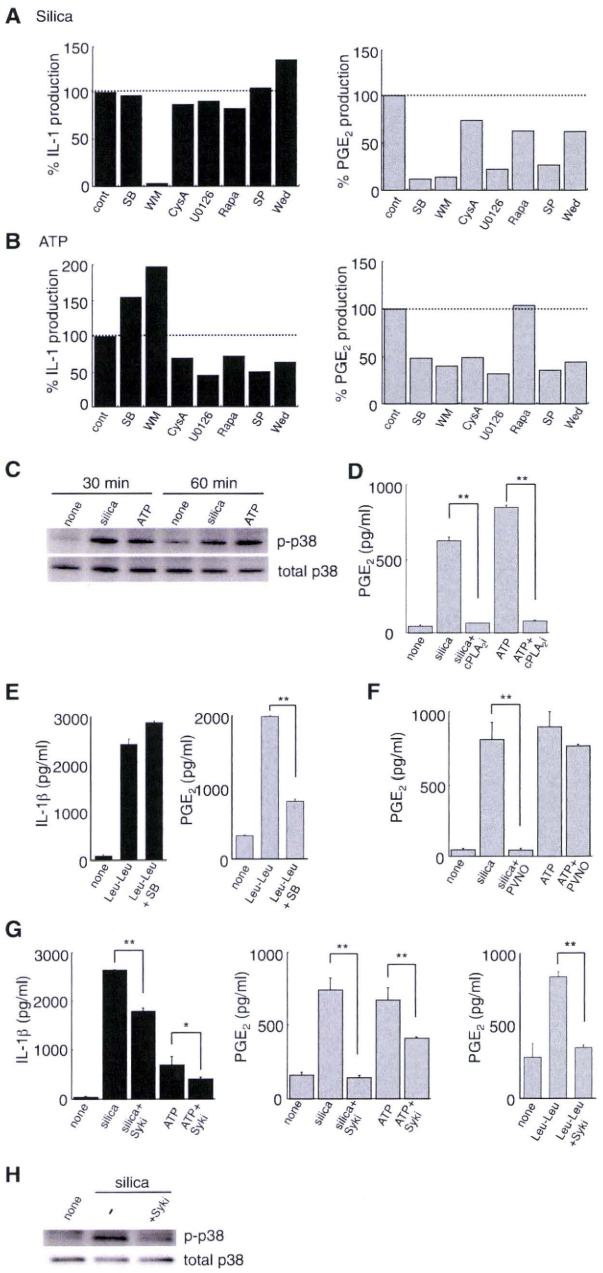
Next, we examined the mechanisms involved in silica-mediated activation of p38 MAP kinase. We have shown that lysosomal rupture by Leu-Leu-OMe activated macrophages to produce PGE_2 (Figure 2E). However, SB203580 significantly suppressed the production of PGE_2 but not IL-1β in Leu-Leu-OMe-treated macrophages (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



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

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 (*$p < 0.05$, **$p < 0.01$).

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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Chapter 12
Immune Recognition of Nucleic Acids and Their Metabolites

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Abstract Recent research suggests that nucleic acids are active modulators of the immune system. RNA and DNA can be detected by specific receptors – the so-called Toll-like receptors, RIG-I-like receptors, and NOD-like receptors. Resultant intra- and intercellular activations of the innate immune system are pivotal in both protective and pathological immune responses during infection and other immunological disorders. Moreover, our immune system is substantially modulated by metabolic intermediates of nucleic acids. Elucidation of such manifold mechanisms involved in immune recognition of nucleic acids and their metabolites offers the possibility of novel nucleic acid-based immunotherapies as well as interventions for nucleic acid-related immune disorders.

12.1 Introduction

Nucleic acids of mammalian cells have long been thought to be immunologically inert as they are normally tightly packed and sequestered within the nucleus. However, recent progress in immunology has shown that nucleic acids and their metabolites are specifically detected by the innate immune receptors, also called Pattern Recognition Receptors (PRRs); these include Toll-like receptors (TLRs), Retinoic Acid Inducible Gene-I (RIG-I)-like Receptors (RLRs), and Nod-like Receptors (NLRs). These receptors are able to distinguish "non-self," such as infectious organisms or damaged host cells, from "self" moieties of host or environmental entities, activating intra- and intercellular signaling pathways of the immune system through receptor-mediated recognition of dangerous "non-self" signatures.

