

Airway peptidoglycan and immunostimulatory DNA exposures have divergent effects on the development of airway allergen hypersensitivities

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Background: Environmental exposures to toll-like receptor (TLR) ligands have been suggested to provide immunologic protection against allergic diseases. However, some TLRs use unique intracellular signaling pathways, suggesting that ambient TLR ligand exposures might induce a range of host responses.

Objective: These investigations compared peptidoglycan (PGN; TLR2)–induced and immunostimulatory sequence DNA oligodeoxynucleotide (ISS-ODN; TLR9)–induced innate responses and determined how airway exposures to these TLR ligands affect adaptive immunity and the asthmatic phenotype.

Methods: In vitro and in vivo studies innate responses to PGN and ISS-ODN were compared. Alternatively, mice were intranasally immunized with ovalbumin (OVA) alone or OVA plus PGN or ISS-ODN, and adaptive immune profiles and responses to airway OVA challenge were assessed.

Results: PGN and ISS-ODN induced divergent innate responses predictive of their having T_H2- and T_H1-biasing adjuvant potential, respectively. Consistent with these findings, mice intranasally immunized with OVA alone had relatively weak adaptive responses, whereas intranasal OVA/PGN- and OVA/ISS-ODN-coimmunized mice had much stronger humoral and cellular responses that were T_H2 and T_H1 biased, respectively. Finally, on airway allergen challenge, mice intranasally immunized with OVA alone had modest T_H2-biased airway hypersensitivity responses, whereas airway responses were greatly exaggerated for intranasal OVA/PGN-immunized mice. In contrast, intranasal OVA/ISS-ODN-immunized mice had little evidence of airway hypersensitivity after airway allergen challenge.

Conclusions: Considered in a larger context, these results suggest that inspired air is likely to contain TLR ligands capable of both preventing and precipitating the asthmatic phenotype. (*J Allergy Clin Immunol* 2004;113:448-54.)

Key words: Toll-like receptor 2, toll-like receptor 9, peptidoglycan, immunostimulatory sequence oligodeoxynucleotide, CpG motif, asthma, intranasal vaccination, hygiene hypothesis

Our present understanding of asthma suggests that a breakdown in the tolerogenic tone of the airways and associated lymphoid tissues underlies its pathogenesis.¹ Although the basis for this immunologic dysregulation is uncertain, several lines of investigation suggest that in addition to aeroallergens themselves, other inhaled factors are likely to contribute to the development of airway tolerance, hypersensitivities, or both.^{2,3} In this respect toll-like receptor (TLR) ligands are of particular interest because they are produced by a wide range of microbes, are present in ambient air, and have robust immunostimulatory activities.⁴⁻⁶

Previously, we and others reported that immunostimulatory oligodeoxynucleotide (ISS-ODN; a synthetic analogue of microbial DNA and TLR9 ligand) is a potent T_H1 adjuvant effective in both preventing and reversing T_H2-biased immune deviation and the asthmatic phenotype in mice.⁷⁻¹⁰ However, there are 10 identified TLRs and a much larger number of TLR ligands.⁴ Furthermore, although TLRs share elements of a common signaling pathway, alternative pathways exist that are not shared by all TLRs.⁴ Moreover, select ligands have been shown to recruit several TLRs to the signaling platform. These observations suggest that TLR ligands have the potential to induce divergent immune responses as a function of divergence in the signaling cascades they activate in responsive cells.

In line with this perspective, published investigations demonstrate that ligands for TLR2 and TLR4 elicit unique dendritic and mast cell responses, with TLR4 stimulation inducing stronger IL-12 production from dendritic cells (DCs), and TLR2 stimulation favoring mast cell degranulation and T_H2 cytokine production.¹¹⁻¹³ Other studies suggest that TLR2 ligands preferentially induce T_H2-biased adaptive responses.^{14,15} Recently, *Escherichia coli* LPS (TLR4) was reported to induce T_H1 and T_H2 responses to intranasally coadministered ovalbumin (OVA) when used at high and low doses, respectively¹⁶; low-dose LPS vaccinations also primed mice for development of the asthmatic phenotype. Consistent with these studies, epidemiologic investigations have correlated high home LPS levels with

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Abbreviations used

APC:	Antigen-presenting cell
BALF:	Bronchoalveolar lavage fluid
BMDDC:	Bone marrow–derived dendritic cell
DC:	Dendritic cell
ISS-ODN:	Immunostimulatory oligodeoxynucleotide
mDC:	Myeloid dendritic cell
OVA:	Ovalbumin
pDC:	Plasmacytoid dendritic cell
PGN:	Peptidoglycan
TLR:	Toll-like receptor

protection from atopic manifestations and low home LPS levels with their development.^{2,3} Along with LPS, TLR2 ligands and microbial DNA are known to be ubiquitous in the environment.^{5,6} However, although LPS and ISS-ODN have been investigated, it remains to be determined whether airway TLR2 ligand exposures can influence the development of aeroallergen hypersensitivities.

