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

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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-1 α 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-1 α 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-1 α 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-1 α adjuvant activity was not dependent on mast cells. However, the ability of IL-1 α 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-1 α 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-1 α to enhance immune responses to codelivered Ags. IL-1 α 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-1 α 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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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; NT₅₀, 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 stimulants, 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/IgG (intranasal and transcutaneous 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-1 α , 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-1 α 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-1 α 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-*Il1r1*^{tm1Imx/J}), CD45.1 congenic (B6.SJL-Ptprc^aPepc^b/BoyJ), *Rag1*^{-/-} (B6;129S7-*Rag1*^{tm1Mom/J}), and C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at 4–6 wk of age. Mast cell-deficient Kit^{W-sh}/HNhrJaeBsmJ mice (SASH) were obtained from Dr. Laura P. Hale (Department of Pathology, Duke University; available from The Jackson Laboratory as strain #005051) (45). 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, tibiae, 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 × 10⁷ 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 μ g LPS (Invivogen *E. coli* 0111:B4) in 10 μ l 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 μ l FACS buffer, centrifuged, and the supernatant removed as before. Samples were blocked with unlabeled mouse IgG2a at 10 μ g/ml in 100 μ l FACS buffer for 30 min on ice. Plates were then washed two times. Specific Abs were added to the wells in 100 μ l (4 μ g/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 μ l 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 μ l (5 μ l per nostril). Nonchimeric mice were vaccinated on days 0, 7, and 21. Chimeric 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 Camell, CA; 4 endotoxin units [EU]/mg), rLF + 4 μ g IL-1 α (400-ML; 0.00144 EU/ μ g; R&D, Minneapolis, MN), and rLF + 1 μ g cholera toxin (CT) (EU/mg unknown; List #100B). For three of the four *Il1r1* chimeric mouse experiments, mice were vaccinated with 25 μ g LF for the day 0, 7, and 21 immunizations and 50 μ g LF on day 42. For the remaining experiment, mice were immunized with 50 μ g LF each time. The calculated endotoxin delivery per mouse was ~0.20 (LF alone) to 0.22 EU (LF + IL-1 α). 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 collected 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 μ g/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/ μ g. 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 ELISA wash buffer and then 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 calculated as the percentage of the titer at the 0 M concentration; for example, (3 M titer/0 M titer)*100%.

Spleen cell restimulation

Splenocytes were restimulated *ex vivo* as previously described by our laboratory (55) with the following exceptions. Cells were plated in 250 μ l media/well at 1×10^7 cells/ml in 48-well plates (CoStar 353078), and 250 μ l T cell media \pm 40 μ g/ml rLF was added to the cells. Plates were incubated at 37°C for 60 h. Supernatants were harvested into 96-well deep-well plates and stored at -80°C. Thawed samples were tested for the presence of specific cytokines (IL-2,-4, -10, -17, IFN- γ) using a multiplexed bead assay from R&D. Values less than the low value of the standard curve were assigned a value of half the low standard. Data shown are the mean Ag-specific cytokine production for each group (i.e., LF-induced cytokine production - unstimulated cell cytokine production).

Lethal toxin neutralization assay

Serum collected from mice on day 42 or 56 postimmunization was used to measure the titer of anthrax lethal toxin-neutralizing Abs in an anthrax macrophage toxicity assay as described previously by our laboratory (56). In brief, a serum dilution series (50 μ l) was incubated with recombinant anthrax protective Ag (rPA) (List #171; 50 μ l; 0.1875 μ g/ml) and rLF (100 μ l; 0.1875 μ g/ml) for 1 h at 37°C. The serum/rPA/rLF mixtures were then added to J774A cells and incubated at 37°C for 4 h. Cell Titer 96 Aqueous One Solution (Promega, Madison, WI) was then used to determine the cell viability. Data shown are NT₅₀, the serum dilution at which neutralization of lethal toxin results in 50% of the plated macrophages remaining viable. Samples with undetectable neutralization activity (<1:64) were below our tested range and were assigned a value of one less than the lowest log₂ dilution (1:32) for graphical and statistical representation.

Rag1^{-/-} mouse reconstitution

Rag1^{-/-} mice (Jax #002096; B6;129S7-Rag1^{tm1Mom}/J) were injected retro-orbitally with 5×10^7 splenocytes and 8×10^5 CLN cells in HBSS (from wild-type [WT] or IL-1R1^{-/-} mice) and rested for 20 d. On day 20, peripheral blood was tested for reconstitution by flow cytometry using allophycocyanin anti-mouse CD3, PE anti-mouse B220, and FITC anti-mouse IgM Abs (1:200) using the earlier described peripheral blood flow cytometry protocol (data not shown). Mice were then vaccinated as described earlier on days 0, 7, 21, and 63 with 25 μ g LF \pm 4 μ g IL-1 α .

Cytokine/chemokine detection

Serum and nasal lavage (NL) samples from WT mice were tested for the presence of cytokines and chemokines using a mouse 23-plex multiplex bead assay from Bio-Rad (Hercules, CA). Based on the profiles seen in WT mice, serum and NL samples from chimeric mice were evaluated using custom kits from Bio-Rad (serum: IL-6, Eotaxin, G-CSF, keratinocyte chemoattractant [KC], and MCP-1; NL: IL-6, IL-10, IL-13, IFN- γ , G-CSF, KC, MCP-1, and TNF- α). Samples with values less than the low value of the standard curve were assigned a value of half the low standard.

Statistical analysis

All analyses were done in GraphPad Prism v. 5.00 (La Jolla, CA). All Ab data were analyzed using ANOVA with Bonferroni's multiple-comparison posttest. Samples with no detectable ELISA titers were assigned a value of

one less than the lowest log₂ dilution for statistical and graphical purposes. All cytokine data were log-transformed and analyzed using ANOVA with a Bonferroni's multiple-comparison posttest. Any Ag-specific cytokine production value equal to 0 was assigned a value of 0.01 before the log transformation to prevent loss of data. To evaluate differences in the number of mice producing mucosal anti-LF IgA, the numbers of "responders" and "nonresponders" were compared between groups using Fisher's exact test with Bonferroni's correction. A *p* value <0.05 was considered significant.

