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Maximal Adjuvant Activity of Nasally Delivered IL-1α Requires Adjuvant-Responsive CD11c⁺ Cells and Does Not Correlate with Adjuvant-Induced In Vivo Cytokine Production

Afton L. Thompson,* Brandi T. Johnson,* Gregory D. Sempowski,*†‡§ Michael D. Gunn,*†‡§ Baidong Hou,* Anthony L. DeFranco,‖ and Herman F. Staats*†‡§

IL-1 has been shown to have strong mucosal adjuvant activities, but little is known about its mechanism of action. We vaccinated IL-1R1 bone marrow (BM) chimeric mice to determine whether IL-1R1 expression on stromal cells or hematopoietic cells was sufficient for the maximal adjuvant activity of nasally delivered IL-1α as determined by the acute induction of cytokine responses and induction of Bacillus anthracis lethal factor (LF)-specific adaptive immunity. Cytokine and chemokine responses induced by vaccination with IL-1α were predominantly derived from the stromal cell compartment and included G-CSF, IL-6, IL-13, MCP-1, and keratinocyte chemoattractant. Nasal vaccination of Il1r1−/− (knock-out [KO]) mice given wild-type (WT) BM (WT→KO) and WT→WT mice with LF + IL-1α induced maximal adaptive immune responses, whereas vaccination of WT mice given Il1r1−/− BM (KO→WT) resulted in significantly decreased production of LF-specific serum IgG, IgG subclasses, lethal toxin-neutralizing Abs, and mucosal IgA compared with WT→KO and WT→WT mice (p < 0.05). IL-1α adjuvant activity was not dependent on mast cells. However, the ability of IL-1α to induce serum LF-specific IgG2c and lethal toxin-neutralizing Abs was significantly impaired in CD11c−/Myd88−/− mice when compared with WT mice (p < 0.05). Our results suggest that CD11c⁺ cells must be directly activated by nasally administered IL-1α for maximal adjuvant activity and that, although stromal cells are required for maximal adjuvant-induced cytokine production, the adjuvant-induced stromal cell cytokine responses are not required for effective induction of adaptive immunity. The Journal of Immunology, 2012, 188: 2834–2846.

Mucosal vaccines have been shown to induce both systemic and mucosal Ag-specific humoral and cell-mediated immunity, and therefore are useful for the induction of protective immunity against pathogens usually encountered first at a mucosal surface, including polio, influenza, cholera, and inhalational anthrax (1–3). The nasally administered, live-attenuated influenza vaccine and the oral live-attenuated poliovirus vaccine are examples of mucosally administered vaccines able to induce both systemic and mucosal immunity in humans (3, 4). However, there are drawbacks to using live-attenuated organ-isms as mucosal vaccines, which is the current standard, because they cannot be used in immunocompromised individuals, and their requirement for cold-chain storage is an additional hindrance to their widespread use (5–8). Vaccines based on weaker immunogens, such as synthetic peptides or protein subunits, would allow mucosal vaccination in immunocompromised individuals but generally require an adjuvant to induce protective immunity (9); however, no adjuvants have been approved for mucosal use with subunit immunogens. Identifying and characterizing safe and effective vaccine adjuvants are top priorities for creating new and effective mucosally administered subunit vaccines.

Because of the negative effects associated with the delivery of toxin-based adjuvants (10–15), we have previously investigated the ability of IL-1α to enhance immune responses to codelivered Ags. IL-1α provides effective adjuvant activity when delivered nasally, enhancing the production of Ag-specific mucosal IgA, serum IgG (16), and serum lethal toxin-neutralizing Abs (17), and it has been shown to be safe and well tolerated in cynomolgus macaques (18) and rabbits (17). Although IL-1α is an effective mucosal vaccine adjuvant, little is known about its mechanism of action in this setting. IL-1 is a pleiotropic cytokine (19) that, among several other activities, can induce the production of numerous cytokines and chemokines (20–24). Although several studies have examined the ability of vaccine adjuvants to induce serum cytokine and chemokine production (25–28), it is unclear whether these responses are actually required for the development of Ag-specific adaptive immune responses. To gain a better understanding of the correlates of adjuvant activity, it is important to determine whether these adjuvant-induced innate responses are an adequate predictor of adjuvant-dependent induction of protective immunity or whether they reflect adjuvant activities that are un-

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The online version of this article contains supplemental material.

Abbreviations used in this article: BM, bone marrow; CBC, carbonate bicarbonate; CT, cholera toxin; DC, dendritic cell; EU, endotoxin unit; KC, keratinocyte chemoattractant; KO, knock-out; LF, lethal factor; NL, nasal lavage; NT₅ₑ, neutralization titer 50% poly I:C, polyinosinic-polycytidylic acid; rLF, recombinant LF; rPA, recombinant anthrax protective Ag; WT, wild-type.

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related to the induction of protective Ag-specific immune responses. This information will be essential for rationally selecting vaccine adjuvants that induce potent adaptive immune responses in the absence of unnecessary inflammatory activity.

The role of the mucosal barrier in the immune response to mucosal vaccination is also understudied. It is understood that the mucosal epithelium plays a role in the immune response to pathogen invasion, both in its function as a barrier and via activation of cellular receptors (e.g., TLR ligands) and the subsequently activated pathways (29, 30). To study the role of the mucosal barrier in other responses, the bone marrow (BM) chimeric mouse model has been used to examine the requirement for specific receptors (e.g., TLRs) on hematopoietic cells or stromal cells in response to various stimuli, such as inhaled endotoxin. Models using external challenge routes with various Ags and pathogens have also demonstrated a requirement for receptor presence in both compartments (31–33), although many of these studies have focused on innate immune responses or the ability to fight infection, not on the generation of adaptive immune responses. However, other studies have evaluated the roles of individual cell types in the response to adjuvant delivery using mouse strains with targeted mutations. Although an absolute requirement has been demonstrated for mast cells in the adjuvant activity of imiquimod and CTA1-DD/GF (intranasal and cutaneous immunization, respectively) (34, 35), a previous study by Hou et al. (36) demonstrated that the adjuvant activity of systemically delivered CpG required adjuvant-responsive CD11c+ cells for maximal adjuvant activity. Elucidating the requirement for an adjuvant-responsive mucosal barrier after vaccination with one potent mucosal vaccine adjuvant, IL-1α, may provide insight into the role of the mucosal barrier and help identify cell types important in the generation of adaptive immune responses to mucosally delivered vaccines.

