

# Endogenous IL-1R1 Signaling Is Critical for Cognate CD4<sup>+</sup> T Cell Help for Induction of In Vivo Type 1 and Type 2 Antipolysaccharide and Antiprotein Ig Isotype Responses to Intact *Streptococcus pneumoniae*, but Not to a Soluble Pneumococcal Conjugate Vaccine<sup>1,2</sup>

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MyD88<sup>-/-</sup> mice exhibit defective innate, diminished CD4<sup>+</sup> T cell-dependent (TD) type 1, but enhanced type 2, humoral immunity in response to intact *Streptococcus pneumoniae* (Pn). Because type 1 IL-1R (IL-1R1) signaling is MyD88 dependent, a role for endogenous IL-1 was determined. IL-1R1<sup>-/-</sup>, in contrast to MyD88<sup>-/-</sup>, mice exhibited relatively intact innate splenic cytokine expression in response to Pn. Nevertheless, IL-1R1<sup>-/-</sup>, like MyD88<sup>-/-</sup>, mice were more sensitive to killing with live Pn relative to wild-type controls. Although IL-1R1<sup>-/-</sup> mice elicited a normal T cell-independent IgM antipolysaccharide (PS) response to heat-killed Pn, the induction of PS- and protein-specific cognate, but not noncognate, TD type 1 and type 2 IgG isotypes were markedly reduced. Additionally, CD4<sup>+</sup> T cells from Pn-primed IL-1R1<sup>-/-</sup> mice failed to elicit IFN- $\gamma$ , IL-5, or IL-13 secretion upon restimulation with Pn in vitro, whereas MyD88<sup>-/-</sup> mice secreted normal levels of IFN- $\gamma$  and enhanced levels of IL-5 and IL-13. In contrast, IgG responses to a soluble, pneumococcal protein-PS conjugate, with or without adjuvant, showed little dependence on IL-1R1 and normal CD4<sup>+</sup> T cell priming. These data are the first to demonstrate a nonredundant role for endogenous IL-1 in TD induction of humoral immune responses to an intact pathogen, although not a pathogen-derived soluble conjugate, suggesting that antigenic context is a key determinant for IL-1 dependence. These data further suggest that IL-1 may be critical for preserving CD4<sup>+</sup> Th2 function in the presence, but not absence, of MyD88-dependent signaling via TLRs. *The Journal of Immunology*, 2006, 177: 6044–6051.

Interleukin-1 $\alpha$  and IL-1 $\beta$  are proinflammatory cytokines that exert largely overlapping biological effects, both inducing cell signaling exclusively through IL-1R1 (1). Soluble IL-1R antagonist also binds to IL-1R1 but does not exert agonistic activity, serving instead to block IL-1 $\alpha$  and IL-1 $\beta$  activity (2). IL-1RII also binds IL-1 but does not mediate signaling. IL-1 plays a key role in mediating innate host protection in response to pathogens, but is also a major inducer of tissue damage during infections and in pathologic conditions such as asthma and various autoimmune diseases (3, 4). IL-1 also exerts effects on cells involved in the adaptive immune response, acting directly as a costimulus for T cells (5), B cells (6), and dendritic cells (DCs)<sup>4</sup> (7). In this regard,

exogenous IL-1 can act as an adjuvant to enhance Ab production to particulate (8) and soluble protein (9) Ags. However, a role for endogenous IL-1 in stimulating Ab responses to a number of distinct Ags has yielded different results from some studies (10, 11) showing no significant effect, whereas other studies (12–15) demonstrated stimulation. Only a single study (11) evaluated a potential role for endogenous IL-1 in regulating Ab production during infection with a pathogen, *Leishmania major*, and demonstrated no significant effect. Thus, collectively these data leave unresolved whether IL-1 plays any nonredundant, physiologic role in regulating an adaptive humoral immune response to an infectious agent, as well as the determinants, whereby IL-1 is critical for inducing Ig responses to an isolated Ag.

Immunization of mice with intact *Streptococcus pneumoniae* (Pn), a Gram-positive extracellular bacterium, elicits T cell-independent (TI) IgM responses specific for the phosphorylcholine (PC) determinant of the cell wall C-polysaccharide (C-PS), the capsular polysaccharide (PPS), and the cell wall protein, pneumococcal surface protein A (PspA) (16, 17). In addition, Pn induces specific IgG responses that are dependent upon CD4<sup>+</sup> T cells (T cell-dependent (TD)), B7-dependent costimulation, and CD40-CD40L interactions (16–18). IgG anti-PspA and anti-PPS responses are dependent on cognate CD4<sup>+</sup> T cell help, whereas the IgG anti-PC response is stimulated by CD4<sup>+</sup> T cells in a noncognate manner (17, 19). Additionally, DCs pulsed with intact Pn and transferred into naive mice elicit both Pn-specific TI IgM and TD IgG anti-PS and antiprotein responses (20). A role for the endogenous DC in stimulating anti-Pn humoral immunity has also been demonstrated (21).

Intact Pn contains a number of TLR ligands that elicit the early release of pro- and anti-inflammatory cytokines, including IL-1

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Received for publication May 15, 2006. Accepted for publication August 1, 2006.

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<sup>1</sup> This study was supported by National Institutes of Health Grant 1R01 AI49192 and the Uniformed Services University of the Health Sciences Dean's Research and Education Endowment Fund.

<sup>2</sup> Opinions and assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of Department of Defense or the Uniformed Services University of the Health Sciences.

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<sup>4</sup> Abbreviations used in this paper: DC, dendritic cell; Pn, intact *Streptococcus pneumoniae*; TI, T cell independent; PC, phosphorylcholine; C-PS, C-polysaccharide; PPS14, purified pneumococcal capsular polysaccharide, type 14; PspA, pneumococcal surface protein A; TD, T cell dependent; PC-KLH, PC-keyhole limpet hemocyanin; SN, supernatant; CpG-ODN, CpG-containing oligodeoxynucleotide; PnP, purified Pn14 protein extract; IRBP, interphotoreceptor retinoid-binding protein.