Here, we show how the host immune system recognizes and responds to "non-self" nucleic acids in abnormal conditions, such as microbial infections as well as to "self" nucleic acids in certain immunological disorders, such as autoimmune diseases. We also illustrate some clinical applications of nucleic acids that utilize their immunogenic potential, such as vaccine adjuvants.

12.2 The Fundamental Mechanism to Avoid False Recognition of Harmless Nucleic Acids: The Case of Food Metabolism

Metabolizing food enables us to generate energy through ATP and acetyl CoA production. It also allows us to synthesize nucleic acids for cell proliferation (de novo pathway) and to recycle nucleic acids within food metabolites (salvage pathway). Nucleic acids in foods are metabolized from polynucleotides to nucleosides to free bases, using several kinds of digestive enzymes, including endonucleases, phosphodiesterases, and nucleoside phosphorylases. For instance, in the case of purine nucleotide metabolism, phosphate and ribose are successively separated from purine bodies in food by the above enzymes in the small intestine,

yielding free bases like guanine and hypoxanthine (Ishii and Akira 2008). Free bases can be absorbed for recycling through special transporters on cell surface such as Nucleobase-Cation-Symport-1 (NCS1), the structure of which was recently published (Weyand et al. 2008). Unnecessary bases are degraded to uric acids and eliminated to urine, via xanthine. Neither polynucleotides nor nucleosides can enter cytoplasm unless they are degraded to free bases. While this mechanism seems inefficient, it is a fundamental means of avoiding misidentification of harmless or self nucleic acids inside host cells (Ishii and Akira 2008).

12.3 The Mechanism to Distinguish Healthy "Self" Nucleic Acids

Recent research has shown several unique receptors to recognize nucleic acids and their metabolites (Table 12.1; Figs. 12.1 and 12.2). Some of these receptors are expressed on cell surfaces and some are in cytosol or on endosomal membranes. Some of these receptors are specialized to recognize Pathogen-Associated Molecular Patterns (PAMPs); others detect Danger-Associated Molecular Patterns (DAMPs), which are endogenous products released from damaged host cells (Matzinger 2002; Kono and Rock 2008).

Here, we review how the immune system senses and responds to exogenous detrimental nucleic acids adequately in the case of microbial infections (3.1), controls reactions to endogenous nucleic acids in the case of host cell damage (3.2; 3.3), and misidentifies endogenous nucleic acids in the case of autoimmune diseases (3.4).

Table 12.1 Pattern recognition receptors for nucleic acids and their metabolites

Receptor family	Location	Major ligands	Receptor adaptor
Toll-like receptors (TLRs)	Endosome	ssRNA	TLR7/8 MyD88
		dsRNA	TLR3 TRIF
		Unmethylated CpG in ssDNA, abnormal DNA	TLR9 MyD88
RIG-I-like receptors (RLRs)	Cytosol	5′-triphosphate ssRNA	RIG-I IPS-1
		dsRNA	MDA5 IPS-1
		dsRNA	LGP2 IPS-1
NOD-like receptors (NLRs)	Cytosol	Microbial and synthetic RNA	NLRP3 ASC
Purinergic receptors	Cell surface	Adenosine	P1 None
		Nucleotides (ATP, UTP, ADP, UDP)	P2X None
		ATP	P2Y None
Others	Cytosol	dsDNA	AIM2 ASC
		dsDNA	DAI Unknown

MyD88 myeloid differentiation primary response gene 88, *TRIF* TLR-domain-containing adapter-inducing interferon-β, *RIG-I* retinoic acid-induced gene I, *IPS-1* interferon-β promoter stimulator-1, *MDA5* melanoma differentiation-associated gene-5, *ASC* apoptosis-associated speck-like protein containing a CARD, *AIM2* absent in melanoma 2