Because the bioactivities of peptidoglycan (PGN) and ISS-ODN have been found to be TLR2 and TLR9 dependent, respectively,^{17–19} in the present investigations we compared the effects of PGN and ISS-ODN on innate and adaptive immunity and host development of airway hypersensitivities. Innate responses to PGN and ISS-ODN were characterized by distinct patterns of cytokine production and costimulatory molecule expression. Moreover, intranasal OVA–immunized mice had weak but T_H2-biased immune and pulmonary hypersensitivity responses, whereas intranasal OVA/PGN–vaccinated mice had substantially stronger T_H2-biased adaptive and airway challenge responses. In contrast, mice intranasally immunized with OVA and ISS-ODN had highly T_H1-biased responses but little evidence of pulmonary hypersensitivity after airway allergen challenge. These results demonstrate that PGN and ISS-ODN induce qualitatively different innate antigen-presenting cell (APC) responses that are predictive of the T_H2- and T_H1-biased adaptive responses induced by these TLR ligands, respectively. Moreover, these findings highlight the potential of some TLR ligands, including PGN, to precipitate the development of asthma.

METHODS

Reagents and mice

HPLC-purified OVA was purchased from Worthington Laboratories, phosphorothioate ISS-ODN (5'-TGACTGTGAACGTTG GAGATGA-3') was synthesized by Trilink Biotechnologies, and purified *Staphylococcus aureus* PGN was purchased from Toxin Technology. The endotoxin contents of the reagents used in these studies were determined with a commercial kit (Bio-Whittaker). Four- to 6-week-old female BALB/c mice (Jackson Laboratories) were maintained in the University of California, San Diego, Animal Facility, which is certified by the American Association for the Accreditation of Laboratory Animal Care.

In vitro derivation of DCs

Mouse femurs were harvested with sterile techniques. Bones were flushed with complete media (RPMI; 1 mmol/L Na pyruvate, penicillin/streptomycin, 10% heat-inactivated [low LPS] FCS, and GM-CSF; R&D Systems) at 5 ng/mL. Cells were spun down,

washed, counted, and resuspended at 2×10^5 cells/mL in media and cultured in large petri dishes. Fresh media and GM-CSF were added on day 3, and bone marrow–derived dendritic cells (BMDDCs) were harvested for experiments on day 6.

PGN- and ISS-ODN–induced cytokine and chemokine responses

BMDDCs were cultured in triplicate at 1×10^6 cells/mL in media alone or supplemented with PGN or ISS-ODN. Supernatants from cultures were harvested at 24 hours for cytokine ELISA. Alternatively, mice were lightly anesthetized (isoflurane, Abbott Laboratories) before intranasal delivery of PGN (100 μ g) or ISS-ODN (10 μ g) in 30 μ L of PBS or PBS alone. Twenty-four hours later, mice were killed, and lungs were lavaged with 800 μ L of PBS. Bronchoalveolar lavage fluid (BALF) samples were centrifuged, and supernatants were collected for analysis. Standard ELISA techniques were used to determine IL-6, IL-12p40 (Pharmingen), and keratinocyte-derived chemokine (KC; R&D Systems) levels in culture and BALF supernatants.

PGN- and ISS-ODN–induced airway inflammation

BALF cell pellets obtained from intranasal PGN- and ISS-ODN–challenged mice were resuspended in 1 mL of PBS, and total cell counts were determined with a hemocytometer. Additionally, aliquots were cytospun onto slides and Wright–Giemsa stained for determination of BALF differentials by a blinded observer counting a minimum of 200 cells per slide from random high-power fields.