It is unclear whether any of the previously described properties of IL-1 activity (37–39) are related to the adjuvant mechanism of action of nasally delivered IL-1 or whether the adjuvant activity of IL-1 is mediated through a particular cellular compartment (e.g., stromal or hematopoietic cells), because it has effects on both hematopoietic (40) and nonhematopoietic (41–44) cells. To examine the requirement for adjuvant-induced cytokines and determine the cellular compartment required to express the IL-1R1 for IL-1 nasal adjuvant activity, we generated mice expressing IL-1R1 on only their stromal (radioresistant) cells or their BM-derived cells using the BM chimeric mouse model, and monitored adjuvant-dependent induction of innate cytokine responses and Ag-specific adaptive immunity. Our results demonstrated that, when IL-1α was delivered nasally, serum cytokine production was dependent on IL-1R expression by stromal cells. However, IL-1R1 expression on only stromal cells was not sufficient for maximal induction of Ag-specific adaptive immunity. By contrast, in mice with IL-1R1 expression on only hematopoietic cells, acute production of cytokines after nasal immunization adjuvanted with IL-1α was not observed despite maximal induction of anti-lethal factor (anti-LF) adaptive immunity. In addition, adjuvant-responsive CD11c+ cells were required for maximal IL-1-adjuvant activity.

Materials and Methods
Mice
Female Il1r1−/− (B6.129S7-Il1r1tm1Imx/J), CD45.1 congenic (B6.SJL-PtprcPepc8BoyJ), Rag1−/− (B6.129S7-Rag1tm1Mom/J), and C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at 4–6 wk of age. Mast cell-deficient KitW/W*HnilJaeBonJ mice (SASH) were obtained from Dr. Laura P. Hale (Department of Pathology, Duke University; available from The Jackson Laboratory as strain #005051). DC-Myd88−/− mice were produced as previously described (36). Mice were housed in filter-top cages and provided with food and water ad libitum. All procedures were approved by the Duke University Institutional Animal Care and Use Committee.

Mouse irradiation and BM transfer
The protocol used was similar to those previously described (32, 46, 47). BM was harvested the morning of the irradiation and transfer. Donor mice were sacrificed using CO2 euthanasia, and marrow was harvested by flushing the femurs, tibias, and humeri with cold medium (RPMI 1640, 10% FBS, 1% Penstrep, 1% HEPES, 1% NEAA, 1% sodium pyruvate, 0.1% 2-ME). The harvested BM was washed twice with sterile, cold PBS and resuspended to 1 × 106 cells/ml in PBS. Cells were stored on ice until transfer. Recipient mice were irradiated in an x-ray irradiator (Xrad 320; Precision X-Ray, North Branford, CT) with 10.5 Gy total body irradiation. Recipients were anesthetized and transferred retro-orbitally with 5 × 106 donor cells. Mice were kept warm and were monitored through recovery from anesthesia. Mice were nasally administered 1 μL LPS (Invivogen E. coli 0111:B4) in 10 μL PBS three times after irradiation on weeks 1, 3, and 5 in an attempt to induce mucosal dendritic cell (DC) turnover and ensure that all DCs in the nasal cavity were of donor origin (48). Mice were rested for a total of 8–10 wk after irradiation, and chimerism was confirmed using flow cytometry for CD45.1 and CD45.2 (data not shown).

Flow cytometry on peripheral blood leukocytes
Whole blood samples were prepared for flow cytometry as previously described (49). Heparinized peripheral blood samples were centrifuged at 500 × g for 8 min and resuspended in 10 ml FACS buffer (PBS without calcium or magnesium, 0.5% FBS, 0.005% EDTA). Samples were centrifuged at 500 × g for 8 min. The supernatant was removed, and samples were transferred to 96-well V-bottom plates and centrifuged at 400 × g for 2 min to pellet the cells. The plate was flicked to remove supernatant and then blotted before vortexing. Samples were washed in 200 μL FACS buffer, centrifuged, and the supernatant removed as before. Samples were blocked with unlabeled mouse IgG2a at 10 μg/ml in 100 μL FACS buffer for 30 min on ice. Plates were then washed two times. Specific Abs were added to the wells in 100 μl (4 μg/ml in FACS buffer) and incubated for 30 min on ice in the dark. PE mouse anti-mouse CD45.1 and allophycocyanin mouse anti-mouse CD45.2 were purchased from BD Pharmingen (San Diego, CA). Plates were washed two times, and 200 μl 1% paraformaldehyde was added to fix cells before moving samples to FACS tubes. The samples were acquired on a four-color FACSCalibur and analyzed in FlowJo (Tree Star, Ashland, OR).

Nasal vaccination
Mice were anesthetized with isoflurane before vaccination. Vaccines were administered intranasally in 10 μl (5 μl per nostril). Nonchimeric mice were vaccinated on days 0, 7, and 21. Cimmeric mice were vaccinated on days 0, 7, 21, and 42. For each chimeric study replicate, mice were divided into groups of 4 to 8. Vaccine groups included PBS alone, recombinant LF (rLF) alone (#172; List Biological Laboratories Cambell, CA; 4 endotoxin units [EU]/mg), rLF + 4 μg IL-1α (400-MU; 0.00144 EU/μg; R&D, Minneapolis, MN), and rLF + 1 μg cholera toxin (CT) (EU/mg unknown; List #100B). For three of the four Il1r1 chimeric mouse experiments, mice were vaccinated with 25 μg LF for the day 0, 7, and 21 immunizations and 50 μg LF on day 42. For the remaining experiment, mice were immunized with 50 μg LF each time. The calculated endotoxin delivery per mouse was ∼0.20 (LF alone) to 0.22 EU (LF + IL-1α). Data from each group for all repeat experiments were combined and analyzed together. Mice vaccinated with LF + CT served as a positive control, and LF + CT-vaccinated mice responded similarly in all four chimeric groups.

Sample collection
Blood samples were collected from unanesthetized mice by the submandibular lancet method (50). Samples were collected into 1.5-ml centrifuge tubes and centrifuged at 13,000 rpm at 4°C for 25 min. The serum supernatant was pipetted off into a new tube. Vaginal lavage and fecal samples were obtained as previously described by our laboratory (51, 52). Nasal lavage samples were collected from euthanized mice. After removing the head from the body, we cut away the lower jaw and collected the nasal lavage samples by instilling 1 ml PBS posteriorly into the nasal cavity. Fluid exiting the nostrils was collected and spun at 13,000 rpm at 4°C for 20 min.