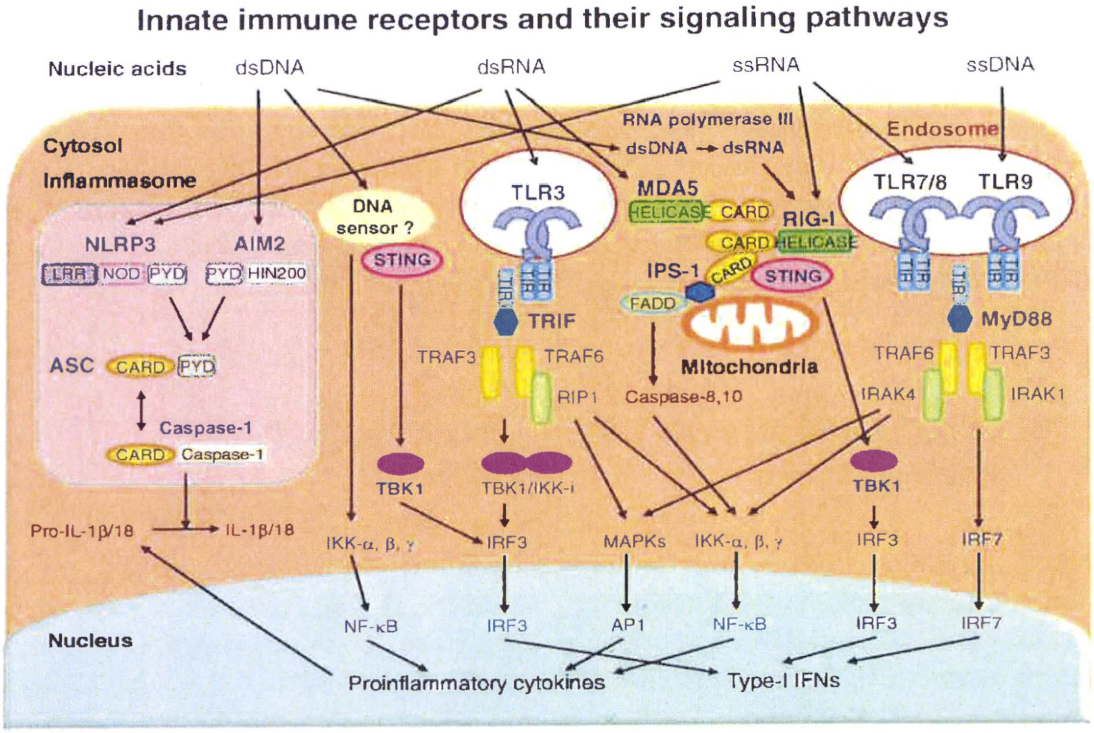

Fig. 12.1 ssRNA and dsRNA are detected by TLR7/8, RIG-I, NLRP3, and TLR3, MDA5, NLRP3, respectively. ssDNA and dsDNA are detected by TLR9 and TBK-dependent unknown DNA sensors, AIM2, RNA polymerase III, respectively. TLR3, TLR7/8, and TLR9 are located in endosomes. TLR3 signals through TRIF. TRIF associates with TRAF3, TRAF6, and RIP1. TRAF3 activates IRF3 via TBK1/ IKK-i, whereas TRAF6 and RIP1 activate MAPKs and NF-κB. TLR7/8 and TLR9 signal through MyD88. MyD88 also associates with TRAF3 and TRAF6. They form a complex with IRAK-4, IRAK-1, and activate IRF7, NF-κB, and MAPKs. RIG-I, MDA5, NLRP3, and AIM2 are located in the cytoplasm. Both RIG-I and MDA5 signal through IPS-1, which interacts with STING and FADD. STING activates IRF3 via TBK1 and FADD activates NF-κB through cleavage of caspase-8/10. NLRP3 and AIM2 associate with ASC. ASC-dependent inflammasome activation induces IL-1β /18 production via caspase-1. RNA polymerase III converts dsDNA into dsRNA and such dsRNA detected by RIG-I. *TBK1* TANK-binding kinase 1, *TRAF* TNF receptor-associated factor, *RIP* receptor-interacting protein, *IRF* Interferon regulatory factor, *IKK* IκB kinase, *NFκB* nuclear factor-kappa B, *MAPKs* mitogen-activated protein kinases, *IRAK* IL-1R-associated kinase; *NFκB* nuclear factor-kappa B; *MAPKs* mitogen-activated protein kinases; primary response gene 88, *STING* stimulator of interferon genes, *FADD* Fas-associated death domain

12.3.1 The Mechanism to Detect Exogenous Nucleic Acids Breaking into Host Cells: The Case of Microbial Infections

The immune response is the host's first-line protection from pathogens. In the case of microbial infections, nucleic acids can be released from microorganisms and infected host cells simultaneously. Therefore, the host has evolved a specialized mechanism to discriminate whether these nucleic acids should be eliminated or ignored, based on their sequences, structures, or modifications (Akira et al. 2006;