## Report Documentation Page

*Form Approved*  
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|   |                                    |                                     |   |   |                                 |
|---|------------------------------------|-------------------------------------|---|---|---------------------------------|
| 1. REPORT DATE<br><b>2006</b>   |                                    | 2. REPORT TYPE                      |   | 3. DATES COVERED<br><b>00-00-2006 to 00-00-2006</b> |                                 |
| 4. TITLE AND SUBTITLE<br><b>Endogenous IL-1R1 Signaling Is Critical for Cognate CD4+ T Cell Help for Induction of In Vivo Type 1 and Type 2 Antipolysaccharide and Antiprotein Ig Isotype Responses to Intact Streptococcus pneumoniae, but Not to a Soluble Pneumococcal Conjugate Vaccine</b> |                                    |                                     |   | 5a. CONTRACT NUMBER                                 |                                 |
|   |                                    |                                     |   | 5b. GRANT NUMBER                                    |                                 |
|   |                                    |                                     |   | 5c. PROGRAM ELEMENT NUMBER                          |                                 |
| 6. AUTHOR(S)  |                                    |                                     |   | 5d. PROJECT NUMBER                                  |                                 |
|   |                                    |                                     |   | 5e. TASK NUMBER                                     |                                 |
|   |                                    |                                     |   | 5f. WORK UNIT NUMBER                                |                                 |
| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)<br><b>Uniformed Services University of the Health Sciences, Department of Pathology, Bethesda, MD, 20814</b>   |                                    |                                     |   | 8. PERFORMING ORGANIZATION REPORT NUMBER            |                                 |
| 9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)   |                                    |                                     |   | 10. SPONSOR/MONITOR'S ACRONYM(S)                    |                                 |
|   |                                    |                                     |   | 11. SPONSOR/MONITOR'S REPORT NUMBER(S)              |                                 |
| 12. DISTRIBUTION/AVAILABILITY STATEMENT<br><b>Approved for public release; distribution unlimited</b>   |                                    |                                     |   |   |                                 |
| 13. SUPPLEMENTARY NOTES   |                                    |                                     |   |   |                                 |
| 14. ABSTRACT  |                                    |                                     |   |   |                                 |
| 15. SUBJECT TERMS   |                                    |                                     |   |   |                                 |
| 16. SECURITY CLASSIFICATION OF:   |                                    |                                     | 17. LIMITATION OF ABSTRACT<br><b>Same as Report (SAR)</b> | 18. NUMBER OF PAGES<br><b>8</b>                     | 19a. NAME OF RESPONSIBLE PERSON |
| a. REPORT<br><b>unclassified</b>  | b. ABSTRACT<br><b>unclassified</b> | c. THIS PAGE<br><b>unclassified</b> |   |   |                                 |

(22–24). Pn-induced cytokine release (22, 25, 26), as well as IL-1R1 signaling (27–30), is dependent on the TLR adaptor protein MyD88. In this regard, both MyD88<sup>-/-</sup> (25, 26) and IL-1R1<sup>-/-</sup> (31–33) mice have decreased innate resistance against pneumococcal meningitis and pneumonia. In addition, MyD88<sup>-/-</sup> mice challenged with intact Pn exhibit a selective reduction in the elicitation of Pn-specific type 1 IgG isotypes (IgG3, IgG2b, and IgG2a), whereas the type 2 IgG isotype, IgG1, is significantly elevated relative to wild-type mice (22), consistent with several study systems (34, 35) in other models. Collectively, these data suggest that the loss of IL-1 activity in MyD88<sup>-/-</sup> mice could account, at least in part, for the MyD88<sup>-/-</sup> functional phenotype in response to intact Pn. Of interest, IL-1 has been shown to play a role in stimulating both type 1 (11, 36–38) and type 2 (14, 15, 39–42) cytokine-mediated immune responses, depending upon the experimental system. Mice genetically deficient in the IL-1R1 (IL-1R1<sup>-/-</sup>) exhibit normal hemopoietic, including lymphoid, development (10, 13, 43), and have normal basal levels of serum Ig isotypes relative to wild-type mice (10). Thus, immunization of IL-1R1<sup>-/-</sup> vs wild-type mice with intact Pn represents an ideal experimental system for determining a potential role for endogenous IL-1 for induction of TI and TD type 1 and type 2 humoral immunity to intact Pn, as well as to soluble, purified Pn-derived Ags.

## Materials and Methods

### Mice

IL-1R1<sup>-/-</sup> (B6.129, and C57BL/6 backgrounds), Rag-1<sup>-/-</sup> (C57BL/6 background), and wild-type B6129SF2/J mice were obtained from The Jackson Laboratory. Wild-type, female C57BL/6 mice were obtained from the National Cancer Institute and were typically used between 6 and 10 wk of age. These studies were conducted in accordance with the principles set forth in the Guide for Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council, revised in 1996, and were approved by the Uniformed Services University of the Health Sciences Institutional Animal Use and Care Committee.

### Reagents

Recombinant PspA was expressed in *Saccharomyces cerevisiae* BJ3505. The supernatant was then passaged through a Q FF column (Amersham Biosciences) and eluted with 0.2 M NaCl solution containing 20 mM Tris (pH 9.0). Eluted PspA was then added to a phenyl HP column (Amersham Biosciences) equilibrated with 40 mM phosphate buffer containing 1.3 M ammonium sulfate (pH 7.0). PspA was then eluted from this column using a 1.3–0.4 M ammonium sulfate gradient. Pooled PspA was dialyzed against a 20 mM sodium acetate (pH 4.7) solution and loaded into an S15 column (Amersham Biosciences). PspA was then eluted with a 0.3 M NaCl solution containing 20 mM of sodium acetate. Eluted PspA was dialyzed and concentrated and found to be >95% pure by Coomassie blue staining. PC-keyhole limpet hemocyanin (PC-KLH) was synthesized as described previously (16). The resulting conjugate had a substitution degree of 19 PC-KLH molecule. Purified PPS14 was purchased from American Type Culture Collection. A protein extract from *S. pneumoniae*, capsular type 14 (Pn14) was prepared using bacterial protein extraction reagent (Pierce).

### Preparation and immunization of *S. pneumoniae*, capsular type 14 (Pn14)

A frozen stock of Pn14 was thawed and subcultured on BBL premed blood agar plates (VWR International). Isolated colonies on blood agar were grown in Todd Hewitt broth (BD Biosciences) to mid-log phase, collected, and heat killed by incubation at 60°C for 1 h. Sterility was confirmed by subculture on blood agar plates. After extensive washings, the bacterial suspension was adjusted with PBS to give an absorbance reading at 650 nm of 0.6, which corresponded to 10<sup>9</sup> CFU/ml. Bacteria were then aliquoted at 10<sup>10</sup> CFU/ml and frozen at -80°C until their use as Ag for mouse immunizations. Mice were immunized i.p. with 2 × 10<sup>8</sup> CFU of heat-killed bacteria in 250 μl of PBS. The Pn14 stock was tested for endotoxin using the *Limulus* amoebocyte lysate assay (QCL-1000) from BioWhittaker. This assay demonstrated that mice injected with 2 × 10<sup>8</sup> CFU equivalents of heat-killed Pn14 receive <20 pg of endotoxin. Serum

samples for measurement of anti-PPS14, anti-PC, and anti-PspA Ab titers were prepared from blood obtained through the tail vein.

### Isolation and culture of peritoneal macrophages and spleen cells

RPMI 1640 (10 ml) was injected into the peritoneal cavity, through the fat pad using a 23-gauge needle. After 3 min, lavage fluid containing the peritoneal cells was withdrawn using an 18-gauge needle. Cells were pelleted by centrifugation at 400 × g for 10 min at room temperature. The cell pellet was suspended in culture medium (RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 0.05 mM 2-ME, 50 μg/ml penicillin, and 50 μg/ml streptomycin) and adjusted to 1 × 10<sup>6</sup> total cells/ml, and 1 ml of cell suspension was added to each well of a 24-well plate and incubated for 3 h at 37°C in a 5% CO<sub>2</sub>-containing incubator. Plates were then washed to remove unbound cells. RBC-lysed splenocytes were obtained using ammonium chloride potassium lysing buffer (Invitrogen Life Technologies). Spleen cells were cultured at 1 × 10<sup>7</sup> cells/ml. Intact heat-killed Pn14 at varying concentrations was added to culture wells containing peritoneal macrophages or spleen cells, and supernatant (SN) was removed 24 h later for measurement of cytokine concentrations by ELISA.