Flow cytometric analyses of PGN- and ISS-ODN–activated DCs

Day 6 BMDDCs were cultured in media alone or with PGN (100 μ g/mL) or ISS-ODN (10 μ g/mL) for 24 hours, and cells were harvested. Alternatively, lungs were harvested from mice 24 hours after intranasal TLR ligand challenge. Lungs were incubated in digestion media (Collagenase VIII-300 U/mL, Sigma; DNase I-1.5 μ g/mL, Sigma) for 1 hour. Mononuclear cells were further purified by means of centrifugation over lympholyte-M (Cederlane Laboratories). TLR ligand–activated cells were stained with APC-linked CD11c, FITC-linked CD11b or B220, and phycoerythrin-linked CD40, CD80, CD86, or OX40L or isotype control mAbs purchased from Pharmingen, except OX40L (eBioscience) and B220 (Caltag) mAbs. Costimulatory molecule expression levels and DC subset frequencies were determined with a FACSCalibur flow cytometry unit, recording 50,000 events per sample.

Immunizations and asthma challenges

Mice were anesthetized and intranasally immunized with OVA (50 μ g) alone or with PGN (10, 100, or 1000 μ g) or ISS-ODN (1, 10, or 100 μ g) in 30 μ L of saline divided equally between the nares. Alternatively, mice were subcutaneously immunized with OVA and alum (1 mg). Mice underwent airway allergen challenge 3 weeks after their last intranasal immunization, as previously described.⁷ Airway allergen challenge consisted of intranasal delivery of OVA (5 μ g) on 2 occasions spaced 5 days apart. Twenty-four hours after the second dose of intranasal OVA, mice were exposed to increasing concentrations of nebulized methacholine for periods of 3 minutes each, followed by measurements of enhanced pause (Penh; mean over the subsequent 3 minutes) in a single-chamber whole-body plethysmograph (Buxco). Methacholine responses are presented as percentages of baseline enhanced pause values when mice were exposed to nebulizer PBS. After methacholine challenge, mice were killed, BALF samples were collected, and total cell counts and differentials were determined.

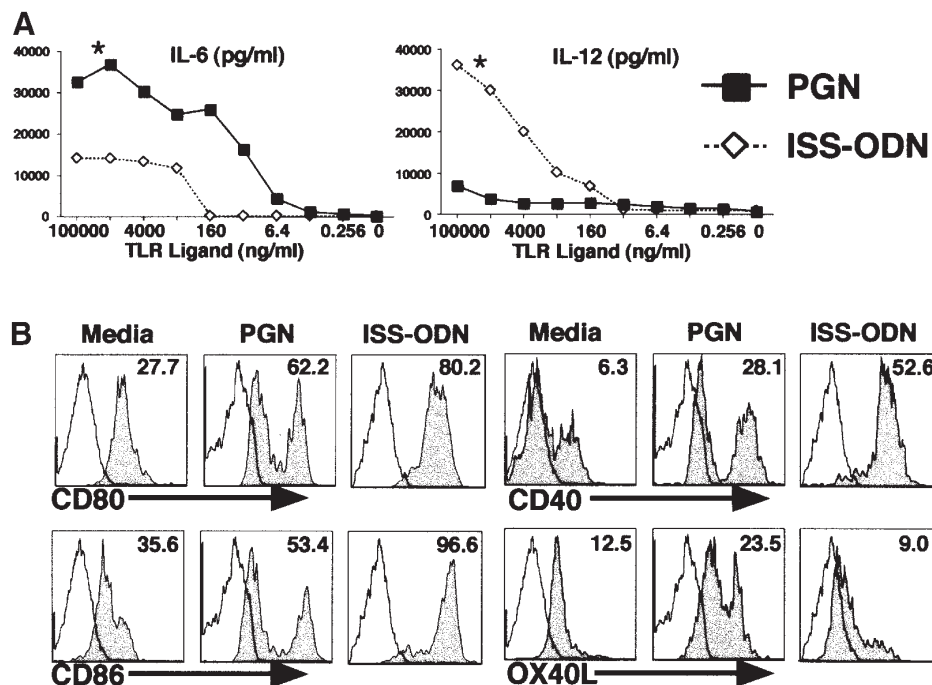


FIG 1. PGN- and ISS-ODN-induced BMDDC responses. **A**, BMDDCs were cultured in media alone or with PGN or ISS-ODN (0.256–100,000 ng/mL). Culture supernatants were harvested at 24 hours for cytokine determinations (* $P < .05$ for PGN vs ISS-ODN). **B**, BMDDCs were cultured in media alone or with PGN (100 μ g/mL) or ISS-ODN (10 μ g/mL). After 24 hours, costimulatory molecule expression on CD11c⁺ cells was quantified as the mean fluorescence intensity ratio (MFI_R), which was calculated by dividing the mean fluorescence intensity for specific staining (shaded) by the mean fluorescence intensity for the isotype control (open). Results are reflective of 3 similar experiments.