ELISA
ELISAs were performed as outlined in Nordone et al. (53), except that ELISA plates were coated with rLF at 2 μg/ml in carbonate bicarbonate (CBC) buffer. The rLF used for coating plates was kindly provided by Dr.
Robert C. Liddington at The Burnham Institute for Medical Research (La Jolla, CA). Sample end-point titers were calculated as the last dilution at which the sample relative light unit reading (560 nm) was 3-fold greater than a similarly diluted naive sample relative light unit reading (560 nm). The log₂ end-point titers were used for statistical analysis. Serum samples were plated at a starting dilution of 1:32. Vaginal and fecal samples were plated at a starting dilution of 1:4. Nasal lavage samples were plated undiluted. Samples with undetectable titers were assigned a titer of one less than the starting plate dilution. Mucosal samples were normalized against the total amount of IgA or IgG present in the sample, as determined using an ELISA. Normalized titers are expressed as titer/μg. Percentage titers in avidity measurements were calculated using the log₂ Ab end-point titer.

**Avidity ELISA**

Avidity ELISAs were performed as described earlier with one exception (54). After overnight incubation with samples, the plates were washed four times with PBS. Washed plates were incubated for 15 min with ammonium thiocyanate (#431354; Sigma, St. Louis, MO) diluted in 15 mM phosphate buffer at concentrations of 0, 1, 2, and 3 M. Plates were then washed four times, and the secondary Ab was added. Sample titers were calculated as described earlier for ELISA titers, and the titers at each NH₄SCN concentration were assigned a value of half the low standard. Values less than the low value of the standard curve were assigned a value of half the low standard. Data shown are NT50, the serum dilution at which neutralization of lethal toxin results in 50% of the plated macrophages remaining viable.

Results

**Nasal vaccination with IL-1α induces the acute production of cytokines and chemokines**

Innate immune responses help shape the adaptive immune response (57–59), and adjuvant delivery is known to induce increased production of a variety of systemic proinflammatory cytokines, including IL-6 and MCP-1, within 3–24 h of vaccination (25, 26). Although IL-1 is known to induce a variety of cytokines/chemokines in tissue culture and after systemic delivery, it is unclear what cytokine/chemokine profile is induced after intranasal delivery and whether the profile is differentially affected by the tissue distribution of IL-1R1. To determine whether the cytokine-inducing activity of IL-1α after nasal immunization was dependent on IL-1R1 expression in only the stromal (radiosensitive) cell compartment, the hematopoietic cell compartment, or both compartments, serum cytokine responses were measured after nasal delivery of IL-1α to IL-1R1 chimeric mice. IL-1R1 mouse BM chimeras were generated by transferring BM cells from Il1r1−/− mice to lethally irradiated mice of the same strains to create four groups of BM chimeras: WT→WT, KO→KO, WT→KO, and KO→WT (donor→recipient). At week 9, BM chimerism was confirmed by flow cytometry (data not shown), and mice were vaccinated in week 10.

Based on the serum cytokine profiles observed in WT mice after nasal delivery of IL-1α (Supplemental Table I), serum samples were taken from chimeric mice at 3 h after vaccination with 25 μg LF alone or LF + 4 μg IL-1α and tested for Eotaxin, MCP-1, KC, G-CSF, and IL-6 (Table I). Nasal vaccination of KO→KO mice with LF + IL-1α did not induce cytokine production above that observed in any mouse group immunized with LF alone, demonstrating that IL-1α cytokine-inducing activity required IL-1R1, and that contaminants were not responsible for the cytokine-inducing activity of IL-1α. Eotaxin was not significantly increased in any of the vaccinated mice. MCP-1, KC, G-CSF, and IL-6 were significantly increased in KO→WT mice vaccinated with LF + IL-1α compared with LF alone-vaccinated mice (p < 0.001) and were equivalent to the levels induced in WT→WT mice, which were significantly increased compared with LF alone (p < 0.001). Conversely, vaccination of WT→KO mice did not result in increased production of those cytokines, and their levels were similar to those in LF alone-vaccinated WT→KO and LF + IL-1α-vaccinated KO→KO mice. Thus, IL-1α appears to be primarily activating the stromal cell compartment to induce systemic cytokine production when used as an adjuvant for nasally delivered vaccines.

Serum cytokine profiles may not reflect local mucosal cytokine production after nasal vaccination. In fact, the cytokine profiles induced by nasal vaccination of WT mice with IL-1α differed in the serum and the nasal lavage (Supplemental Tables I and II, respectively). We therefore evaluated local mucosal cytokine production after nasal immunization of IL-1R1 chimeric mice with IL-1α. Three hours after vaccination with PBS or 4 μg IL-1α, nasal lavage samples were examined for the presence of IL-6, IL-10, IL-13, IFN-γ, G-CSF, KC, MCP-1, and TNF-α (Table II), based on profiles observed in WT mice (Supplemental Table II).
Similar to the results observed in serum samples, increases in cytokine production were the lowest in WT → KO mice, whereas those in KO → WT mice were similar to WT → WT mice. No increases were observed in any group for IL-10, IFN-γ, or TNF-α. At 3 h after vaccination, IL-1α-induced significant increases in IL-6, KC, G-CSF, and MCP-1 in KO → WT and WT → WT mice compared with naive (p < 0.05); all but G-CSF were significantly increased compared with WT → KO (p < 0.05). In WT → KO mice, only G-CSF and KC were significantly increased compared with naive (p < 0.05), although G-CSF was 2-fold lower and KC was significantly lower (40-fold) than in KO → WT and WT → WT mice (p < 0.05). In addition, IL-13 production in KO → WT and WT → WT mice was significantly increased compared with WT → KO mice, but not naive animals. These data indicate that, when used as a nasal vaccine adjuvant, IL-1α induces potent innate immune responses both systemically and in the nasal cavity. In addition, the local cytokine responses were primarily dependent on IL-1R1 expression by stromal (radioresistant) cell populations, although IL-1R1+ hematopoietic cells were able to mediate some KC and G-CSF production.