### Measurement of cytokine concentrations in peritoneal macrophage and spleen cell culture SN

The concentrations of specific cytokines released into the medium of spleen cell and peritoneal macrophage cultures to which Pn14 was added were measured using optimized standard sandwich ELISA. Recombinant cytokines used as standards, as well as the capture mAbs, biotinylated mAbs used for detection, and streptavidin-alkaline phosphatase were purchased from BD Pharmingen. Streptavidin-alkaline phosphatase was used in combination with *p*-nitrophenyl phosphate disodium (Sigma-Aldrich) as substrate to detect the specific binding. Standards were included in every plate and the samples were tested in duplicate.

### Real-time RT-PCR for measurement of splenic cytokine-specific mRNA

Total RNA was extracted from isolated spleen cell populations after immunization with 2 × 10<sup>8</sup> CFU of heat-killed Pn14 using RNAzol B (Tel-Test). Total RNA was then reverse-transcribed using the Superscript II preamplification system for first-strand cDNA synthesis (Invitrogen Life Technologies) according to the manufacturer's instructions. RNA (180 ng) was subsequently used as a template for each real-time PCR. All PCR for cytokine-specific mRNA were performed on an ABI PRISM 7700 Sequence Detector System (PE Applied Biosystems) using proprietary cytokine-specific primers and probes from ABI Applied Biosystems. Relative cytokine mRNA levels were determined by normalization of signal with that for ribosomal RNA. In initial studies, 2-fold dilutions of cDNA generated a linear signal curve over at least a 30-fold range of cDNA concentrations.

### Measurement of serum Ag-specific Ig isotype titers

Immulon 4 ELISA plates (Dydx Technologies) were coated (50 μl/well) with PC-KLH (5 μg/ml), PPS14 (5 μg/ml), or PspA (5 μg/ml) in PBS for 1 h at 37°C or overnight at 4°C. Plates were washed three times with PBS plus 0.1% Tween 20 and were blocked with PBS plus 1% BSA for 30 min at 37°C or overnight at 4°C. Three-fold dilutions of serum samples, starting at a 1/50 serum dilution, in PBS plus 0.05% Tween 20 were then added for 1 h at 37°C or overnight at 4°C, and plates were washed three times with PBS plus 0.1% Tween 20. Alkaline phosphatase-conjugated polyclonal goat anti-mouse IgM or IgG Abs (200 ng/ml final concentration) in PBS plus 0.05% Tween 20 were then added, and plates were incubated at 37°C for 1 h. Plates were washed five times with PBS plus 0.1% Tween 20. Substrate (*p*-nitrophenyl phosphate, disodium; Sigma-Aldrich) at 1 mg/ml in TM buffer (1 M Tris plus 0.3 mM MgCl<sub>2</sub>, pH 9.8) was then added for color development. Color was read at an absorbance of 405 nm on a Multiskan Ascent ELISA reader (Labsystems).

### CD4<sup>+</sup> T cell priming assay

Mice were immunized i.p. with 2 × 10<sup>8</sup> CFU heat-killed Pn14 in saline or PPS14-PspA plus C-PS-PspA (conjugates) adsorbed on 13 μg of alum (Alhydrogel 2%; Brenntag Biosector) mixed with 25 μg of a 30-mer CpG-containing oligodeoxynucleotide (CpG-ODN) (44) and similarly boosted on day 14. On day 28, spleen cell suspensions were prepared from individual mice and RBC were removed using ammonium chloride potassium lysing buffer (Invitrogen Life Technologies). Spleen cells (1 × 10<sup>7</sup>) from either naive or primed wild-type or mutant mice were suspended in 2 ml of

culture medium in 24-well plates and cultured at 37°C in an atmosphere of 7% CO<sub>2</sub> and 95% humidity. After a 30-min incubation, 30 μg of Pn14-derived protein extract (Pn14 primed) or purified PspA (conjugate primed) was added to each culture well. At 24, 48, 72, and 96 h, SN was obtained from separate wells for measurement of secreted cytokine concentrations by ELISA. Cytokine secretion was eliminated when spleen cells were first depleted of CD4<sup>+</sup> T cells using anti-CD4-coated magnetic beads (45).

#### Lethality study

Live Pn14 was grown to mid-log phase. Bacterial numbers were determined by colony counts on blood agar plates. Mice were injected i.p. with either 1 × 10<sup>8</sup> or 4 × 10<sup>7</sup> CFU of live Pn14 in 250 μl of PBS. Mice were observed twice daily for 5 days following infection.

#### Statistics

For Ag-specific serum Ig isotype titers, data were expressed as geometric means ± SEM of the individual serum Ig isotype titers. For concentrations of cytokine-specific protein and mRNA, data were expressed as arithmetic means ± SEM. Significance was determined by Student's *t* test. Values of *p* < 0.05 were considered to be statistically significant.

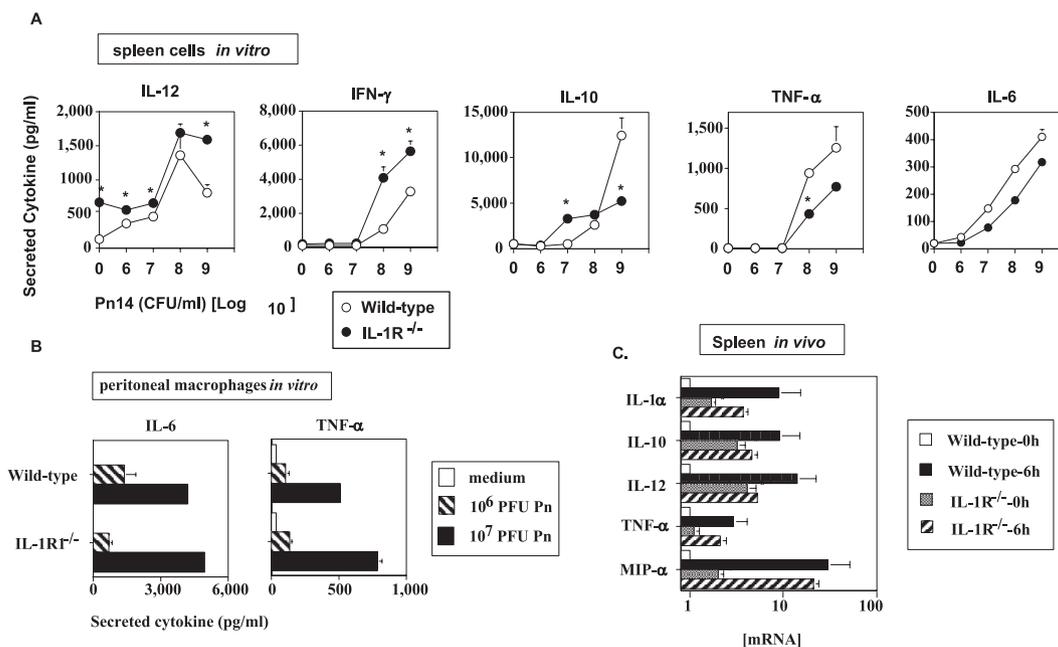
## Results

*IL-1R1<sup>-/-</sup> mice exhibit a largely intact innate cytokine response to Pn14 relative to wild-type mice, but are more sensitive to killing following i.p. infection*