OVA-specific Ig and cytokine responses

Blood samples were obtained 3 weeks after the last intranasal immunization for OVA-specific IgG2a and IgE determinations by ELISA, as previously described.^{7,20} All samples were compared with high-titer anti-ova IgG2a and IgE standards (end point titration greater than 50,000 for IgG2a and equal to 1024 in the case of IgE) given arbitrary concentrations of 20,000 U/mL for IgG2a and 1000 U/mL for IgE. OVA-specific cytokine responses were also determined by using previously described methods.^{7,20} Briefly, splenocytes were prepared from immunized mice by gently teasing spleens to make single-cell suspensions. Splenocytes were cultured in triplicate at 2.5×10^6 cells/mL in complete media with or without OVA (50 μ g/mL) for 72 hours before harvesting supernatants. IL-4, IL-5, IL-13, and IFN- γ levels in culture supernatants were determined by ELISA with Pharmingen reagents.

Statistics

Statistical analyses were conducted with Statview software (Abacus Concepts). Two-tailed unpaired Student *t* tests were conducted to analyze all data except for TLR dose-response cytokine profiles and methacholine hypersensitivity, for which ANOVA was used.

RESULTS

PGN and ISS-ODN induce divergent innate responses in vitro

Initially, we determined whether PGN and ISS-ODN induced qualitatively different cytokine responses (Fig 1, A). PGN-stimulated BMDDCs produced up to 3-fold more IL-6 and BMDDCs cultured with ISS-ODN pro-

duced up to 5-fold more IL-12 over a wide range of TLR ligand concentrations. Similar results were obtained with PGN- and ISS-ODN-activated splenocytes (Fig E1 in OnLine Repository at www.mosby.com/jaci). In addition, ISS-ODN-activated, but not PGN-activated, splenocytes produced IFN- γ (Fig E1). Studies of costimulatory molecule expression demonstrated that compared with unstimulated BMDDCs, BMDDCs activated by PGN expressed higher levels of CD80, CD86, and CD40, and their expression levels were higher still on ISS-ODN-activated BMDDCs (Fig 1, B). In contrast, PGN induced higher levels of BMDDC OX40L expression than ISS-ODN. The innate responses just described were unlikely to be due to LPS contamination because endotoxin was not detected in 100,000 ng/mL solutions of PGN and ISS-ODN, the highest concentrations used. In additional studies, we found that BMDDCs produced from TLR2 and TLR9 knockout mice did not synthesize cytokines when cultured with PGN and ISS-ODN, respectively (data not shown), confirming previous reports of PGN being TLR2 dependent and ISS-ODN being TLR9 dependent in their immunostimulatory activities.^{17–19}

PGN and ISS-ODN induce divergent innate responses in vivo

Naive mice were intranasally challenged with PGN, ISS-ODN, or vehicle alone, and 24 hours later, BALF and lung tissue were collected for analysis. Compared