Results

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

Innate immune responses help to 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-1 α after nasal immunization was dependent on IL-1R1 expression in only the stromal (radioresistant) cell compartment, the hematopoietic cell compartment, or both compartments, serum cytokine responses were measured after nasal delivery of IL-1 α to IL-1R1 chimeric mice. IL-1R1 mouse BM chimeras were generated by transferring BM cells from *Il1r1*^{-/-} (knock-out [KO]) or CD45.1 congenic (WT) mice into lethally irradiated mice of the same strains to create four groups of BM chimeras: WT \rightarrow WT, KO \rightarrow KO, WT \rightarrow KO, and KO \rightarrow WT (donor \rightarrow 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-1 α (Supplemental Table I), serum samples were taken from chimeric mice at 3 h after vaccination with 25 μ g LF alone or LF + 4 μ g IL-1 α and tested for Eotaxin, MCP-1, KC, G-CSF, and IL-6 (Table I). Nasal vaccination of KO \rightarrow KO mice with LF + IL-1 α did not induce cytokine production above that observed in any mouse group immunized with LF alone, demonstrating that IL-1 α cytokine-inducing activity required IL-1R1, and that contaminants were not responsible for the cytokine-inducing activity of IL-1 α . Eotaxin was not significantly increased in any of the vaccinated mice. MCP-1, KC, G-CSF, and IL-6 were significantly increased in KO \rightarrow WT mice vaccinated with LF + IL-1 α compared with LF alone-vaccinated mice (*p* < 0.001) and were equivalent to the levels induced in WT \rightarrow WT mice, which were significantly increased compared with LF alone (*p* < 0.001). Conversely, vaccination of WT \rightarrow KO mice did not result in increased production of those cytokines, and their levels were similar to those in LF alone-vaccinated WT \rightarrow KO and LF + IL-1 α -vaccinated KO \rightarrow KO mice. Thus, IL-1 α 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-1 α 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-1 α . Three hours after vaccination with PBS or 4 μ g IL-1 α , 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).

Table I. Serum cytokine production (pg/ml) in IL-1R1 chimeric mice 3 h after vaccination with LF or LF + IL-1 α

Cytokine/Chemokine	Naive ^a	KO/KO		WT/KO		KO/WT		WT/WT	
		LF	IL-1	LF	IL-1	LF	IL-1	LF	IL-1
IL-6	2	5	4	6	9	6	257 ^{b,c}	7	222 ^{b,c}
Eotaxin	272	521	313	562	572	336	837	479	1128
G-CSF	82	45	70	124	175	104	13,439 ^{b,c}	44	8257 ^{b,c}
KC	35	57	62	62	85	69	3,763 ^{b,c}	71.27	1939 ^{b,c}
MCP-1	158	222	186	447	311	207	3,440 ^{b,c}	262	2831 ^{b,c}

^aNaive animals were WT/WT.

^bSignificantly greater than naive and LF alone within the same chimeric group ($p < 0.001$).

^cSignificantly greater than KO/KO and WT/KO IL-1 ($p < 0.001$).

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-1 α 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 (~6.5-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-1 α 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 and whether the responses correlated with cytokine/chemokine production, we vaccinated chimeric mice on days 0, 7, 21, and 42 with LF alone or LF + 4 μ g IL-1 α . Mice vaccinated with LF + 1 μ g CT served as controls. On day 56 after the initial vaccination, serum samples were collected and tested for the presence of LF-specific IgG (Fig. 1). Mice vaccinated with LF alone produced little anti-LF IgG (<1:80). Mice vaccinated with

LF + CT produced significantly greater amounts of LF-specific IgG than mice immunized with LF alone in all four chimeric groups ($p < 0.001$). Anti-LF serum IgG titers in chimeric mice vaccinated with LF + CT did not significantly differ from each other (range: 1:7,798,000–45,360,000). KO→KO mice vaccinated with LF + IL-1 α did not produce anti-LF IgG titers greater than mice vaccinated with LF alone (<1:80), confirming that the adjuvant activity of IL-1 α was dependent on the presence of IL-1R1 and not due to contaminants, such as LPS. However, the other three chimeric groups had significantly increased serum anti-LF IgG titers ($p < 0.001$) when vaccinated with LF + IL-1 α versus mice immunized with LF alone within the same chimeric group. WT→WT mice produced significantly more anti-LF IgG than KO→WT mice (1:18,720,000 versus 1:392,772, respectively), whereas WT→WT and WT→KO (1:2,731,000) mice did not differ significantly. These data indicated that IL-1R1 expression in either cellular compartment provided significant adjuvant activity after nasal delivery of IL-1 α 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-1 α .

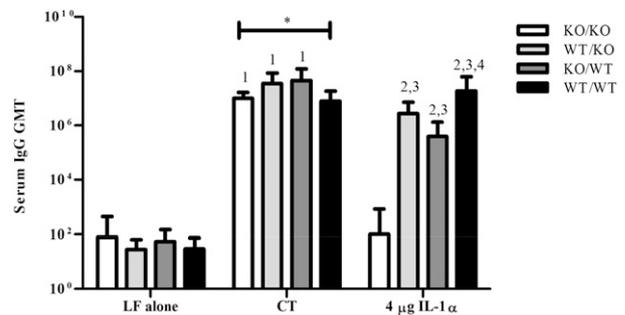


FIGURE 1. Serum anti-LF IgG titers are significantly decreased after vaccination with LF + IL-1 α in the absence of IL-1R1 on hematopoietic cells. Mice were vaccinated on days 0, +7, +21, and +42 after a 10-wk rest period after BM reconstitution. Serum samples taken on day +56 were tested by ELISA. Bars represent the geometric mean titers for each group on day +56 for all replicates, with error bars representing the 95% confidence level. Asterisk indicates 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-1 α within the same chimeric group ($p < 0.001$). 2, Significantly greater than LF alone ($p < 0.001$). 3, Significantly greater than KO/KO LF + IL-1 α ($p < 0.001$). 4, Significantly greater than KO/WT LF + IL-1 α ($p < 0.001$). 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-1 α (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-1 α ; $n = 20$ for WT/KO LF + CT; $n = 21$ for WT/KO LF + IL-1 α and KO/WT LF alone; $n = 23$ for KO/WT LF + CT; $n = 24$ for KO/WT LF + IL-1 α .