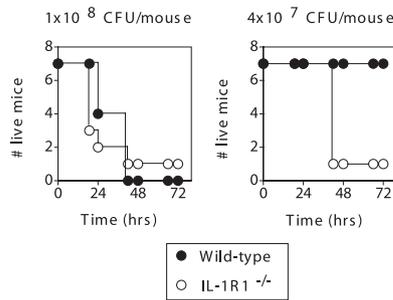
Cytokine induction in vitro and in vivo in response to Pn is almost completely abrogated in MyD88<sup>-/-</sup> mice (22, 25, 26). Because IL-1R1 signaling is dependent on MyD88 (27–30), we wished to determine the relative role of IL-1R1 in mediating Pn-induced, MyD88-dependent immunity. Spleen cells from wild-type and IL-1R1<sup>-/-</sup> mice were stimulated in vitro for 24 h in the absence or presence of varying concentrations of intact, heat-killed Pn14,

upon which the concentrations of several secreted cytokines were measured by ELISA. Modest increases in secreted IL-12 and IFN-γ and decreases in IL-10, TNF-α, and IL-6 were observed in IL-1R1<sup>-/-</sup> spleen cells relative to wild-type controls, but these differences did not always reach statistical significance (Fig. 1A). Furthermore, unelicited peritoneal macrophages from wild-type and IL-1R1<sup>-/-</sup> mice stimulated for 24 h with Pn14 in vitro secreted comparable amounts of IL-6 and TNF-α (Fig. 1B). We previously (46) demonstrated that i.p. injection with Pn14 induces multiple pro- and anti-inflammatory cytokine- and chemokine-specific mRNAs in spleen, peaking between 2 and 6 h postimmunization. As illustrated in Fig. 1C, spleen cells from naive (0 h) IL-1R1<sup>-/-</sup> mice showed a significant (*p* < 0.05) increase, relative to wild-type controls, in mRNA expression specific for IL-1α, IL-12, IL-10, and MIP-1α, with no significant difference in TNF-α. The relative induction of cytokine mRNAs, with the exception of TNF-α, 6 h postimmunization, was blunted in IL-1R1<sup>-/-</sup> mice but the absolute levels at this time point were comparable to that observed in Pn14-immunized wild-type mice. Similar results were observed at 2 h after Pn14 immunization (data not shown). Collectively, these data strongly suggest that IL-1R1<sup>-/-</sup> mice, in striking contrast to MyD88<sup>-/-</sup> mice, are capable of eliciting a largely intact early innate cytokine response to i.p. challenge with Pn14.

Both MyD88<sup>-/-</sup> (25, 26) and IL-1R1<sup>-/-</sup> (31–33) mice have decreased innate resistance against pneumococcal meningitis and pneumonia. We previously demonstrated that MyD88<sup>-/-</sup> mice are also more sensitive to killing following i.p. infection with live Pn14 (22). We now demonstrate that IL-1R1<sup>-/-</sup> mice also show a



**FIGURE 1.** IL-1R1<sup>-/-</sup> mice exhibit a largely intact innate cytokine response to Pn14. **A**, Spleen cells from naive IL-1R1<sup>-/-</sup> or wild-type (B6129SF2/J) mice (five per group) were cultured separately at 1 × 10<sup>7</sup> cells/ml in the absence or presence of the indicated concentrations of intact heat-killed Pn14. Culture SN was removed 24 h later for measurement of cytokine concentrations by ELISA. **B**, Unelicited peritoneal macrophages from naive IL-1R1<sup>-/-</sup> or wild-type (B6129SF2/J) mice (five per group) were pooled and cultured in triplicate at 1 × 10<sup>6</sup> cells/ml in the absence or presence of the indicated concentrations of intact heat-killed Pn14. Culture SN was removed 24 h later for measurement of cytokine concentrations by ELISA. **C**, IL-1R1<sup>-/-</sup> and wild-type (B6129SF2/J) mice (five per group) were immunized i.p. with 2 × 10<sup>8</sup> CFU/mouse of intact heat-killed Pn14. Spleens were removed from either unimmunized mice (0 h) or 6 h following immunization, and total RNA was purified separately from individual mice. Real-time RT-PCR was performed for measurement of relative levels of cytokine- or chemokine-specific mRNA. As a reference, an arbitrary value of 1 was assigned to the arithmetic mean of the wild-type 0-h set of samples. The three other sets of samples (6-h wild-type and 0- and 6-h IL-1R1<sup>-/-</sup>) are expressed in relation to this value. All data are presented as the arithmetic mean ± SEM. \*, *p* < 0.05 by Student's *t* test. The data illustrated are each from a single experiment that is representative of two independent experiments.



**FIGURE 2.** IL-1R1<sup>-/-</sup> mice are more sensitive to killing following i.p. infection. IL-1R1<sup>-/-</sup> and wild-type (B6129SF2/J) mice (seven per group) were injected i.p. with live Pn14 at a dose of either 1 × 10<sup>8</sup> CFU/mouse or 4 × 10<sup>7</sup> CFU/mouse. The higher and lower doses have been found to be lethal and sublethal, respectively, in wild-type mice (22). Mice were observed every 12 h for 5 days. Data are from one experiment that is representative of two independent experiments.