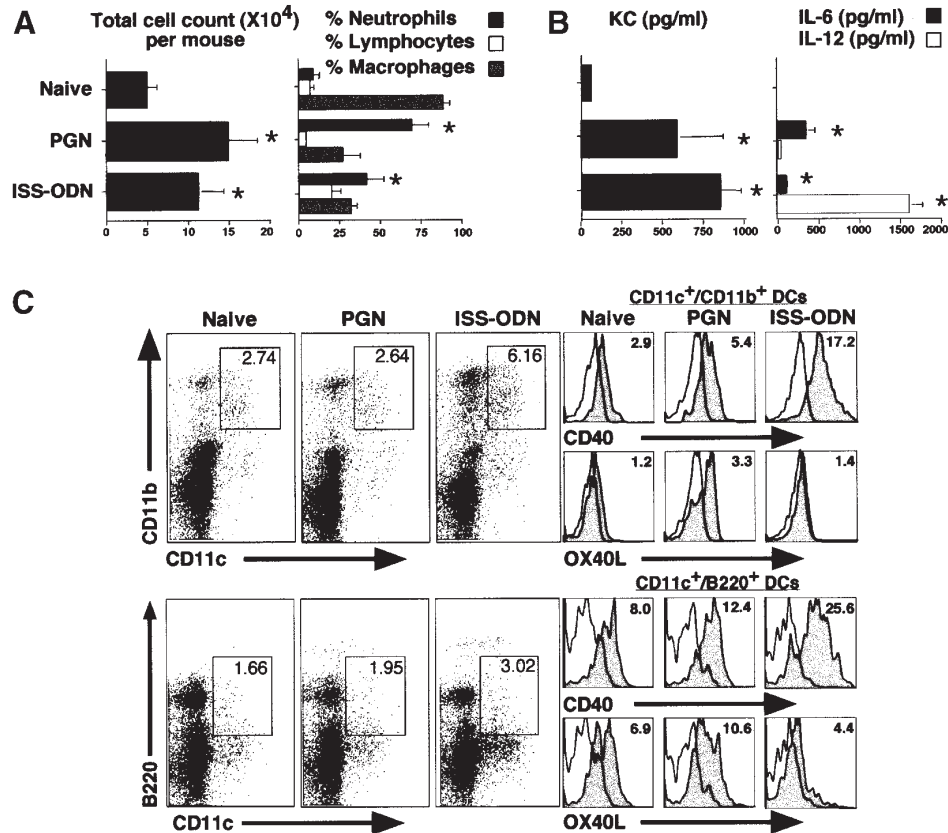


FIG 2. PGN- and ISS-ODN-induced airway responses. Mice (4 per group) received intranasal PGN (100 μ g) or ISS-ODN (10 μ g) or vehicle alone. Twenty-four hours later they were killed, and BALF total cell counts and differentials (**A**) and BALF KC and cytokine concentrations (**B**) were determined (* P < .05 for TLR ligand-treated vs control mice). **C**, Lung digest mononuclear cells were stained with fluorescence-conjugated CD11c, CD11b, or B220 and CD40, OX40L, or appropriate isotype control mAbs. DC subtype frequencies are presented in dot plots (left), whereas mean fluorescence intensity ratios (described in Fig 1) for DC costimulatory molecule expression are presented in the histograms on the right (specific mAb, shaded; isotype control mAb, open).

with BALF from control mice, BALF from intranasal PGN- and ISS-ODN-exposed mice had 2- to 3-fold more cells (Fig 2, A) and averaged 69% and 41% neutrophils, respectively, whereas BALF recovered from control mice had 8% neutrophils. In addition, BALF from intranasal PGN and ISS-ODN treated mice had markedly increased levels of KC (Fig 2, B), a known neutrophil chemoattractant.²¹ BALF samples from intranasal PGN treated mice were also found to have 4-fold higher levels of IL-6 than BALF from intranasal ISS-ODN treated mice, whereas IL-12 was consistently detected only in the BALF of intranasal ISS-ODN treated mice.

Additional analysis of lung digests revealed that on average, lungs of control mice ($n = 3$) yielded 7.2×10^6 cells, whereas after intranasal PGN and ISS-ODN delivery, cell yields increased to 17.1×10^6 and 19.7×10^6 per mouse, respectively. Moreover, although the relative lung frequencies of myeloid (CD11c⁺/CD11b⁺) and plasmacytoid (B220⁺/CD11c⁺) DCs (mDCs and pDCs, respectively) in intranasal vehicle and PGN treated mice were similar, their frequencies were consistently higher in

intranasal ISS-ODN treated mice (Fig 2, C). mDC and pDC identities were confirmed in these studies by their lack of Gr1 (expressed on granulocytes) and CD19 (expressed on B cells) expression, respectively (data not shown). Finally, consistent with our in vitro studies, lung mDCs and pDCs from mice exposed to intranasal PGN were found to express increased levels of CD40 compared with DCs from control mice, and CD40 expression levels were higher still on lung mDCs and pDCs from intranasal ISS-ODN treated mice. In contrast, lung mDCs and pDCs from intranasal PGN treated mice expressed higher levels of OX40L than their counterparts from intranasal vehicle- and ISS-ODN treated mice.