**IL-1R1 is required on BM-derived cells for maximal serum Ab responses after nasal vaccination in IL-1R1 chimeric mice**

Although several studies have examined the ability of vaccine adjuvants to induce cytokines after vaccination, it is unclear whether adjuvant-induced cytokine production is related to Ag-specific adaptive immunity. To determine which IL-1–responsive cellular compartment was required to induce Ag-specific humoral responses, it is unclear whether the responses correlated with cytokine/adjuvant-induced cytokine production. After vaccination, serum samples were collected and tested for the presence of LF-specific IgG by nasally delivered IL-1α as determined by enhanced serum anti-LF IgG titers. However, IL-1R1 expression on only hematopoietic cells was sufficient for the maximal induction of serum anti-LF IgG by nasally delivered IL-1α.

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**Table II. Nasal lavage cytokine production (pg/ml) in IL-1R1 chimeric mice 3 h after vaccination with IL-1α alone**

<table>
<thead>
<tr>
<th>Cytokine/Chemokine</th>
<th>Naive*</th>
<th>WT/KO</th>
<th>KO/WT</th>
<th>WT/WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>IL-10</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>IL-13</td>
<td>13</td>
<td>1</td>
<td>61c</td>
<td>72c</td>
</tr>
<tr>
<td>G-CSF</td>
<td>41</td>
<td>656b</td>
<td>1484b</td>
<td>1896b</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>KC</td>
<td>18</td>
<td>130b</td>
<td>859b</td>
<td>887b</td>
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<tr>
<td>MCP-1</td>
<td>5</td>
<td>7</td>
<td>84b</td>
<td>120b</td>
</tr>
<tr>
<td>TNF-α</td>
<td>2</td>
<td>2</td>
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</table>

*aNaive animals were WT/WT.
*bSignificantly greater than naive (p < 0.05).
*cSignificantly greater than WT/KO (p < 0.05).
To further examine the response to vaccination, we examined vaccine-induced, Ag-specific serum IgG subclass production in the chimeric mice. Day 56 serum samples were tested for the presence of LF-specific IgG1, IgG2b, IgG2c, and IgG3 (Fig. 2). KO→KO mice vaccinated with LF + IL-1α did not produce significantly greater amounts of any IgG subclass than LF alone (≤1:128 versus ≤1:128, respectively). The IgG1 responses were very similar to the IgG responses in all examined groups. When vaccinated with LF + IL-1α, WT→WT mice produced significantly more anti-LF IgG1 than KO→WT mice (p < 0.01), but anti-LF IgG1 production did not differ significantly between WT→KO and KO→WT mice. Differences between the chimeric groups were more pronounced for anti-LF IgG2c, IgG2b, and IgG3 production. All three groups with at least one IL-1–responsive cellular compartment produced significantly greater amounts of anti-LF IgG2c and IgG2b than LF alone-vaccinated mice (p < 0.05), but only WT→KO and WT→WT mice produced significantly greater amounts of anti-LF IgG3 (p < 0.001). Both WT→KO and WT→WT mice produced significantly greater amounts anti-LF IgG2c, IgG2b, and IgG3 than KO→WT mice (p < 0.001 and p < 0.01, respectively). These data support the BM-dependent requirement for maximal IL-1α responsiveness observed in the Ag-specific serum IgG data.

The goal of vaccination is to induce protective immunity against the pathogen of interest, but serum Ag-specific ELISA titers have been shown not to correlate with protection against an aerosolized Bacillus anthracis spore challenge (60). To evaluate the induction of protective humoral immunity after nasal immunization of chimeric mice, we measured serum anthrax lethal toxin-neutralizing Ab titers, which have been shown to correlate with protection against an aerosolized Bacillus anthracis spore challenge (60) (Fig. 3). Serum samples were collected on day 56. Serum from LF alone-vaccinated mice did not neutralize lethal toxin, whereas serum from LF + CT-vaccinated mice induced 50% lethal toxin-neutralizing titers (NT50) ranging between 1:403 and 1:1354 (p < 0.001 versus LF alone). Similar to our results measuring anti-LF IgG responses, KO→KO mice vaccinated with LF +IL-1α did not respond to vaccination with the production of lethal toxin-neutralizing serum Abs. Vaccination of KO→WT mice with LF + IL-1α induced an NT50 of only 1:64, which was not significantly increased compared with neutralizing Abs induced by immunization with LF alone. WT→KO and WT→WT mice vaccinated with LF + IL-1α induced significantly greater amounts of toxin-neutralizing Abs (1:221 and 1:299, respectively) than those induced in LF alone-vaccinated mice (p < 0.001) and KO→WT mice vaccinated with LF + IL-1α (p < 0.01).

Binding avidity of Ab for its specific Ag is an additional Ab property that has been shown to positively correlate with protective activity (e.g., virus or toxin neutralization) (61–63). As such, avidity was measured using an NH4SCN elution assay (Fig. 4). Ab titers declined in all vaccine groups and all chimeric groups with increasing concentrations of NH4SCN (0–3 M). Mice vaccinated