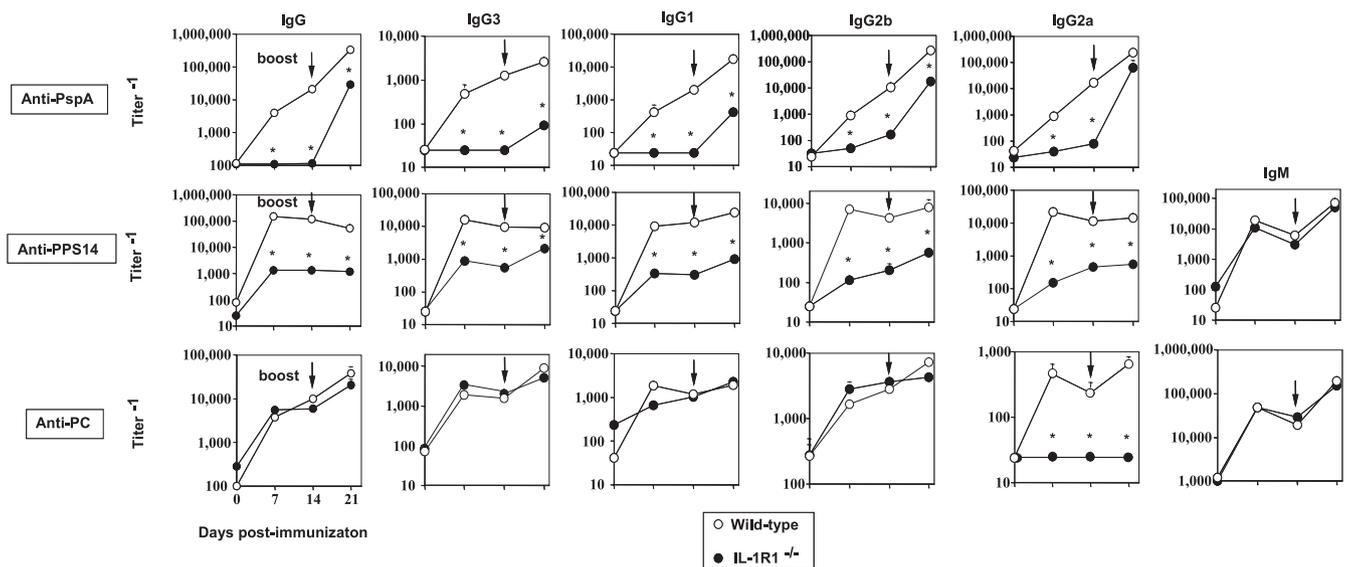
similar sensitivity. Thus, six of seven IL-1R1<sup>-/-</sup> mice, but none of seven wild-type mice, challenged i.p. with 4 × 10<sup>7</sup> CFU/mouse of live Pn14, died by 48 h postinfection (Fig. 2). All wild-type mice and the one surviving IL-1R1<sup>-/-</sup> mouse were still alive after 5 days (data not shown). Collectively, these and the published data strongly suggest that a defect in IL-1R1 signaling alone is a major contributor to the vulnerability of MyD88<sup>-/-</sup> mice to infection with Pn, independent of several other key proinflammatory mediators.

*IL-1R1<sup>-/-</sup> mice exhibit a marked defect in both type 1 and type 2 IgG anti-PspA and IgG anti-PPS14 isotypes, but not IgG anti-PC responses following i.p. immunization with Pn14*

We previously demonstrated (22) that MyD88<sup>-/-</sup> mice challenged i.p. with intact Pn14 were markedly defective in their induction of type 1 (IgG3, IgG2b, and IgG2a) isotypes specific for a number of pneumococcal proteins (i.e., PspA, PspC, and PsaA) as well as polysaccharides (i.e., PPS14 and PC determinant of C-PS),

whereas IgM responses were at best modestly reduced. In contrast, type 2 (IgG1) responses in MyD88<sup>-/-</sup> mice were significantly elevated relative to wild-type controls. In light of the direct costimulatory effects of IL-1 on cells important for adaptive immunity (i.e., DC, B cells, and T cells), we wished to determine the potential contribution of defective IL-1R1 signaling to the altered Pn14-dependent humoral immune responses observed in MyD88<sup>-/-</sup> mice. Wild-type and IL-1R1<sup>-/-</sup> mice were immunized i.p. with heat-killed Pn14 and boosted in a similar manner 14 days later. The TI IgM anti-PPS14 and IgM anti-PC responses to Pn14 in wild-type and IL-1R1<sup>-/-</sup> mice were comparable (Fig. 3), suggesting that B cell function in the absence of IL-1R1 signaling was intact. In contrast, the primary IgG anti-PspA and IgG anti-PPS14 responses in IL-1R1<sup>-/-</sup> mice were markedly reduced, whereas the IgG anti-PC response was similar to that observed in wild-type mice. Further analysis of IgG isotypes revealed that the reduction in serum titers of IgG anti-PspA and IgG anti-PPS14 was due to comparable decreases in both type 1 (IgG3, IgG2b, and IgG2a) and type 2 (IgG1) isotypes. Of note, only IgG2a anti-PC, a small contributor to the total IgG anti-PC response, was reduced in IL-1R1<sup>-/-</sup> mice (Fig. 3).

Pn14 induces memory for IgG anti-PspA, but not for IgG anti-PPS14, responses (17). In this regard, secondary immunization with Pn14 resulted in a proportionately greater boost in serum titers of total IgG anti-PspA and all IgG anti-PspA isotypes, especially IgG2b and IgG2a, in IL-1R1<sup>-/-</sup> relative to wild-type mice, in relation to the primary response (Fig. 3). However, the secondary titers of total IgG anti-PspA and IgG anti-PspA isotypes, except for IgG2a, in IL-1R1<sup>-/-</sup> mice were still significantly below that observed in wild-type mice. These data are in striking contrast to that observed in Pn14-immunized MyD88<sup>-/-</sup> mice, in which a selective decrease in type 1, and elevation in type 2, IgG isotypes specific for both PspA, PPS14, and PC were observed, despite the inability to signal through IL-1R1 (22). Thus, IL-1 may be critical for preserving CD4<sup>+</sup> Th2 function in the presence, but not absence, of MyD88-dependent signaling via TLRs.

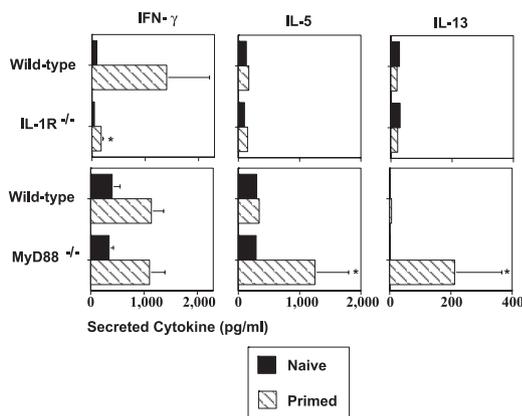


**FIGURE 3.** IL-1R1<sup>-/-</sup> mice exhibit a marked defect in both type 1 and type 2 IgG anti-PspA and IgG anti-PPS14 isotypes, but not IgG anti-PC responses following i.p. immunization with Pn14. IL-1R1<sup>-/-</sup> and control B6129SF2/J mice (seven per group) were immunized i.p. with 2 × 10<sup>8</sup> CFU of intact, heat-killed Pn14 and similarly boosted on day 14. Sera were collected on the indicated days for measurement of serum titers of Ag-specific IgM and IgG isotypes by ELISA. Data are presented as geometric mean ± SEM. \*, *p* < 0.05 by Student's *t* test. The data are from one experiment that is representative of three independent experiments. Two experiments used IL-1R1<sup>-/-</sup> mice on B6129 background and one experiment was on the C57BL/6 background with similar results.