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

Cytokine/Chemokine	Naive ^a	WT/KO	KO/WT	WT/WT
IL-6	2	74	595 ^{b,c}	852 ^{b,c}
IL-10	1	1	1	1
IL-13	13	1	61 ^c	72 ^c
G-CSF	41	656 ^b	1484 ^b	1896 ^b
IFN- γ	1	2	2	3
KC	18	130 ^b	859 ^{b,c}	887 ^{b,c}
MCP-1	5	7	84 ^{b,c}	120 ^{b,c}
TNF- α	2	2	2	2

^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 \rightarrow KO mice vaccinated with LF + IL-1 α did not produce significantly greater amounts of any IgG subclass than LF alone ($\leq 1:128$ versus $\leq 1:128$, respectively). The IgG1 responses were very similar to the IgG responses in all examined groups. When vaccinated with LF + IL-1 α , WT \rightarrow WT mice produced significantly more anti-LF IgG1 than KO \rightarrow WT mice ($p < 0.01$), but anti-LF IgG1 production did not differ significantly between WT \rightarrow KO and KO \rightarrow 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 \rightarrow KO and WT \rightarrow WT mice produced significantly greater amounts of anti-LF IgG3 ($p < 0.001$). Both WT \rightarrow KO and WT \rightarrow WT mice produced significantly greater amounts anti-LF IgG2c, IgG2b, and IgG3 than KO \rightarrow WT mice ($p < 0.001$ and $p < 0.01$, respectively). These data support the BM-dependent requirement for maximal IL-1 α 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 serum IgG responses, KO \rightarrow KO mice vaccinated with LF + IL-1 α did not respond to vaccination with the production of lethal toxin-neutralizing serum Abs. Vaccination of KO \rightarrow WT mice with LF + IL-1 α induced an NT₅₀ of only 1:64, which was not significantly increased compared with neutralizing Abs induced by immunization with LF alone. WT \rightarrow KO and WT \rightarrow WT mice vaccinated with LF + IL-1 α 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 \rightarrow WT mice vaccinated with LF + IL-1 α ($p < 0.01$).

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

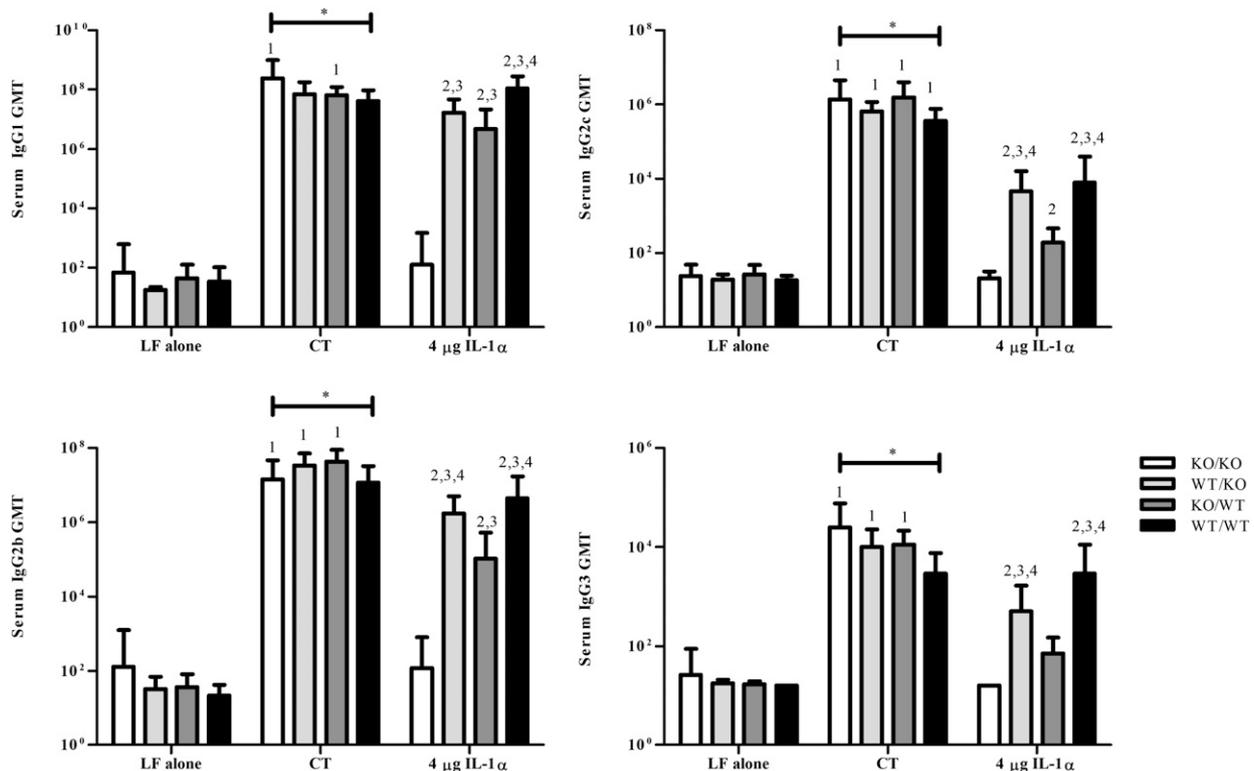


FIGURE 2. Serum anti-rLF IgG subclass profiles are significantly altered after vaccination with LF + IL-1 α in the absence of IL-1R1 expression on hematopoietic cells. Mice were vaccinated on days 0, +7, +21, and +42 after a 10-wk rest period after BM reconstitution. Serum samples taken on day +56 were 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-1 α 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-1 α ($p < 0.001$). 4, Significantly greater than KO/WT LF + IL-1 α ($p < 0.01$). 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-1 α (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-1 α ; $n = 20$ for WT/KO LF + CT. IgG1 and IgG2c: $n = 21$ for WT/KO LF + IL-1 α and KO/WT LF alone; $n = 23$ for KO/WT LF + CT; $n = 25$ for KO/WT LF + IL-1 α . IgG2b and IgG3: $n = 20$ for WT/KO LF + IL-1 α ; $n = 21$ for and KO/WT LF alone; $n = 22$ for KO/WT LF + CT; $n = 25$ for KO/WT LF + IL-1 α (IgG2b); and $n = 24$ for KO/WT LF + IL-1 α (IgG3).