**FIGURE 2.** Serum anti-rLF IgG subclass profiles are significantly altered after vaccination with LF + IL-1α in the absence of IL-1R1 expression on hematopoietic cells. Mice were vaccinated on days 0, +7, +21, and +42 after a 10-wk rest period after BM reconstitution. Serum samples taken on day +56 were tested by ELISA. Bars represent the geometric mean titers for each group on day +56 for all replicates, with error bars representing the 95% confidence level. Asterisks indicate CT groups do not differ significantly from each other and are significantly greater than LF alone (p < 0.001). 1, Significantly greater than LF + IL-1α within the same chimeric group (p < 0.01). 2, Significantly greater than LF alone within the same chimeric group (p < 0.05). 3, Significantly greater than KO/KO LF + IL-1α (p < 0.001). 4, Significantly greater than KO/WT LF + IL-1α (p < 0.01). Four replicates unless noted; IgG1, IgG2c, IgG2b, IgG3: n = 7 for KO/KO LF alone (two replicates); n = 8 for KO/KO LF + CT (two replicates); n = 11 for KO/KO LF + IL-1α (two replicates); n = 18 for WT/WT LF alone; n = 19 for WT/KO LF alone, WT/WT LF + CT, and WT/WT LF + IL-1α; n = 20 for WT/KO LF + CT. IgG1 and IgG2c: n = 21 for WT/KO LF + IL-1α and KO/WT LF alone; n = 23 for KO/WT LF + CT; n = 25 for KO/WT LF + IL-1α. IgG2b and IgG3: n = 20 for WT/KO LF + IL-1α; n = 21 for and KO/WT LF alone; n = 22 for KO/WT LF + CT; n = 25 for KO/WT LF + IL-1α (IgG2b); and n = 24 for KO/WT LF + IL-1α (IgG3).
FIGURE 3. LF + IL-1α–induced protection against lethal toxin is significantly impaired by the absence of IL-1R1 on hematopoietic cells. Mice were vaccinated on days 0, +7, +21, and +42 after a 10-wk rest period after BM reconstitution. Serum samples taken on day +56 were tested using an anthrax macrophage toxicity assay. Bars represent the geometric mean titers for each group on day +56 for all replicates, with error bars representing the 95% confidence level. Asterisk denotes CT groups do not differ significantly from each other and are significantly greater than LF alone (p < 0.001). 1, Significantly greater than LF + IL-1α within the same chimeric group (p < 0.05). 2, Significantly greater than LF alone within the same chimeric group (p < 0.001). 3, Significantly greater than KO/KO LF + IL-1α (p < 0.001). 4, Significantly greater than KO/WT LF + IL-1α (p < 0.01). Four replicates unless noted; n = 7 for KO/KO LF alone (two replicates); n = 8 for KO/KO LF + CT (two replicates); n = 11 for KO/KO LF + IL-1α (two replicates); n = 18 for WT/WT LF alone; n = 19 for WT/KO LF alone, WT/WT LF + CT, and WT/WT LF + IL-1α; n = 20 for WT/KO LF + CT; n = 21 for WT/KO LF + IL-1α and KO/WT LF alone; n = 23 for KO/WT LF + CT; n = 25 for KO/WT LF + IL-1α.

FIGURE 4. LF + IL-1α–induced Ab avidity is significantly decreased by the absence of IL-1R1 on hematopoietic cells. Mice were vaccinated on days 0, +7, +21, and +42 after a 10-wk rest period after BM reconstitution. Serum samples taken on day +56 were tested using an NH4SCN elution ELISA. Lines represent titers as a percentage of the titer at 0 M NH4SCN. Asterisks denote significantly less than WT/KO and WT/WT (p < 0.05). Four replicates: n = 10 for KO/WT LF alone; n = 13 for WT/KO LF alone; n = 14 for WT/WT LF alone; n = 19 for WT/WT LF + CT, WT/WT LF + IL-1α, KO/WT LF + CT, and KO/WT LF + IL-1α; n = 22 for WT/KO LF + IL-1α; n = 25 for WT/KO LF + CT.

with LF + CT had relatively high Ab avidity compared with those in other groups. At 3 M NH4SCN, serum IgG titers in CT-vaccinated mice were still >40% of the titer at 0 M NH4SCN in all three chimeric groups examined. All chimeric groups vaccinated with LF + CT had similar Ab avidity at 3 M NH4SCN (range: 42–52%). These data indicate that the absence of IL-1R1 in one compartment did not affect the ability of the immune system to generate high-avidity Abs. However, KO→WT mice vaccinated with LF + IL-1α had significantly lower percentage titers at 1, 2, and 3 M NH4SCN (47, 20, and 12%, respectively) than WT→KO (70, 40, and 32%, respectively) and WT→WT (78, 46, and 36%, respectively) mice (p < 0.05). Therefore, the ability of IL-1α to induce the production of high-avidity anti-LF Abs was affected by the absence of IL-1R1 on hematopoietic cells.

Taken together, these results demonstrate a compartment-specific requirement for IL-1α—responsiveness for the induction of maximal Ag-specific humoral immunity, with IL-1R1 KO→WT mice having decreased serum immune responses to vaccination with LF + IL-1α when compared with WT→KO and WT→WT. It is also evident from the chimeric mouse studies that serum and local cytokine/chemokine responses 3 h after vaccination with LF + IL-1α do not correlate with the adaptive immune response. Mucosal Ab responses to vaccination with LF + IL-1α are impaired in IL-1R1 KO→WT mice

One of the strengths of mucosal vaccination is the ability to induce Ag-specific mucosal IgA production, and when used as nasal vaccine adjuvants, IL-1α and IL-1β have been shown to enhance Ag-specific mucosal IgA production (16, 17). We therefore investigated mucosal Ab production in the chimeric mouse model to determine whether the IL-1R1 expression requirement for mucosal Ab responses followed the requirement observed for serum Ab responses. Fecal, vaginal, and nasal lavage samples were examined for the presence of Ag-specific and total IgA. Mice vaccinated with LF alone produced little anti-LF mucosal IgA (<0.5 tier/µg; Fig. 5). Mucosal Ab production was highly variable between animals for all groups, and fecal IgA production was low in all groups (range of group averages: 0–5 tier/µg). Nasal and vaginal LF-specific IgA production was elevated in all groups vaccinated with LF + CT (nasal: 23.8–41.5 tier/µg; vaginal: 36.2–77.4 tier/µg), but no group had significantly increased nasal or vaginal anti-LF IgA titers compared with mice immunized with LF alone. However, KO→WT mice vaccinated with LF + CT had significantly increased fecal IgA production (p < 0.05). WT→KO and WT→WT mice vaccinated with LF + IL-1α had significantly increased nasal anti-LF IgA compared with KO→KO mice (p < 0.01) and mice vaccinated with LF alone within the same chimeric group (p < 0.05). WT→WT mice vaccinated with LF + IL-1α also had significantly increased vaginal anti-LF IgA compared with KO→WT, WT→KO, and LF alone-vaccinated WT→WT mice (p < 0.05). KO→WT mice vaccinated with LF + IL-1α produced detectable vaginal and nasal anti-LF IgA (16.5 and 74.2 tier/µg, respectively), but the titers were not significantly increased over LF alone-vaccinated KO→WT or KO→KO mice vaccinated with LF + IL-1α. WT/WT mice vaccinated with LF + IL-1α produced significantly more anti-LF vaginal IgA than WT/WT mice vaccinated with LF + CT (p < 0.05), and WT→KO mice vaccinated with LF + IL-1α produced significantly more anti-LF nasal IgA than WT→KO mice vaccinated with LF + CT. In addition to confirming that IL-1α can more potently induce...
Because of the variability in mucosal Ab production, the number of mice responding to vaccination with anti-LF IgA production was also analyzed (Table III). Including an adjuvant in the vaccine formulation increased the number of animals per group with detectable anti-LF IgA production, and overall, the general pattern resembled that observed in the serum responses (Figs. 2–5). Comparing the number of mice producing vaginal and nasal IgA demonstrated that fewer KO→WT mice (48 and 54%, respectively) produced IgA than WT→KO (79 and 100%, respectively) and WT→WT (81 and 88%, respectively).