CD4<sup>+</sup> T cells from Pn14-immunized IL-1R1<sup>-/-</sup> mice fail to exhibit priming for either type 1 or type 2 cytokine production, whereas those from MyD88<sup>-/-</sup> mice have normal type 1 and elevated type 2 responses

CD4<sup>+</sup> T cells obtained 28 days following two immunizations (days 0 and 14) of wild-type mice with intact heat-killed Pn14 secrete elevated levels of IFN- $\gamma$ , but not IL-4, IL-5, or IL-13 upon restimulation with a Pn14 protein extract (PnP) *in vitro*, relative to naive mice (45). As illustrated in Fig. 4, CD4<sup>+</sup> T cells from IL-1R1<sup>-/-</sup> mice immunized twice with Pn14 *i.p.* failed to release IFN- $\gamma$ , as well as IL-5 or IL-13 upon *in vitro* restimulation with PnP. In striking contrast, CD4<sup>+</sup> T cells from Pn14-immunized MyD88<sup>-/-</sup> mice secreted normal amounts of IFN- $\gamma$  and elevated levels of IL-5 and IL-13, upon *in vitro* restimulation with PnP, relative to wild-type mice (Fig. 4). IL-4 was not detected in any of the groups (data not shown). Thus, the differences observed in CD4<sup>+</sup> T cell priming in IL-1R1<sup>-/-</sup> and MyD88<sup>-/-</sup> mice immunized with Pn14 corresponded to the striking differences in the elicited IgG1 response.

We previously demonstrated that the CD4<sup>+</sup> T cell help for the IgG anti-PspA and IgG anti-PPS14 response to Pn14 was cognate in nature (17, 19). In contrast, an as yet uncharacterized form of TCR-nonspecific CD4<sup>+</sup> T cell help markedly promotes the IgG anti-PC response to Pn14. Thus, the normal IgG anti-PC response in IL-1R1<sup>-/-</sup> mice, in contrast to IgG anti-PspA and IgG anti-PPS14, suggests that IL-1R1 signaling was critical for the cognate, but not noncognate, CD4<sup>+</sup> T cell help observed in this model. To reinforce this idea, we directly determined whether the IgG anti-PC response in IL-1R1<sup>-/-</sup> mice was indeed CD4<sup>+</sup> T cell dependent, similar to what we observed in wild-type mice (16). Thus, 1 day before Pn14 immunization, wild-type and IL-1R1<sup>-/-</sup> mice were injected with an anti-CD4<sup>+</sup> T cell mAb (clone GK1.5) (47), which depletes CD4<sup>+</sup> T cells *in vivo*. Similar to that illustrated in Fig. 3, the IgG anti-PspA and IgG anti-PPS14, but not IgG anti-PC, response to Pn14 was significantly ( $p < 0.05$ ) reduced in



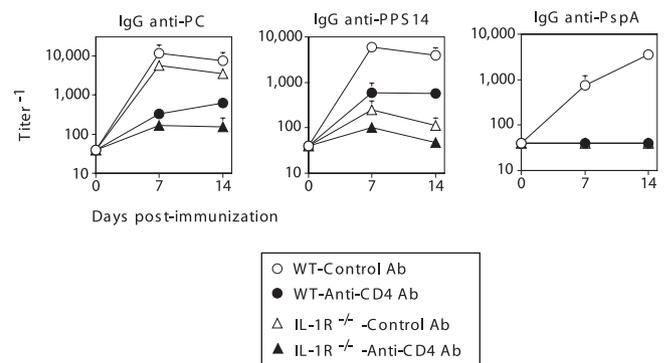
**FIGURE 4.** CD4<sup>+</sup> T cells from Pn14-immunized IL-1R1<sup>-/-</sup> mice fail to exhibit priming for either type 1 or type 2 cytokine production, whereas those from MyD88<sup>-/-</sup> mice have normal type 1 and elevated type 2 responses. IL-1R1<sup>-/-</sup>, MyD88<sup>-/-</sup>, and wild-type (C57BL/6) mice (seven per group) were immunized *i.p.* with  $2 \times 10^8$  CFU of intact, heat-killed Pn14 and similarly boosted on day 14. Spleens were removed on day 28, and spleen cells were cultured at  $5 \times 10^6$ /ml in the absence or presence of 30  $\mu$ g/ml PnP. Culture SN was removed 48 h later for measurement of IFN- $\gamma$ , IL-5, and IL-13 concentrations by ELISA. Data are presented as arithmetic mean  $\pm$  SEM. \*,  $p < 0.05$  by Student's *t* test. The data are from one experiment each that is representative of two independent experiments. The second experiment used IL-1R1<sup>-/-</sup> mice on the B6129 background and wild-type B6129SF2/J mice, with similar results.

IL-1R1<sup>-/-</sup> relative to wild-type mice injected with control rat IgG Ab (Fig. 5). Injection of anti-CD4 mAb not only significantly ( $p < 0.05$ ) reduced the IgG responses specific for PspA, PPS14, and PC in wild-type mice, as we previously reported (17), but produced a comparable, significant ( $p < 0.05$ ) reduction in the IgG anti-PC responses in IL-1R1<sup>-/-</sup> mice. These data thus demonstrate that IL-1R1 signaling is critical for cognate, but not noncognate, CD4<sup>+</sup> T cell help in Pn14-immunized mice.

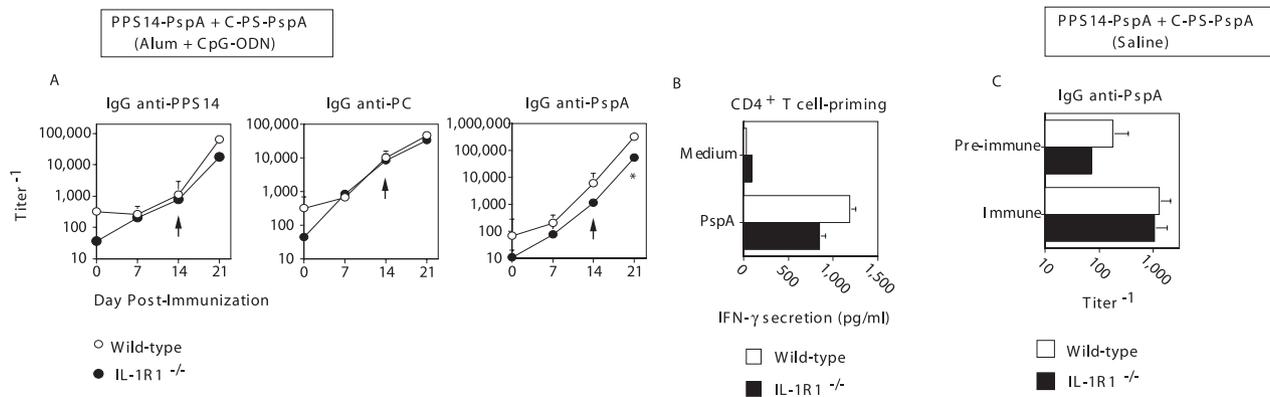
*A soluble conjugate of pneumococcal PPS14 and PspA, in the presence or absence of adjuvant, elicits a normal, or only modestly reduced, IgG anti-PspA and anti-PPS14 response in IL-1R1<sup>-/-</sup> mice*

Conjugation of PS Ag to an immunogenic protein (conjugate vaccine) converts the anti-PS response from TI-2 to TD, recruiting cognate CD4<sup>+</sup> T cell help for augmented primary induction of Ig, and the development of memory for both the anti-PS and antiprotein response (48, 49). We recently observed (45) distinct differences in the regulation of the IgG anti-PPS14 and IgG anti-PC responses to conjugates of PPS14-PspA and C-PS-PspA, when compared with similar responses elicited by intact Pn14. Thus, the IgG anti-PPS14 and IgG anti-PC responses to conjugate, in contrast to intact Pn14, exhibited more prolonged kinetics of primary induction, and striking increases in secondary titers following boosting. We thus wished to determine whether the IgG anti-PspA and IgG anti-PPS14 responses to conjugate, in the absence or presence of adjuvant, were also dependent on IL-1R1<sup>-/-</sup> signaling. Coinjection of conjugate with a TLR ligand can markedly enhance the subsequent humoral immune response (45, 50, 51), which could, in theory, influence its relative dependence on costimulation with endogenous IL-1.