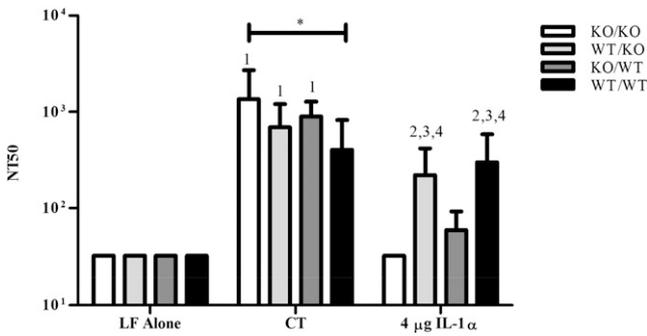


FIGURE 3. LF + IL-1 α -induced protection against lethal toxin is significantly impaired by the absence of IL-1R1 on hematopoietic cells. Mice were vaccinated on days 0, +7, +21, and +42 after a 10-wk rest period after BM reconstitution. Serum samples taken on day +56 were tested using an anthrax macrophage 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-1 α 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-1 α ($p < 0.001$). 4, Significantly greater than KO/WT LF + IL-1 α ($p < 0.01$). 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-1 α (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-1 α ; $n = 20$ for WT/KO LF + CT; $n = 21$ for WT/KO LF + IL-1 α and KO/WT LF alone; $n = 23$ for KO/WT LF + CT; $n = 25$ for KO/WT LF + IL-1 α .

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 \rightarrow WT mice vaccinated with LF + IL-1 α had significantly lower percentage titers at 1, 2, and 3 M NH₄SCN (47, 20, and 12%, respectively) than WT \rightarrow KO (70, 40, and 32%, respectively) and WT \rightarrow WT (78, 46, and 36%, respectively) mice ($p < 0.05$). Therefore, the ability of IL-1 α 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-1 α -responsiveness for the induction of maximal Ag-specific humoral immunity, with IL-1R1 KO \rightarrow WT mice having decreased serum immune responses to vaccination with LF + IL-1 α when compared with WT \rightarrow KO and WT \rightarrow WT. It is also evident from the chimeric mouse studies that serum and local cytokine/chemokine responses 3 h after vaccination with LF + IL-1 α do not correlate with the adaptive immune response.

Mucosal Ab responses to vaccination with LF + IL-1 α are impaired in IL-1R1 KO \rightarrow 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-1 α and IL-1 β 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

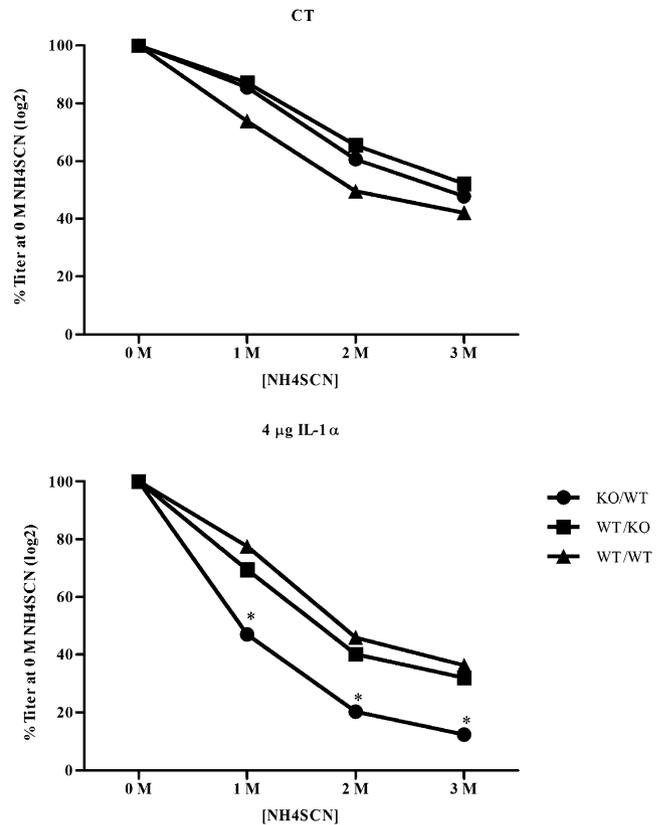


FIGURE 4. LF + IL-1 α -induced Ab avidity is significantly decreased by the absence of IL-1R1 on hematopoietic cells. Mice were vaccinated on days 0, +7, +21, and +42 after a 10-wk rest period after BM reconstitution. Serum samples taken on day +56 were tested using an NH₄SCN elution ELISA. Lines represent 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-1 α , KO/WT LF + CT, and KO/WT LF + IL-1 α ; $n = 22$ for WT/KO LF + IL-1 α ; $n = 25$ for WT/KO LF + CT.