These data demonstrate that the anti-LF mucosal IgA titers were highly variable within groups. However, the general pattern of production and the number of animals responding to vaccination with production of Ag-specific mucosal IgA were similar to that observed in the serum responses, as responses in KO→WT mice were impaired compared with WT→KO and WT→WT mice vaccinated with LF + IL-1α.

Vaccine-induced, Ag-specific splenocyte cytokine production is not impacted in IL-1R1 chimeric mice

Given that the IL-1α–induced innate cytokine/chemokine response and IgG subclass profile were significantly altered in IL-1R1 chimeric mice, it was important to determine whether Ag-specific cytokine responses were also impacted by the tissue distribution of IL-1R1. We restimulated splenocytes with LF and tested the supernatants for the presence of Ag-specific IL-2, IL-4, IL-10, IL-17, and IFN-γ (Fig. 6). Groups vaccinated with LF alone produced small amounts of each tested cytokine. Cells from LF + CT-vaccinated groups generally produced the highest levels of all tested cytokines, the amounts of which did not differ significantly between chimeric groups. Cells from KO→KO mice vaccinated with LF + IL-1α did not produce significantly greater amounts of any of the five cytokines compared with those from KO→KO LF-alone-vaccinated mice. Cells from KO→WT mice vaccinated with any of the adjuvanted formulations and from KO→KO mice vaccinated with LF + CT produced less IL-17 than those from WT→KO or WT→WT mice, but these differences were not significant. Cells from WT→KO, KO→WT, and WT→WT mice vaccinated with LF + IL-1α did not produce significantly different amounts of any of the five cytokines examined, although those from KO→WT mice produced 2-fold less IFN-γ than did WT→WT mice (338.5 versus 718 pg/ml, respectively). However, cells from WT→WT mice in this vaccine group produced significantly greater amounts of IL-4 than LF alone (24.59 versus 1.94 pg/ml, respec-

Table III. Percentage responders to vaccination and total number of animals per group by chimeric group and vaccine formulation

<table>
<thead>
<tr>
<th>Group</th>
<th>Vaginal IgA</th>
<th>Fecal IgA</th>
<th>Nasal IgA</th>
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</thead>
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<tr>
<td></td>
<td>%</td>
<td>n</td>
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</tr>
<tr>
<td>LF alone</td>
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<td>WT/KO</td>
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<tr>
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<td>0</td>
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<tr>
<td>LF + CT</td>
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<td></td>
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<tr>
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<tr>
<td>WT/WT</td>
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<td>89</td>
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<tr>
<td>LF + 4 µg IL-1α</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>KO/WT</td>
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<tr>
<td>WT/WT</td>
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<td>16</td>
<td>88</td>
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</tbody>
</table>

Compared using Fisher’s exact test with Bonferroni’s correction.

*Significantly greater than LF alone within the same chimeric group.

Significantly greater than KO/KO LF + IL-1α.
tively; \( p < 0.01 \), whereas those from KO→WT (18.13 pg/ml) and WT→KO (17.55 pg/ml) mice did not; this difference was not reflected in cells from mice vaccinated with LF alone, LF + 1 \( \mu \)g CT, or LF + 4 \( \mu \)g IL-1\( \alpha \) (\( p < 0.05 \)). Four replicates unless noted; \( n = 5 \) for KO/ KO LF alone, LF + CT, and LF + IL-1\( \alpha \) (two replicates); \( n = 12 \) for WT/KO LF alone, WT/WT LF alone, and WT/WT LF + IL-1\( \alpha \); \( n = 14 \) for WT/WT LF + CT; \( n = 15 \) for WT/KO LF + CT and WT/KO LF + IL-1\( \alpha \); \( n = 17 \) for KO/WT LF alone and KO/WT LF + CT; \( n = 18 \) for KO/WT LF + IL-1\( \alpha \). NS, not significantly increased over LF alone within the same chimeric group.

**FIGURE 6.** Ag-specific cytokine production after splenocyte restimulation with rLF is not impacted in IL-1R1 chimeric mice. Splenocytes were removed after the last sample collection on day +56 after four vaccinations. Bars represent the mean cytokine production for each group for all replicates minus the average production by unstimulated cells, with error bars representing the 95% confidence level. 1, Significantly greater than KO/KO LF + 4 \( \mu \)g IL-1\( \alpha \) (\( p < 0.05 \)); 2, Significantly greater than KO/WT LF + 4 \( \mu \)g IL-1\( \alpha \) (\( p < 0.05 \)). Four replicates unless noted; \( n = 5 \) for KO/ KO LF alone, LF + CT, and LF + IL-1\( \alpha \) (two replicates); \( n = 12 \) for WT/KO LF alone, WT/WT LF alone, and WT/WT LF + IL-1\( \alpha \); \( n = 14 \) for WT/WT LF + CT; \( n = 15 \) for WT/KO LF + CT and WT/KO LF + IL-1\( \alpha \); \( n = 17 \) for KO/WT LF alone and KO/WT LF + CT; \( n = 18 \) for KO/WT LF + IL-1\( \alpha \). NS, not significantly increased over LF alone within the same chimeric group.

**IL-1\( \alpha \) adjuvant activity is not impaired in Rag1\(^{-/-}\) mice adoptively transferred with Il1r1\(^{-/-}\) splenocytes**

The earlier chimeric mouse data demonstrate that IL-1\( \alpha \) adjuvant activity is the most depressed in mice with Il1r1\(^{-/-}\) hematopoietic cells. Because IL-1 has been shown to have a direct effect on T cells (64), we chose to evaluate the requirement for IL-1–responsive T and B cells in the response to vaccination using an adoptive transfer model. Rag1\(^{-/-}\) mice were reconstituted with WT or Il1r1\(^{-/-}\) splenocytes and vaccinated with 25 \( \mu \)g LF alone, LF + 1 \( \mu \)g CT, or LF + 4 \( \mu \)g IL-1\( \alpha \) on days 0, 7, 21, and 63. No significant differences in anti-LF IgG Ab titers were observed between any of the groups vaccinated with LF + adjuvant, and all groups were significantly increased over LF alone (\( p < 0.01 \); Fig. 7). Serum lethal toxin NT\(_{50}\) responses also did not significantly differ between groups vaccinated with LF + CT or LF + IL-1\( \alpha \) (data not shown). These data suggest that the nasal adjuvant activity of IL-1\( \alpha \) did not require T and B cell expression of IL-1R1.