Wild-type and IL-1R1<sup>-/-</sup> mice were immunized *i.p.* with PPS14-PspA plus C-PS-PspA either in saline or adsorbed to alum in the presence of a stimulatory CpG-ODN, a TLR9 ligand (52). Mice were similarly boosted 14 days later. As illustrated in Fig. 6A, when using conjugate with alum plus CpG-ODN, no significant differences were observed in the primary IgG anti-PspA, PPS14, or PC responses when comparing wild-type and IL-1R1<sup>-/-</sup> mice. Upon boosting, enhanced secondary IgG responses for all three Ags were observed in both wild-type and IL-1R1<sup>-/-</sup> mice, although a modest, but significantly higher secondary IgG anti-PspA response was observed in wild-type mice (Fig. 6A). In



**FIGURE 5.** Anti-CD4 mAb (clone GK1.5) inhibits the IgG anti-PC response in both wild-type and IL-1R1<sup>-/-</sup> mice. IL-1R1<sup>-/-</sup> and control B6129SF2/J mice (seven per group) were injected *i.p.* with 1 mg each of either control rat IgG or anti-CD4 mAb (clone GK1.5). One day later, mice were immunized *i.p.* with  $2 \times 10^8$  CFU of intact, heat-killed Pn14. Sera were collected on the indicated days for measurement of serum titers of Ag-specific IgG isotypes by ELISA. Data are presented as geometric mean  $\pm$  SEM. A single experiment was performed.



**FIGURE 6.** A soluble conjugate of pneumococcal PPS14 and PspA, in the presence or absence of adjuvant, elicits a normal, or only modestly reduced, IgG anti-PspA and anti-PPS14 response in IL-1R1<sup>-/-</sup> mice. *A*, IL-1R1<sup>-/-</sup> and control C57BL/6 mice (seven per group) were immunized i.p. with 1  $\mu$ g each of PPS14-PspA plus C-PS-PspA adsorbed to alum plus CpG-ODN mice were similarly boosted on day 14. Sera were collected on the indicated days for measurement of serum titers of Ag-specific IgG by ELISA. *B*, Spleens were removed on day 28 following Pn14 immunization on days 0 and 14 from the mice in *A*, and spleen cells were cultured at  $5 \times 10^6$ /ml in the absence (medium) or presence (PspA) of 30  $\mu$ g/ml PspA. Culture SN were removed 48 h later for measurement of IFN- $\gamma$  concentrations by ELISA. *C*, IL-1R1<sup>-/-</sup> and control C57BL/6 mice (seven per group) were immunized i.p. with 1  $\mu$ g each of PPS14-PspA plus C-PS-PspA in saline and similarly boosted on day 14 similar to *A*. Data are presented as geometric mean (serum titers) or arithmetic mean (IFN- $\gamma$  concentrations)  $\pm$  SEM. \*,  $p < 0.05$  by Student's  $t$  test. The data are from a single experiment that is representative of two independent experiments.

contrast to that observed using intact Pn14, CD4<sup>+</sup> T cells from conjugate-immunized IL-1R1<sup>-/-</sup> mice showed comparable priming for IFN- $\gamma$  production upon in vitro restimulation with PspA, relative to wild-type mice (Fig. 6*B*), consistent with the relatively intact TD humoral immune response. Although Ig responses to conjugate in saline were significantly lower, relative to those using alum plus CpG-ODN, comparable secondary IgG anti-PspA responses in wild-type and IL-1R1<sup>-/-</sup> mice were also observed (Fig. 6*C*). Collectively, these data suggest that the physical form and/or context of the Ag, as opposed to the presence or absence of adjuvant, may contribute to the relative dependence on endogenous IL-1 for costimulating humoral immunity.

## Discussion

We demonstrate, for the first time, a critical role for endogenous IL-1 in costimulating CD4<sup>+</sup> T cell-dependent type 1 and type 2 IgG isotype responses to an intact pathogen, but not in response to a soluble conjugate vaccine derived from this pathogen. This dichotomy did not appear to be secondary to the presence or absence of adjuvant, but rather suggested a role for the physical form or context of the specific Ag. These data further reveal a hitherto unappreciated interplay between IL-1R1 and TLR signaling, via MyD88, in cytokine-dependent, IgG isotype regulation. Thus, IL-1 may be critical for preserving CD4<sup>+</sup> Th2 function in the presence, but not absence, of MyD88-dependent signaling via TLRs. In light of the largely intact early innate cytokine response to Pn14 in IL-1R1<sup>-/-</sup> mice, the observed IL-1R1-mediated effects on both innate and adaptive immunity to this pathogen suggest that IL-1 plays a relatively unique and nonredundant role in these responses.

Mice genetically deficient in the IL-1R1 (IL-1R1<sup>-/-</sup>) exhibit normal hemopoietic, including lymphoid, development (10, 13, 43) and have normal basal levels of serum Ig isotypes relative to wild-type mice (10), thus indicating no major developmental abnormality in the humoral immune system. Although exogenous IL-1 can act as an adjuvant to enhance Ab production to particulate (8) and soluble protein (9) Ags, several studies of its endogenous role in mediating humoral immunity have generated contrasting results. Thus, IL-1R1<sup>-/-</sup> mice immunized i.p. with the soluble TI-2 Ag, DNP-Ficoll in saline or the TD Ag, TNP-KLH in alum elicit serum titers of IgM anti-DNP and IgG1 and IgE anti-TNP,

respectively, that are comparable to wild-type mice (10). Similarly, IL-1R1<sup>-/-</sup> mice made roughly comparable Ag-specific IgG1 and IgG2a responses to *L. major* infection or to immunization s.c. with alum-precipitated KLH in CFA plus *Corynebacterium parvum* (11). In contrast, IL-1 $\alpha$ -deficient (IL-1<sup>-/-</sup>) mice immunized i.p. with SRBC in PBS exhibited reduced serum titers of SRBC-specific IgM and both type 1 and type 2 IgG isotypes as well as IgE (13). Consistent with these data, IL-1 receptor antagonist knockout mice exhibited an elevated SRBC-specific response relative to wild-type mice, although the specific IgM response was normal. In a separate (12) study, it was observed that IL-1 $\beta$ <sup>-/-</sup>, but not IL-1 $\alpha$ <sup>-/-</sup>, mice also had defective IgM and IgG anti-SRBC response. Additionally, in this latter study, IL-1 $\alpha$  $\beta$ <sup>-/-</sup> mice exhibited defective IgM and IgG anti-TNP responses to the TI-2 Ag, TNP-Ficoll, but normal responses to the TI-1 Ag, TNP-LPS. Finally, IL-1  $\alpha\beta$ <sup>-/-</sup> mice exhibited decreased serum titers of OVA-specific IgG1 and IgE in a Th2 OVA-induced model of airway hypersensitivity (14).