Intranasal OVA vaccinations with PGN and ISS-ODN lead to divergent immune responses

In studies of adaptive immunity, intranasal OVA immunization without TLR ligand induced weak but relatively T_H2 -biased immune responses (Table I). In com-

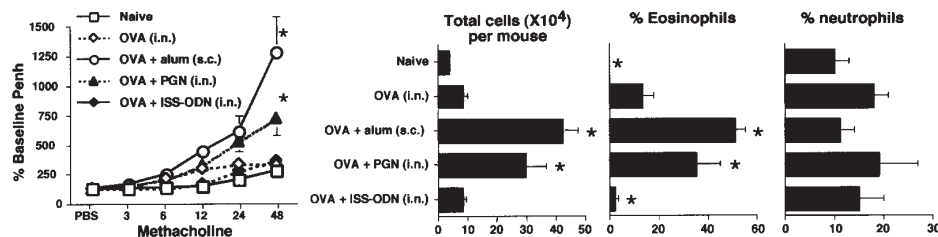


FIG 3. Airway challenge responses of intranasal OVA/PGN- and intranasal OVA/ISS-ODN-immunized mice. Mice (4 per group) were intranasally immunized with OVA (50 μ g) alone or with OVA plus PGN (100 μ g) or ISS-ODN (10 μ g) or were subcutaneously immunized with OVA and alum (1 mg). Three weeks after their last immunizations, mice were intranasally challenged with OVA. Methacholine sensitivity was determined 24 hours after the last intranasal OVA challenge, and BALF cell counts and differentials were determined with samples collected immediately after methacholine challenge. Results are reflective of 4 similar experiments (* P < .05 for intranasal OVA-vaccinated mice vs all other groups).

TABLE I. Intranasal OVA/PGN and OVA/ISS-ODN immunization-induced systemic responses

Adjuvant	IgG2a (U/mL)	IgE (U/mL)	IFN- γ (pg/mL)	IL-4 (pg/mL)	IL-5 (pg/mL)	IL-13 (pg/mL)
None	12 $\pm$ 8	262 $\pm$ 84	ND	32 $\pm$ 23	ND	211 $\pm$ 92
Alum (1 mg)	209 $\pm$ 90	4885 $\pm$ 1755*	ND	474 $\pm$ 145*	2100 $\pm$ 392*	6780 $\pm$ 1738*
PGN (10 μ g)	23 $\pm$ 23	ND	325 $\pm$ 216	36 $\pm$ 17	483 $\pm$ 277	ND
PGN (100 μ g)	29 $\pm$ 19	1498 $\pm$ 293*	137 $\pm$ 42	179 $\pm$ 49	1218 $\pm$ 651*	1802 $\pm$ 216*
PGN (1000 μ g)	68 $\pm$ 42	1694 $\pm$ 248*	ND	302 $\pm$ 86*	938 $\pm$ 243*	5050 $\pm$ 1731*
ISS-ODN (1 μ g)	45,325 $\pm$ 3465*	86 $\pm$ 86	1971 $\pm$ 601*	ND	ND	ND
ISS-ODN (10 μ g)	28,007 $\pm$ 7750*	ND	5424 $\pm$ 590*	ND	ND	ND
ISS-ODN (100 μ g)	39,385 $\pm$ 2129*	ND	11,872 $\pm$ 4547*	ND	ND	35 $\pm$ 23

Mice (4 per group) were intranasally immunized with OVA alone or with PGN or ISS-ODN or were subcutaneously sensitized with OVA and alum on 3 occasions spaced 1 week apart. Sera and splenocytes were collected for analyses 3 weeks after the last immunization. Results are reflective of 4 similar experiments.

ND, None detected.

* P < .05 for intranasal OVA-vaccinated mice versus all other groups.

parison, intranasal immunizations with OVA and PGN, particularly at the 2 higher doses, or OVA and ISS-ODN at all 3 doses induced much stronger but T_H2 - and T_H1 -biased systemic (Table I) and mucosal (Fig E2) responses, respectively. In this regard, although intranasal vaccination with both TLR ligands induced equivalent BALF IgA responses, only intranasal OVA/PGN-immunized mice had consistently detectable serum levels of OVA-specific IgE, and intranasal OVA/ISS-ODN-immunized mice had 1000-fold higher IgG2a levels. Furthermore, splenocytes and bronchial lymph node mononuclear cells from intranasal OVA- and PGN-vaccinated mice produced high amounts of IL-4, IL-5, and IL-13 but little IFN- γ when cultured with OVA, whereas mononuclear cells from intranasal OVA/ISS-ODN-immunized mice produced high levels of IFN- γ and negligible amounts of IL-4, IL-5, and IL-13.