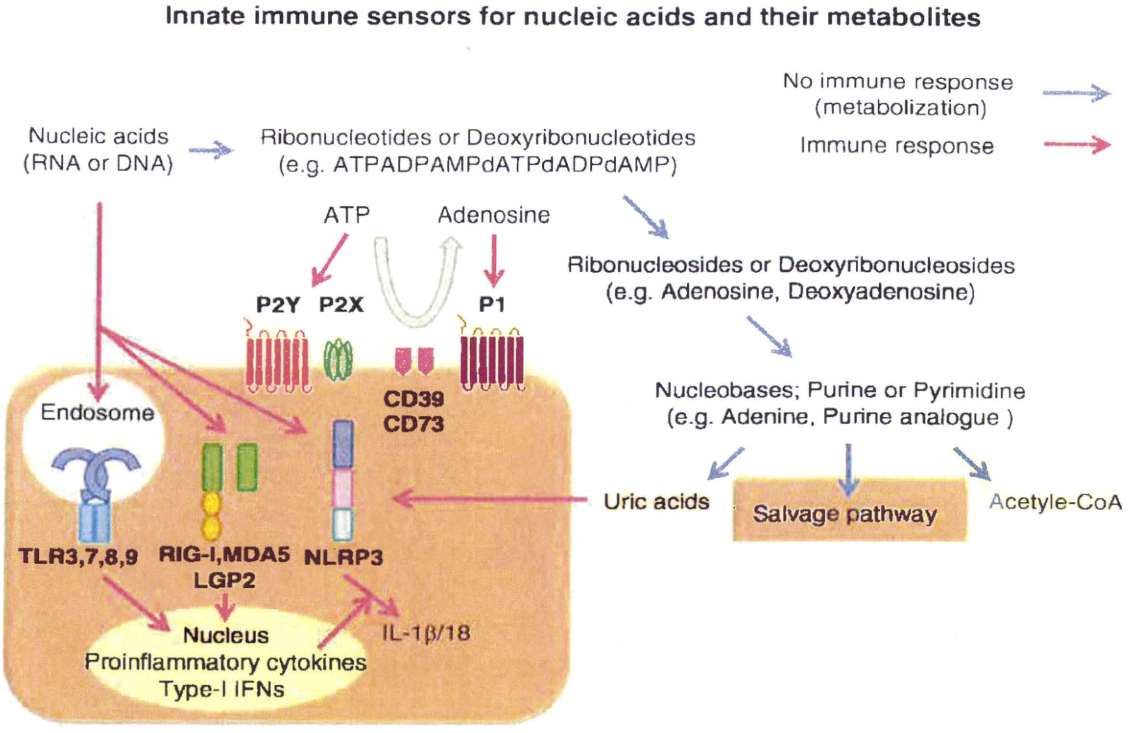

Fig. 12.2 Metabolization (*blue arrow*): Nucleic acids in foods are metabolized from polynucleotides to nucleosides to free bases, using several kinds of digestive enzyme. Free bases can be used for acetyl CoA production (energy) or recycling (salvage pathway) or degraded to uric acids and eliminated to urine. Immune response (red arrow): Nucleic acids and their metabolites can be released from damaged or dying host cells in the case of infection or tissue injury. For example, viral RNA or DNA can be directly detected by endosomal TLRs and cytoplasimic RLRs and NLRs. In addition, aberrant high concentration of extracellular nucleotides incuding ATP, adenosine, and their metabolic end products, uric acids, can be detected by innate immune sensors containing NLRs, ATP-gated P2 receptor, and G protein-coupled P1 receptors as danger signals. These responses mainly culminate in IL-1β/18 production via inflammasome activation. Adenosine is degraded from ATP through a cascade of ectonucleotidases, including nucleoside triphosphate diphosphorylase (NTPDase, also called CD39) and 5′-ectonucleotidase (Ecto50NTase, also called CD73)

Ishii et al. 2008b). Here, we illustrate several systems involved in the recognition of exogenous nucleic acids through immune receptors, including TLRs, RLRs, and NLRs.