titer/ μ g; 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 titer/ μ g). Nasal and vaginal LF-specific IgA production was elevated in all groups vaccinated with LF + CT (nasal: 23.8–41.5 titer/ μ g; vaginal: 36.2–77.4 titer/ μ g), but no group had significantly increased nasal or vaginal anti-LF IgA titers compared with mice immunized with LF alone. However, KO \rightarrow WT mice vaccinated with LF + CT had significantly increased fecal IgA production ($p < 0.05$). WT \rightarrow KO and WT \rightarrow WT mice vaccinated with LF + IL-1 α had significantly increased nasal anti-LF IgA compared with KO \rightarrow KO mice ($p < 0.01$) and mice vaccinated with LF alone within the same chimeric group ($p < 0.05$). WT \rightarrow WT mice vaccinated with LF + IL-1 α also had significantly increased vaginal anti-LF IgA compared with KO \rightarrow WT, WT \rightarrow KO, and LF alone-vaccinated WT \rightarrow WT mice ($p < 0.05$). KO \rightarrow WT mice vaccinated with LF + IL-1 α produced detectable vaginal and nasal anti-LF IgA (16.5 and 74.2 titer/ μ g, respectively), but the titers were not significantly increased over LF alone-vaccinated KO \rightarrow WT or KO \rightarrow KO mice vaccinated with LF + IL-1 α . WT/WT mice vaccinated with LF + IL-1 α produced significantly more anti-LF vaginal IgA than WT/WT mice vaccinated with LF + CT ($p < 0.05$), and WT \rightarrow KO mice vaccinated with LF + IL-1 α produced significantly more anti-LF nasal IgA than WT \rightarrow KO mice vaccinated with LF + CT. In addition to confirming that IL-1 α can more potently induce

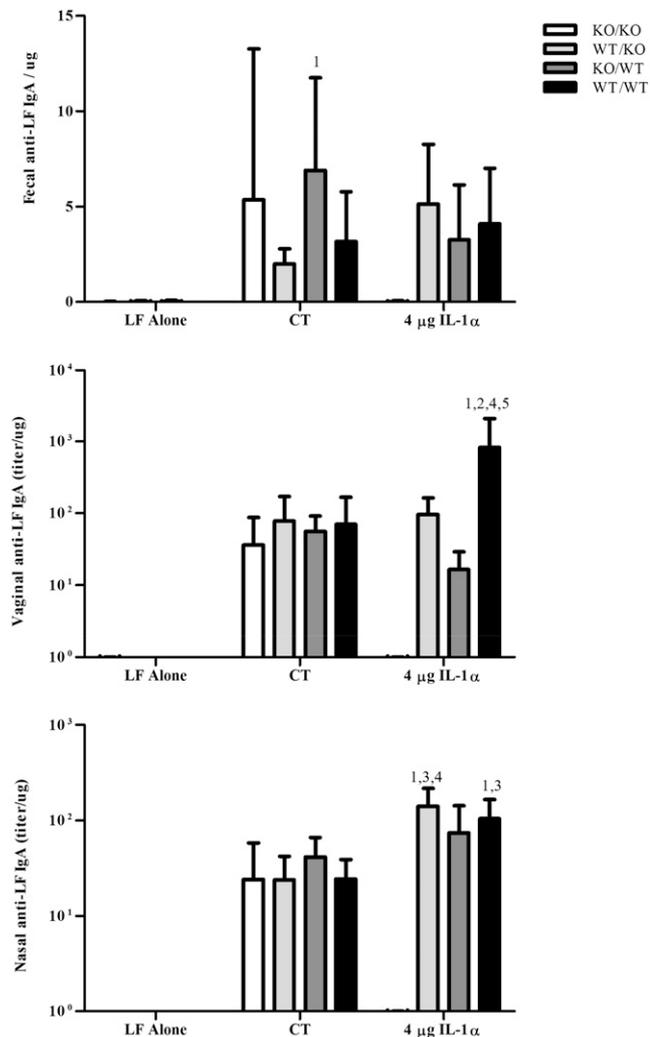


FIGURE 5. Vaginal, not nasal, anti-LF IgA titers are significantly affected by the absence of IL-1R1 on hematopoietic and stromal cells. Mice were vaccinated on days 0, +7, +21, and +42 after a 10-wk rest period after BM reconstitution. Bars represent the mean IgA titer/ μ g on day +56 (vaginal) or day +66 or +82 (nasal lavage), with error bars representing the 95% confidence level. 1, Significantly greater than LF alone within the same chimeric group ($p < 0.01$). 2, Significantly greater than KO/WT LF + IL-1 α ($p < 0.05$). 3, Significantly greater than KO/KO LF + IL-1 α ($p < 0.01$). 4, Significantly greater than LF + CT within the same chimeric group ($p < 0.05$). 5, Significantly greater than WT/KO LF + IL-1 α ($p < 0.05$). Four replicates unless noted: fecal IgA: $n = 7$ for KO/KO LF alone (two replicates); $n = 8$ for KO/KO LF + CT (two replicates); $n = 11$ for KO/KO LF + IL-1 α (two replicates); $n = 16$ for WT/WT LF + IL-1 α ; $n = 17$ for WT/WT LF alone; $n = 18$ for WT/WT LF + CT; $n = 19$ for WT/KO LF + IL-1 α and LF + CT; $n = 20$ for WT/KO LF alone, KO/WT LF + CT, and WT/KO LF + CT; $n = 24$ for KO/WT LF + IL-1 α . Vaginal IgA: $n = 7$ for KO/KO LF alone and KO/KO LF + CT (two replicates); $n = 11$ for KO/KO LF + IL-1 α (two replicates); $n = 16$ for WT/WT LF + IL-1 α ; $n = 17$ for WT/WT LF alone; $n = 18$ for WT/WT LF + CT; $n = 19$ for WT/KO LF + IL-1 α and LF + CT; $n = 20$ for WT/KO LF alone; $n = 21$ for KO/WT LF alone; $n = 23$ for KO/WT LF + CT; $n = 25$ for KO/WT LF + IL-1 α . Nasal IgA (two replicates): $n = 7$ for KO/KO LF alone and WT/WT LF alone; $n = 8$ for KO/KO LF + CT and WT/WT LF + IL-1 α ; $n = 9$ for WT/WT LF + CT; $n = 10$ for WT/KO LF alone and LF + CT; $n = 11$ for KO/KO LF + IL-1 α and WT/KO LF + IL-1 α ; $n = 12$ for KO/WT LF alone; $n = 13$ for KO/WT LF + CT and LF + IL-1 α .

mucosal IgA production than CT (16), these data suggest that IL-1R1 is required on both hematopoietic and stromal cells for maximal production of vaginal, not nasal, IgA.

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 \rightarrow WT mice (48 and 54%, respectively) produced IgA than WT \rightarrow KO (79 and 100%, respectively) and WT \rightarrow 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 \rightarrow WT mice were impaired compared with WT \rightarrow KO and WT \rightarrow WT mice vaccinated with LF + IL-1 α .