To compare the T_H2 adjuvant potential of PGN with a more familiar T_H2 adjuvant, control mice were subcutaneously vaccinated with OVA and alum (Table I and Fig E2). Subcutaneous OVA/alum vaccination consistently induced stronger serum IgE and T_H2 cytokine responses from OVA-stimulated splenocytes and bronchial lymph node mononuclear cells than intranasal OVA/PGN vaccination, but differences between these vaccination groups were generally less than 3-fold. Of note, the OVA prepa-

ration used in these studies had very low but detectable LPS contamination (0.027 EU/ μ g or 0.0027 ng/ μ g protein). However, mice receiving the 50- μ g OVA vaccination dose only received 1.35 EU (0.135 ng) of endotoxin, a murine dose well below that previously associated with adjuvant activity in our laboratory (unpublished data) and others.¹⁶

Intranasal coimmunization with PGN but not ISS-ODN primes mice for T_H2 -biased hypersensitivity responses in the airways

In a final series of experiments, immunized mice described in Table I underwent airway allergen challenge, and methacholine-induced bronchospasm and airway inflammation were assessed (Fig 3). Compared with naive mice, intranasal OVA-immunized and airway-challenged mice did not display significant increases in methacholine sensitivity, but on average, BALF cellularity and neutrophil frequency increased by more than 80%. Moreover, eosinophils in BALF exudates increased from less than 1% in naive mice to 13% in intranasal OVA-immunized and challenged mice. In contrast to intranasal vaccination with OVA alone, intranasal OVA/PGN vaccination primed mice for exaggerated methacholine responses after airway allergen challenge (Fig 3). Moreover, on average,

intranasal OVA/PGN-immunized mice had 4-fold increases in their total BALF cellularity and 3-fold increases in their frequency of BALF eosinophils compared with BALF from intranasal OVA-immunized mice. However, subcutaneous OVA/alum-immunized mice consistently had stronger airway hypersensitivity responses than intranasal OVA/PGN-immunized mice. Interestingly, despite development of T_H1 -biased humoral and cellular responses, intranasal OVA/ISS-ODN-immunized mice did not have increased methacholine sensitivity nor did they have strong airway inflammatory responses after airway allergen challenge. In particular, although BALF from intranasal OVA-vaccinated mice was relatively eosinophil rich, eosinophilia was not evident in BALF recovered from intranasal OVA/ISS-ODN-immunized mice.

DISCUSSION

In addition to allergens themselves, other elements in inspired air have the potential to affect the immunologic status of the lungs.^{2,3} In this regard TLR ligands are ubiquitous and have potent immunostimulatory activities.^{4,6} However, there is mounting evidence that ligands for different TLRs can induce a range of immunologic responses.⁴ PGN, a TLR2 ligand, is an essential structural component of virtually all bacterial cell walls, but in general, the cell walls of gram-negative bacteria are covered by an LPS-rich outer membrane, whereas for gram-positive bacteria, the PGN-rich cell wall remains exposed.²² In contrast to differences in their contents of PGN and LPS, CpG motifs are equally frequent in the genomes of most gram-positive and gram-negative bacteria. Although LPS and ISS-ODN have previously been identified as having antiallergic immunologic potential, the immunologic effect of TLR2 ligand exposures on the allergic phenotype has yet to be considered. Therefore in the present series of investigations, we compared the immunologic activities of *S aureus* PGN and synthetic ISS-ODN.

In studies of innate immunity, PGN induced stronger IL-6 and weaker IL-12 and IFN- γ responses than ISS-ODN (Figs 1, A; 2, B; and E1). In addition, although both TLR ligands upregulated DC expression of costimulatory molecules (Figs 1, B, and 2, C), ISS-ODN favored CD80, CD86, and CD40 expression, whereas PGN favored OX40L expression. Moreover, although resident pulmonary DCs exist primarily in an immature and inactivated state,^{23,24} airway exposures to both PGN and ISS-ODN induced the recruitment of activated mDCs and pDCs to the lungs (Fig 2, B). Other investigators have reported that APCs producing high levels of IL-6^{25,26} favor T_H2 cell development, whereas IFN- γ ²⁷ and IL-12^{28,29} exposures early in CD4 cell differentiation favor T_H1 differentiation. Analogously, CD40 engagement on APCs by its ligand on CD4 cells has been found to stimulate IL-12 production and T_H1 differentiation,^{30,31} whereas APC OX40L engagement by OX40-expressing CD4 cells has been shown to favor T_H2 differentiation.^{32,33} Therefore our results suggested that PGN and ISS-ODN might favor T_H2 - and T_H1 -