12.3.1.1 Endosomal Recognition for Exogenous Nucleic Acids via TLRs

Currently, 11 members of the TLR family have been identified as functional receptors in mammals. Among them, TLR-3, -7, -8, and -9 have been shown to recognize nucleic acids within early endosomes (Takeda and Akira 2005). TLRs have ectodomains that contain variable numbers of Leucine-Rich-Repeat (LRR) motifs and a cytoplasmic signaling domain termed the Toll/IL-1R homology (TIR) domain. LRR motifs are responsible for sensing nucleic acids; the crystal structures

of TLR-3 (but not TLR-7, -8 or -9) have been shown to have horseshoe-like solenoid shapes (Choe et al. 2005; Leonard et al. 2008). TLR-3 recognizes double-stranded (ds) RNAs, which can be intermediates for single-stranded (ss) RNA viral replication, symmetrical transcription byproducts of DNA viruses, or synthetic compounds (e.g., poly IC) (Alexopoulou et al. 2001). TLR-3 is expressed in immune cells, including Dendritic Cells (DCs) and macrophages; it is also expressed in nonimmune cells such as epithelial cells, and it is inducible through type-I IFN response (Alexopoulou et al. 2001; Tissari et al. 2005). Double-stranded RNA recognition via TLR-3 can occur when virus-infected cells are phagocytosed by immune cells (Schulz et al. 2005). TLR-3 signals through the adaptor molecule TIR domain containing adaptor inducing IFN-β (TRIF, also known as TICAM1) (Yamamoto et al. 2003; Oshiumi et al. 2003). TRIF associates with tumor necrosis factor-receptor associated factor (TRAF)-3, TRAF-6, and receptor-interacting protein (RIP)-1. This complex activates NF-κB via IκK-complex, AP-1 via MAPK, and Interferon Response Factor (IRF)-3 via a complex of the TRAF family member-associated NK-κB activator-Binding Kinase 1 (TBK1) with Inducible IκB Kinase (IκKi), initiating pathways that culminate in proinflammatory cytokines and IFN-β production (Akira and Takeda 2004) (Fig. 12.1).

TLR-7 and -8 recognize uridine-rich or uridine/guanosine-rich ssRNA of virus (e.g., influenza virus, human immunodeficiency virus) and synthetic compounds (e.g., imidazoquinolines) (Diebold et al. 2004; Jurk et al. 2002). The expression of TLR-7 is limited to B cells and DCs in human and mice, while TLR-8 functions primarily in human monocytes and myeloid DCs (Hornung et al. 2008). Both genes are located on the X chromosome. TLR-9 preferentially recognizes unmethylated CpG motifs (lack of cytocine methylation) in microbial DNA, which is thus discernable from highly methylated CpG motifs in mammalian DNA (Hemmi et al. 2000; Krieg 2002; Wagner 2004; Klinman 2004); TLR-9 binding to its cognate ligand induces a conformational change that activates the downstream intracellular signaling pathway (Latz et al. 2007). Sequence restriction of TLR-9 recognition varies as unmethylated CpG motifs are needed to activate TLR-9 only in ssDNA with phosphorothionate backbones, whereas natural phosphodiester DNA can activate TLR-9 independently of CpG motifs (Haas et al. 2008). In addition, TLR-9 activation by synthetic oligonucleotides (ODNs) is dependent on cell types (Ishii et al. 2004) and secondary structures of ODNs, such as aggregated forms (Kerkmann et al. 2005; Hou et al. 2008). The expression of TLR-9 is restricted to Plasmacytoid DCs (pDCs) and B cells in humans, while expression of mouse TLR-9 is broader, as TLR-9 is highly expressed in murine myeloid DCs and macrophages (Kadowaki et al. 2001; Bauer et al. 2001; Hornung et al. 2002).

The proteins TLR-7, -8, and -9 share a common signaling pathway through a TIR domain – containing adaptor molecule, Myeloid Differentiation Factor 88 (MyD88). MyD88 associates with TRAF3/6 and interleukin-1-receptor-associated kinase (IRAK)-1/4. This complex activates NF-κB, AP-1, and IRF7 via IκK-complex, MAPK and IκKα, respectively, and culminates in proinflammatory cytokines and IFN-α/β production (Akira and Takeda 2004). IRF7 is constitutively expressed in pDCs and is especially involved in inducing massive type-I IFNs response via