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

Given that the IL-1 α -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 \rightarrow KO mice vaccinated with LF + IL-1 α did not produce significantly greater amounts of any of the five cytokines compared with those from KO \rightarrow KO LF alone-vaccinated mice. Cells from KO \rightarrow WT mice vaccinated with any of the adjuvanted formulations and from KO \rightarrow KO mice vaccinated with LF + CT produced less IL-17 than those from WT \rightarrow KO or WT \rightarrow WT mice, but these differences were not significant. Cells from WT \rightarrow KO, KO \rightarrow WT, and WT \rightarrow WT mice vaccinated with LF + IL-1 α did not produce significantly different amounts of any of the five cytokines examined, although those from KO \rightarrow WT mice produced 2-fold less IFN- γ than did WT \rightarrow WT mice (338.5 versus 718 pg/ml, respectively). However, cells from WT \rightarrow 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

Group	Vaginal IgA		Fecal IgA		Nasal IgA	
	%	<i>n</i>	%	<i>n</i>	%	<i>n</i>
LF alone						
WT/KO	0	20	5	21	0	10
KO/WT	0	21	22	18	0	12
WT/WT	0	17	0	15	0	7
LF + CT						
WT/KO	72 ^a	18	100 ^a	20	80 ^a	10
KO/WT	74 ^a	23	95 ^a	20	92 ^a	13
WT/WT	61 ^a	18	89	18	89 ^a	9
LF + 4 μ g IL-1 α						
WT/KO	79 ^{a,b}	19	79 ^{a,b}	19	100 ^{a,b}	11
KO/WT	48 ^a	25	79 ^a	24	54	13
WT/WT	81 ^{a,b}	16	88 ^{a,b}	16	88 ^{a,b}	8

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

^aSignificantly greater than LF alone within the same chimeric group.

^bSignificantly greater than KO/KO LF + IL-1 α .

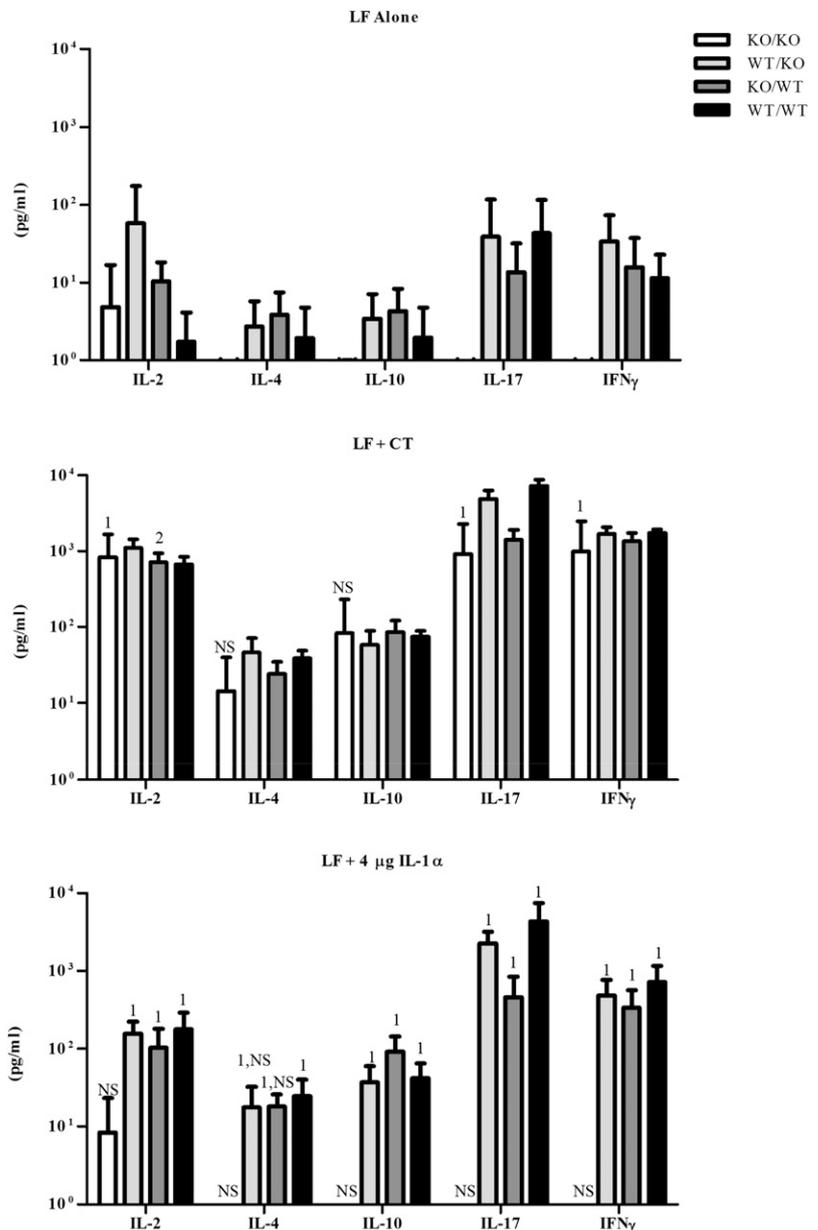


FIGURE 6. Ag-specific cytokine production after splenocyte restimulation with rLF is not impacted in IL-1R1 chimeric mice. Splenocytes were removed after the last sample collection on day +56 after four vaccinations. Bars represent the mean cytokine production for each group for all replicates minus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

tively; $p < 0.01$), whereas those from KO \rightarrow WT (18.13 pg/ml) and WT \rightarrow KO (17.55 pg/ml) mice did not; this difference was not reflected in cells from mice vaccinated with LF + CT, in which cells from WT \rightarrow KO, KO \rightarrow WT, and WT \rightarrow WT mice all produced significantly greater amounts of IL-4 than those from LF alone-vaccinated mice. These data indicate that spleen cell restimulation cytokines are not significantly impacted by the absence of IL-1R1 in either compartment, suggesting that vaccine-induced splenocyte cytokine secretion profiles in IL-1R1 chimeric mice vaccinated with IL-1 α do not directly correlate with the innate cytokine/chemokine profile or the IgG subclass profile observed in previous experiments.