Our study, combined with the limited studies discussed above, suggest that, at least for systemic TD Ig responses, endogenous IL-1 could play a more critical role during responses to particulate (e.g., Pn14 and SRBC) as opposed to soluble (pneumococcal conjugate, KLH, or TNP-KLH) Ags. Ig responses to the soluble TI Ags (DNP-Ficoll and TNP-LPS) also occurred independently of IL-1, similar to the TI IgM responses to Pn14 described in this study, although a single study found a stimulatory role for IL-1 in the Ig response to TNP-Ficoll. Although IL-1 has been shown to have a direct costimulatory effect on B cells (6), the data on TI responses appear to indicate that this property is not critical in vivo. The role of IL-1 in TD responses could reflect its ability to directly costimulate T cells (5), as well as indirectly through costimulation of Ag-presenting DC (7). The ability of IL-1 to costimulate chemokine release (53, 54) may also help to promote TD immunity. Thus, IL-1 enhances CCL21 expression on endothelial cells and promotes entry of DC into lymph nodes with subsequent enhanced T cell priming (55). Clearly, in some experimental systems, redundant mechanisms also exist that can substitute for these effects of IL-1.

We observed that both type 1 and type 2 IgG isotype and/or T cell cytokine responses to intact Pn14 were inhibited in IL-1R1<sup>-/-</sup>

mice. These data were surprising given that MyD88<sup>-/-</sup> mice, which also lack IL-1R1 signaling (27–29), exhibit an elevated type 2 IgG isotype and T cell cytokine response to Pn14, associated with decreased type 1 immunity (22). These data suggest that IL-1 may be critical for preserving CD4<sup>+</sup> Th2, function in the presence, but not absence, of MyD88-dependent signaling via TLRs, as well as playing a critical role in costimulating CD4<sup>+</sup> Th1 function. Activation of TLRs on DCs typically stimulates the release of IL-12 that ordinarily inhibits type 2 cytokine differentiation (56). Although, Pn14-primed CD4<sup>+</sup> T cells in wild-type mice did not secrete detectable IL-4 upon restimulation, mice deficient in IL-4 have a defective IgG1 response to Pn14 (46) indicating the presence of an endogenous type 2 component.

The role of IL-1 in CD4<sup>+</sup> T cell differentiation into either type 1 or type 2 cytokine-secreting cells is complex, and may depend upon the experimental system. Thus, IL-1 has been shown to be a critical costimulus for IL-12-dependent Th1 differentiation in Th2-prone BALB/c although not Th1-prone C57BL/6 mice (36). IL-1 also synergizes with IL-12 for induction of IFN- $\gamma$  from human tonsillar CD4<sup>+</sup> T cells (37). The ability of IL-1 to costimulate IL-12-dependent IFN- $\gamma$  from NK cells (57) and  $\gamma\delta$  T cells (58) may further serve to drive Th1 differentiation. In this regard, blockade of endogenous IL-1 signaling during infections with *Mycobacterium tuberculosis* or *L. major*, in which Th1 responses are protective, resulted in decreased IFN- $\gamma$  production from primed T cells (11, 38).

IL-1 may also promote Th2 responses. Thus, IL-1 costimulates proliferation (39–41) and IL-9 secretion (42) in differentiated Th2, but not Th1 cells. IL-1 also costimulates mast cell secretion of Th2 cytokines such as IL-5 and IL-9 (59). Consistent with these data, IL-1  $\alpha\beta$ <sup>-/-</sup> mice exhibited reduced production of IL-4 and IL-5 in a Th2 OVA-induced model of airway hypersensitivity (14). IL-1R1 signaling is dependent on the TLR adaptor protein, MyD88 (27–30). In this regard, a recent study (60) demonstrated that both MyD88<sup>-/-</sup> and IL-1R1<sup>-/-</sup> mice were resistant to experimental autoimmune uveitis, induced by s.c. immunization with interphoretic retinoid-binding protein (IRBP) in CFA. Surprisingly, whereas, lymph node cells from IRBP-primed MyD88<sup>-/-</sup> mice secreted decreased Th1 and enhanced Th2 cytokines upon in vitro rechallenge with IRBP, IRBP-primed IL-1R1<sup>-/-</sup> mice exhibited defects in both the Th1 and Th2 cytokine response, similar to what we observed using intact Pn14.

A potential requirement for IL-1 in costimulating both Th1 and Th2 responses may also, in part, reflect its role in inducing CD40L and OX40 on CD4<sup>+</sup> T cells. Thus, as stated above, IL-1<sup>-/-</sup> mice immunized i.p. with SRBC in PBS exhibited reduced serum titers of SRBC-specific IgM and both type 1 and type 2 IgG isotypes as well as IgE (13). This was associated with decreased CD4<sup>+</sup> T cell expression of CD40L and OX40 due to absent IL-1 release by APCs (13). CD40L, induced on activated CD4<sup>+</sup> T cells, stimulates DC to express OX40L (61) which, in turn, costimulates activated CD4<sup>+</sup> T cells expressing OX40. Although OX40-OX40L interactions appear to favor the release of Th2 cytokines (62), it also promotes CXCR5 expression on CD4<sup>+</sup> T cells (63), important for their localization into B cell follicles (64, 65), where either Th1 or Th2 cells can deliver help for B cell Ab production (66). Finally, combined signaling of CD4<sup>+</sup> T cells via OX40 and CD28 is synergistic for stimulating their proliferation and survival (67). The ability of agonistic anti-OX40 mAb to enhance both Th1 and Th2 responses to Ag-pulsed DC suggests that IL-1-costimulated expression of OX40, may amplify an ongoing immune response, regardless of Th differentiation (68). Of note, the TD IgG, but not IgM, anti-PspA, PPS14, as well as PC responses to Pn14 are inhibited by injection of a blocking anti-CD40L mAb (clone MR1)

(17). However, anti-CD40L mAb would presumably also block the TD responses to conjugate (49), which, like the IgG anti-PC response to Pn14, are not inhibited in IL-1R1<sup>-/-</sup> mice.

## Acknowledgments

We thank Dr. Andrew Lees (Biosynexus) for provision of PC-KLH, purification of PspA, and for making the PPS14-PspA and C-PS-PspA conjugates.

## Disclosures

The authors have no financial conflict of interest.

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