**Mast cells are not required for the adjuvant activity of IL-1\( \alpha \)**

IL-1\( \alpha \) acts on a variety of cell types, and it has been shown to induce mast cells to secrete IL-6 without causing degranulation (20). In the previous studies, nasal lavage IL-6 production was slightly increased in WT→KO mice given 0.25 or 4 \( \mu \)g IL-1\( \alpha \) nasally, and hematopoietic cell expression of IL-1R1 correlated with maximal adjuvant activity. In addition, mast cells have been reported to be required for the maximal adjuvant activities of imiquimod (35) and CTA1-DD/IgG (34). Therefore, we chose to evaluate the re-
requirement for mast cells in the nasal adjuvant activity of IL-1α. SASH mice have an inversion of the transcriptional regulatory elements upstream of the c-kit gene and do not produce mature mast cells (65, 66). SASH and WT mice were vaccinated on days 0, 7, and 21 with LF alone, LF + 1 μg CT, or LF + 4 μg IL-1α. Two weeks after the final vaccination (day 35), both WT and SASH mice vaccinated with LF + CT or 4 μg IL-1α had significantly increased serum anti-LF IgG titer compared with LF alone-vaccinated mice (p < 0.001; Fig. 8). WT and SASH mice vaccinated with LF + CT had serum anti-LF IgG geometric mean titers of 1:38,540,000 and 1:29,210,000, respectively (NS). WT and SASH mice vaccinated with LF + 4 μg IL-1α had serum anti-LF IgG geometric mean titer of 1:44,280,000 and 1:134,200,000, respectively (NS). Serum anti-LF IgG1 and IgG2c production was also similar between WT and SASH mice vaccinated with either LF + CT or LF + 4 μg IL-1α (data not shown). These data demonstrate that mast cells are not required for the adjuvant activity of nasally delivered IL-1α.

**Discussion**

In this study, we demonstrated that vaccination of IL-1R1 chimeric mice with LF + IL-1α induced significantly increased serum anti-LF Ab production in mice expressing IL-1R1 in either the stromal or BM-derived cellular compartments. However, the responses induced by vaccination were not equal across the three chimeric groups; IL-1R1 KO → WT mice produced significantly less Ag-specific serum IgG, serum IgG subclasses, and LeTx-neutralizing Abs than WT → KO and WT → WT mice. In addition, KO → WT mice had significantly lower Ab avidity than WT → KO and WT → WT mice. Therefore, maximal adjuvant activity of IL-1α after nasal immunization was maintained in mice expressing IL-1R1 on only hematopoietic cells, although T and B cells and mast cells were not required to be IL-1 responsive. Interestingly, DC expression of MyD88 was required for maximal IL-1α adjuvant activity. Conversely, IL-1α–induced innate cytokine and chemokine production required IL-1–responsive stromal cells, indicating that IL-1α–induced cytokine production was not required for IL-1α–induced anti-LF adaptive immune responses. In addition, although previous reports by our laboratory (16) and others (69) have suggested that CT may mediate its adjuvant activity by inducing IL-1, our data clearly show that CT maintains mucosal adjuvant activity in II1r1 Δ/Δ mice, demonstrating that CT-induced IL-1 is not required for the mucosal adjuvant activity of CT.

Many studies have examined the early (0–48 h) cytokine and chemokine profiles induced by specific adjuvants (25–28), but few have compared these profiles with the adjuvant-induced adaptive immune response (47, 70). Because of the ability of the chimeric model to assign adjuvant-induced responses to an adjuvant-responsive compartment, we examined the cytokine and chemokine profiles induced by nasal vaccination with IL-1α. Other studies have examined early serum cytokine production after i.m., s.c., or i.p. administration of vaccine adjuvants to mice and have shown different profiles of cytokine/chemokine induction (25, 71–73). In keeping with the results in the literature, we demonstrated an adjuvant-specific profile of cytokine production after intranasal vaccination, because IL-1α induced the production of IL-6, G-CSF, KC, and MCP-1. However, similar to the other studies, which observed peak cytokine production at 3–6 h, IL-1α–induced cytokine/chemokine production peaked at 3 h after vaccination. It
is noteworthy that the timing of sample collection therefore affects which cytokines are detected. Although IL-1α induced some cytokines/chemokines that were similar to the other adjuvants described, its profile was distinct.

To our knowledge, no other group has directly investigated the manner in which IL-1α mediates its adjuvant activity, although others have evaluated the role of endogenous IL-1 in the induction of host immune responses. Such studies have demonstrated that IL-1β and IL-1R1 have activities on CD4+ T cell expansion (64), and IL-1 is known to induce neutrophil migration (21, 22). In our study, the neutrophil chemokine KC and the granulopoiesis cytokine G-CSF were produced primarily by the nonhematopoietic cells in the serum and nasal lavage. Although G-CSF and KC remained increased in the nasal lavage of WT→KO mice, which did have anti-LF immune responses similar to WT→WT mice, their presence in KO→WT mice did not correlate with anti-LF adaptive immune responses equivalent to those measured in WT→WT mice. Although neutrophils have been shown to be important in the clearance of many pathogens (74, 75), they were recently demonstrated to inhibit Ag presentation and Ag-specific immune responses after vaccination with several Ags (i.e., HEL, OVA, or listeriolysin O) and adjuvants (i.e., IFA, CFA, alum) when comparing neutrophil-depleted or G-CSFR2/2 mice with WT (76). Therefore, it is unlikely that G-CSF, KC, or neutrophils play a large role in the nasal adjuvant activity of IL-1α.