IL-1 α adjuvant activity is not impaired in Rag1 $^{-/-}$ mice adoptively transferred with Il1r1 $^{-/-}$ splenocytes

The earlier chimeric mouse data demonstrate that IL-1 α 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 μ g LF alone, LF + 1 μ g CT, or LF + 4 μ g IL-1 α 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 NT₅₀ responses also did not significantly differ between groups vaccinated with LF + CT or LF + IL-1 α (data not shown). These data suggest that the nasal adjuvant activity of IL-1 α did not require T and B cell expression of IL-1R1.

Mast cells are not required for the adjuvant activity of IL-1 α

IL-1 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 \rightarrow KO mice given 0.25 or 4 μ g IL-1 α 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-

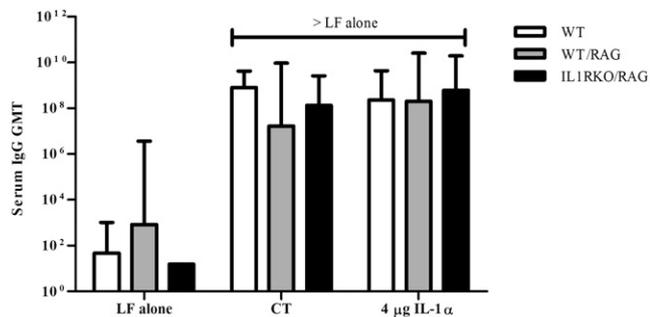


FIGURE 7. IL-1-responsive T and B cells are not required for IL-1 α nasal adjuvant activity. Mice were vaccinated on days 0, +7, +21, and +63 after a 20-d rest period after adoptive transfer of splenocytes. Serum samples taken on day +77 were tested by ELISA. Bars represent the geometric mean titers for each group on day +77, with error bars representing the 95% confidence level. One replicate; $n = 4$ for KO/RAG LF alone and LF + CT; $n = 5$ for all other groups.

requirement for mast cells in the nasal adjuvant activity of IL-1 α . 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 μ g CT, or LF + 4 μ g IL-1 α . Two weeks after the final vaccination (day 35), both WT and SASH mice vaccinated with LF + CT or 4 μ g IL-1 α had significantly increased serum anti-LF IgG titers 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 μ g IL-1 α had serum anti-LF IgG geometric mean titers 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 μ g IL-1 α (data not shown). These data demonstrate that mast cells are not required for the adjuvant activity of nasally delivered IL-1 α .

IL-1 must directly activate DCs for maximal induction of adaptive immunity after nasal vaccination

Previous studies using mice with adjuvant-unresponsive DCs have demonstrated the requirement for direct stimulation of DCs to induce the production of Ag-specific IgG2b and IgG2a/c after systemic immunization with a soluble TLR ligand adjuvant (36,

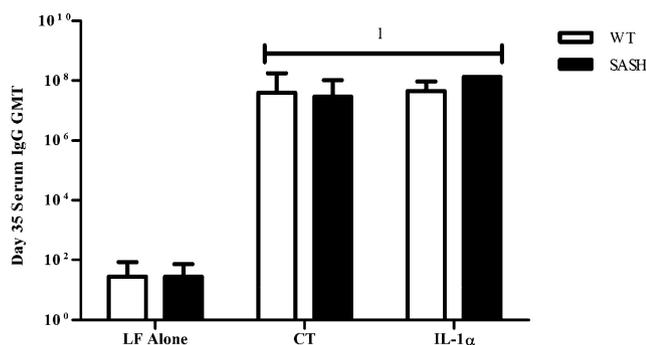


FIGURE 8. Mast cells are not required for the adjuvant activity of nasally delivered IL-1 α . Serum anti-rLF IgG titers on day +35 after intranasal immunization with rLF with or without adjuvant. Mice were vaccinated on days 0, +7, and +21. Serum samples taken on day +35 were tested by ELISA. Bars represent the geometric mean titers for each group on day +35, with error bars representing the 95% confidence level. 1, Significantly greater than LF alone within the same mouse group ($p < 0.001$). One replicate; $n = 5$ for all groups.

67). To evaluate the requirement of direct DC stimulation for IL-1 α adjuvant activity, we nasally vaccinated DC-*Myd88*^{-/-} mice, which have a DC-specific deletion of the *Myd88* gene that is required for IL-1 signaling (68), and WT, *Il1r1*^{-/-}, and *Myd88*^{-/-} mice with 25 μ g LF + 4 μ g IL-1 α on days 0, 7, 21, and 35. On day 42, LF + IL-1 α induced serum LF-specific IgG titers of 1:2,854,000 and 1:524,288 in WT and DC-*Myd88*^{-/-} mice, respectively, which were significantly increased when compared with *Il1r1*^{-/-} and *Myd88*^{-/-} mice ($< 1:105$; Fig. 9A). Similar results were seen for LF-specific IgG1 (Fig. 9B). However, LF + IL-1 α -induced serum IgG2c differed significantly between WT and DC-*Myd88*^{-/-} mice (1:3,649 and 1:588, respectively; $p < 0.05$), although IgG2c was significantly increased in both groups when compared with *Il1r1*^{-/-} and *Myd88*^{-/-} mice ($p < 0.01$).