biased adaptive responses by virtue of the divergent innate responses they elicit. Moreover, by increasing pro-inflammatory cytokine levels in the local milieu and recruiting activated DCs to the lungs, the present results suggest a general mechanism by which microbial products like PGN and ISS-ODN might break immunologic tolerance in the airways.

In additional studies, mice were intranasally immunized with OVA alone or with PGN or ISS-ODN, and adaptive responses were analyzed. Both PGN and ISS-ODN proved to be effective mucosal adjuvants (Table I and Fig E2). Moreover, as predicted, PGN favored T_H2 -biased Ig and cellular responses, whereas ISS-ODN favored T_H1 -biased responses. Considered in context with the APC responses induced by these TLR ligands, it is reasonable to suggest that the divergent innate responses induced by PGN and ISS-ODN are likely to contribute to their divergent effects on adaptive immunity. However, further investigation will be required to fully elucidate the complex and redundant mechanisms by which PGN- and ISS-ODN-activated mDCs, pDCs, and other APC subsets shape adaptive responses.

TLR ligands in ambient air have been proposed to be protective against asthma.^{2,3,6} Nonetheless, whereas intranasal OVA/ISS-ODN immunization induced T_H1 -biased responses, intranasal OVA/PGN vaccination primed mice for T_H2 -biased responses, suggesting that these mice might also be predisposed toward T_H2 -biased airway hypersensitivity responses. We found that although intranasal OVA-immunized mice had weak T_H2 -biased hypersensitivity responses on airway allergen challenge, responses were far stronger for intranasal OVA/PGN-immunized mice (Fig 3). In contrast, despite the robust nature of their T_H1 -biased adaptive responses, intranasal OVA/ISS-ODN-immunized mice had little evidence of airway hypersensitivity after intranasal OVA challenge.

High-dose and low-dose airway LPS exposures have been shown to prevent and induce the development T_H2 -biased allergen hypersensitivities and asthma, respectively.¹⁶ However, we do not believe that differential dose-response effects explain the divergent T_H -biasing activities of PGN and ISS-ODN for several reasons. First, we found that PGN and ISS-ODN induced consistently divergent innate cytokine responses over a wide range of TLR ligand concentrations (Figs 1 and E1). Furthermore, although we found that the magnitude of their adaptive responses were influenced, intranasal OVA/PGN- and OVA/ISS-ODN-immunized mice had T_H2 - and T_H1 -biased responses no matter what TLR ligand and vaccination dose was used (Table I). Finally, although some intracellular signaling elements are shared by all TLRs, some TLRs are known to use additional signaling pathways not used by other members of this receptor family⁴; these later observations provide a molecular basis for ligands of different mammalian TLRs having both shared and divergent immunologic activities.

On the basis of the presented results, it is tempting to speculate that physiologic exposures to PGN and ISS-

ODN in ambient air might be provocative for and protective against asthma pathogenesis, respectively. However, our experimental model falls short of mimicking physiologic airway TLR ligand exposures in several important ways. First, inspired air is likely to generate much lower TLR ligand concentrations in the respiratory tract than those achieved by means of intranasal bolus, but physiologic TLR ligand exposures are also likely to be relatively continuous. Furthermore, although we compared individual TLR ligands in controlled experiments, inspired air is likely to contain ligands for multiple TLRs,^{5,6} as well as ligands for other immunocyte receptors. Finally, TLR ligands have the capacity to induce both self-tolerance and cross-tolerance, leading to decreased responsiveness with repeated exposures.^{4,34} These and other considerations will require further investigation before the effects of environmental TLR ligand exposures on the natural history of asthma are fully appreciated. Nonetheless, the experimental results contained herein demonstrate the highly divergent immunologic potential of ligands for TLR2 and TLR9 and add a new layer of complexity to our understanding of the molecular means by which microbes can influence the asthmatic phenotype.

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