Despite the significant induction of cytokines in serum and mucosal secretions after nasal immunization with IL-1α, our studies demonstrated that adjuvant-dependent early cytokine and chemokine production did not correlate with the induction of adaptive immune responses induced by vaccination. Our results are in agreement with others that adjuvant-induced cytokine production was not required for the induction of adaptive immunity. Sanders et al. (70) recently demonstrated that, when vaccinating mice i.p. with flagellin, serum cytokine and chemokine induction and splenic DC maturation were largely impaired in TLR5−/− mice, although serum IgG responses to flagellin were not impaired. A recent study by Longhi et al. (73) examined the ability of polyinosinic-polycytidylic acid (poly I:C) to induce serum cytokine production when given i.p., and although they demonstrated increased production of six cytokines (IL-6, IL-12(p40), TNF-α, IFN-α, IFN-β, IFN-γ), only IFN-α and IFN-β correlated with Ag-specific CD4+ T cell development. Although we vaccinated mice intranasally, our results and the published data from others suggest that many of the frequently used markers of innate immunity induction do not correlate with the induction of adaptive immune responses to the vaccine Ag. Therefore, studies of adjuvant mechanism should also focus on other areas of cooperation between the innate and adaptive immune response.

Several other studies have also used the BM chimeric mouse model to evaluate the adaptive immune responses to many different
Ag after codelivery with adjuvant. These studies have demonstrated that the induction of Ag-specific adaptive immune responses is dependent on both an adjuvant-responsive hematopoietic compartment and an adjuvant-responsive nonhematopoietic compartment (47, 73, 77). Two recent studies evaluated the requirements for TLR3 and MDA5 in poly I:C-induced Ag-specific IFN-γ CD4+ T (73) and CD8+ T cell (77) responses when delivered i.p. to mice with HIV gag p24 or OVA, respectively. Unlike IL-1, which can only signal through IL-1R1, poly I:C signals through both MDA5 and TLR3, but it is unclear which receptor(s) mediate poly I:C-induced immune responses (77). Together, the two studies indicate that poly I:C requires signaling capabilities in both compartments: TLR3 on hematopoietic cells and MDA5 on stromal cells. Remarkably, all of these studies used different routes of challenge/immunization but still demonstrated the requirement for Ag/adjuvant responsiveness in both compartments. In contrast with those studies, our results demonstrate that only IL-1R1 expression on hematopoietic cells was sufficient to induce maximal adaptive immune responses. However, we primarily evaluated humoral immune responses and did not examine CTL or T cell proliferative responses, which may explain the discrepancy between the studies.

IL-1 has been shown to play a role in the development of Th immune responses, including increased production of TH2 cytokines in Il1r1−/− mice when compared with WT mice (38). However, we did not observe any significant differences in Ag-specific Th cytokine production between chimeric mice vaccinated with LF + CT or LF + IL-1α. It is possible that this is a reflection of the route of delivery, as they delivered Ag s.c. and it has been shown that different routes of immunization induce different Th biases (78). In addition, serum Ag-specific IgM, IgG1, and IgG2a titers have not been shown to significantly differ between WT and Il1r1−/− mice (38, 79). Our results after immunization with LF + CT agree with the data of both studies, because we did not observe any significant differences in anti-LF IgG subclass production between chimeric groups. However, we did demonstrate that IL-1R1 chimerism significantly impacted the ability of IL-1α to enhance anti-LF IgG subclass production, indicating that the addition of exogenous IL-1 may have effects not seen when comparing the effects of endogenous levels of IL-1 in WT and Il1r1−/− mice.

Other studies have demonstrated the importance of direct DC stimulation with the adjuvant CpG to induce the production of Ag-specific IgG2b and IgG2a/c after systemic immunization (36, 67). Similar to the results in our study, the production of proinflammatory cytokines by non-DCs was able to partially compensate for the lack of TLR ligand responsiveness, inducing DC maturation (67) and, in one study, the production of significantly greater amounts of total IFN-γ CD4+ T cells compared with Mysd88−/− mice (80). These studies demonstrate that adjuvant-responsive DCs were required for maximal adjuvant activity after systemic immunization. Our results also demonstrate that the direct activation of DCs by IL-1α is required for maximal Ab production after nasal immunization using IL-1α as the adjuvant. Similar to the previously published studies, we observed significantly impaired production of Ag-specific IgG2b and IgG2c. However, further studies are required to determine the exact role of IL-1 stimulation of DCs in B cell Ab production after vaccination. In addition, these studies do not indicate whether IL-1-responsive DCs are sufficient for IL-1 adjuvant activity. However, we must note that the CD11c-Cre mutation used to generate the DC-Mysd88−/− mice also results in a loss of Myd88 expression on alveolar macrophages. Nonetheless, because of the small volume used for vaccination (5 μl/nostril), it is unlikely that any of the vaccine formulation reached the alveoli (81).

In summary, this study demonstrated that IL-1–responsive hematopoietic cells are sufficient for the maximal induction of Ag-specific adaptive immunity after vaccination with the adjuvant IL-1α, and that the direct action of IL-1α on DCs is required for maximal adjuvant activity. Although the Il1r1−/− stromal (radiation-resistant) cell compartment was able to produce cytokines/chemokines after nasal immunization with IL-1α and enhance the induction of Ag-specific adaptive responses, it was not sufficient for inducing maximal anti-LF adaptive immune responses in the absence of IL-1R1 on hematopoietic cells. Future studies are planned to optimize the nasal delivery of IL-1α–adjuvanted vaccines to maximize local adjuvant activity by direct activation of DCs.

Acknowledgments

We are grateful for the assistance provided by Melissa Ventevogel when creating the IL-1R1 mouse BM chimeras.

Disclosures

The authors have no financial conflicts of interest.

References


Supplementary Table I. Serum cytokine induction in WT mice after nasal vaccination with LF + IL-1α

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<th>3 Hours</th>
<th>6 Hours</th>
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<tbody>
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<td>LF</td>
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<td>282</td>
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<sup>a</sup> Significantly increased over naïve at the same time point (p < 0.05)

<sup>b</sup> Significantly increased over LF at the same time point (p < 0.05)
Supplementary Table II. Nasal lavage cytokine production (pg/ml) in WT mice at 3, 6, and 24 hours after vaccination with LF or IL-1α

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<sup>a</sup>Significantly increased vs. naïve at the same time point (p < 0.05)
<sup>b</sup>Significantly increased vs. LF at the same time point (p < 0.05)