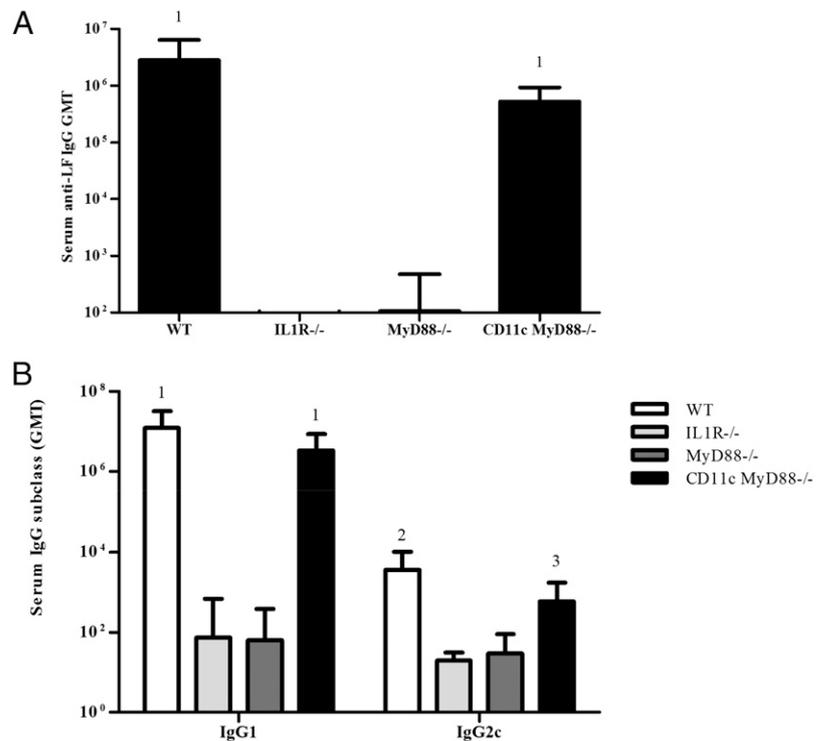
The ability of LF + IL-1 α to induce serum lethal toxin-neutralizing Abs in all four strains of mice was also examined (Fig. 10). Lethal toxin-neutralizing Ab production was significantly increased in WT mice (NT₅₀ 1:189) when compared with all other groups ($p < 0.01$). Vaccination of DC-*Myd88*^{-/-} mice induced an NT₅₀ of 1:89, which was not significantly increased when compared with *Il1r1*^{-/-} or *Myd88*^{-/-} mice (undetectable). These data indicate that direct adjuvant stimulation of DCs is required for maximal nasal adjuvant activity of IL-1 α .

Discussion

In this study, we demonstrated that vaccination of IL-1R1 chimeric mice with LF + IL-1 α 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 \rightarrow WT mice produced significantly less Ag-specific serum IgG, serum IgG subclasses, and LeTx-neutralizing Abs than WT \rightarrow KO and WT \rightarrow WT mice. In addition, KO \rightarrow WT mice had significantly lower Ab avidity than WT \rightarrow KO and WT \rightarrow WT mice. Therefore, maximal adjuvant activity of IL-1 α 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-1 α adjuvant activity. Conversely, IL-1 α -induced innate cytokine and chemokine production required IL-1-responsive stromal cells, indicating that IL-1 α -induced cytokine production was not required for IL-1 α -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 *Il1r1*^{-/-} 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-1 α . 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-1 α 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-1 α -induced cytokine/chemokine production peaked at 3 h after vaccination. It

FIGURE 9. IL-1-responsive DCs are required for the maximal adjuvant activity of nasally delivered IL-1 α . **(A)** Serum anti-rLF IgG titers on day +42 after intranasal immunization with rLF + IL-1 α . **(B)** Serum anti-rLF IgG1 and IgG2c titers day +42 after intranasal immunization with rLF + IL-1 α . Serum samples taken on day +42 were tested by ELISA. Bars represent the geometric mean titers for each group on day +42, with error bars representing the 95% confidence level. 1, Significantly greater than *Il1r1*^{-/-} and *Myd88*^{-/-} mice ($p < 0.001$). 2, Significantly greater than all other groups ($p < 0.05$). 3, Significantly greater than *Il1r1*^{-/-} and *Myd88*^{-/-} mice ($p < 0.01$). One replicate; $n = 18$ for WT; $n = 10$ for all other groups.



is noteworthy that the timing of sample collection therefore affects which cytokines are detected. Although IL-1 α 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-1 α 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-1 β 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 \rightarrow KO mice, which did have anti-LF immune responses similar to WT \rightarrow WT mice, their presence in KO \rightarrow WT mice did not correlate with anti-LF adaptive immune responses equivalent to those measured in WT \rightarrow 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-CSFR^{-/-} 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-1 α .

Despite the significant induction of cytokines in serum and mucosal secretions after nasal immunization with IL-1 α , 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

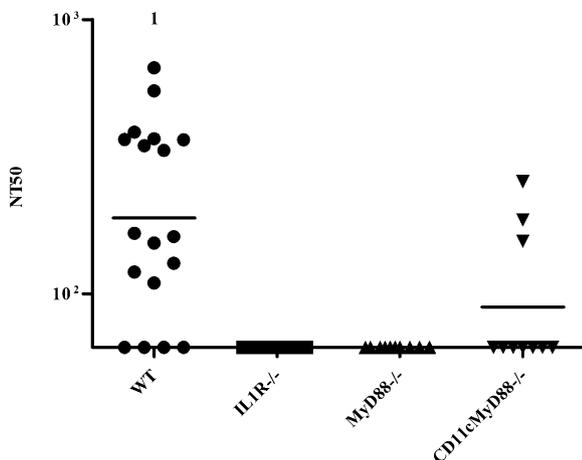


FIGURE 10. IL-1-responsive DCs are required for the maximal induction of serum lethal toxin-neutralizing Abs after vaccination with LF + IL-1 α . Serum samples taken on day +42 were tested using an anthrax macrophage toxicity assay. Lines represent the geometric mean titer within each group, with circles representing the titer for individual mice within each group. 1, Significantly increased compared with all other groups ($p < 0.01$). One replicate; $n = 18$ for WT; $n = 10$ for all other groups.

Ag_s 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-1 α . 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-1 α 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 *Myd88*^{-/-} 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-1 α is required for maximal Ab production after nasal immunization using IL-1 α 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-*Myd88*^{-/-} mice also results in a loss of MyD88 expression on alveolar macrophages. Nonetheless, because of the small volume used for vaccination (5 μ l/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-1 α , and that the direct action of IL-1 α 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-1 α 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-1 α -adjuvanted vaccines to maximize local adjuvant activity by direct activation of DCs.

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Disclosures

The authors have no financial conflicts of interest.

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