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Serum Amyloid A Activates the NLRP3 Inflammasome and Promotes Th17 Allergic Asthma in Mice

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IL-1 β is a cytokine critical to several inflammatory diseases in which pathogenic Th17 responses are implicated. Activation of the NLRP3 inflammasome by microbial and environmental stimuli can enable the caspase-1–dependent processing and secretion of IL-1 β . The acute-phase protein serum amyloid A (SAA) is highly induced during inflammatory responses, wherein it participates in systemic modulation of innate and adaptive immune responses. Elevated levels of IL-1 β , SAA, and IL-17 are present in subjects with severe allergic asthma, yet the mechanistic relationship among these mediators has yet to be identified. In this study, we demonstrate that *Saa3* is expressed in the lungs of mice exposed to several mixed Th2/Th17-polarizing allergic sensitization regimens. SAA instillation into the lungs elicits robust TLR2-, MyD88-, and IL-1–dependent pulmonary neutrophilic inflammation. Furthermore, SAA drives production of IL-1 α , IL-1 β , IL-6, IL-23, and PGE₂, causes dendritic cell (DC) maturation, and requires TLR2, MyD88, and the NLRP3 inflammasome for secretion of IL-1 β by DCs and macrophages. CD4⁺ T cells polyclonally stimulated in the presence of conditioned media from SAA-exposed DCs produced IL-17, and the capacity of polyclonally stimulated splenocytes to secrete IL-17 is dependent upon IL-1, TLR2, and the NLRP3 inflammasome. Additionally, in a model of allergic airway inflammation, administration of SAA to the lungs functions as an adjuvant to sensitize mice to inhaled OVA, resulting in leukocyte influx after Ag challenge and a predominance of IL-17 production from restimulated splenocytes that is dependent upon IL-1R signaling. *The Journal of Immunology*, 2011, 187: 64–73.

The IL-1 family of cytokines is critical to the host response to infection, playing a variety of functions not only in the acute-phase response from the liver, but also in alterations in metabolism, induction of fever, and lymphocyte activation (1). Overproduction of IL-1 β , in particular, is thought to be responsible for a variety of autoinflammatory syndromes such as familial Mediterranean fever and Muckle-Wells syndrome and is also a contributing factor in osteoarthritis, rheumatoid arthritis, gout, multiple sclerosis (experimental autoimmune encephalomyelitis), colitis, diabetes, and Alzheimer's disease (2–9). Setting IL-1 β apart from other acute-phase cytokines such as IL-6 and TNF- α is the requirement for processing from an inactive proform to an active secreted form by caspase-1 cleavage, which itself is activated by the assembly of a cytoplasmic inflammasome com-

plex. The NLRP3 inflammasome is not only critical for IL-1 β release in response to a variety of stimuli, but has also been implicated in several of the same autoimmune and autoinflammatory disorders in which IL-1 β plays a causative role. Key to these models of inappropriate adaptive immune responsiveness is the development of a Th17-biased phenotype, and recent research has highlighted the importance of IL-1 β in Th17 development (10, 11). IL-1 β , in conjunction with IL-6 and TGF- β , is critical in humans for the development of the Th17 lineage. Not only do Th17 cells upregulate mRNA expression of the IL-1R compared with Th1 and Th2 cells, but lack of this receptor on polyclonally stimulated T cells results in significant reduction of IL-17A, IL-17F, IL-21, and IL-22 production (10, 12).

Long known to be a biomarker of inflammation in a multitude of diseases (13), serum amyloid A (SAA) is also a critical mediator of disease pathogenesis. SAA can stimulate cells via TLR2 to elicit a robust signaling cascade in human monocytes (14) and mouse macrophages (15), whereas it can also signal through the formyl-peptide receptor (FPR)-like 1/FPR2 to promote neutrophil chemotaxis and activation (16–18). In addition, SAA induces expression of matrix metalloproteinases and collagenases that are pivotal in tissue remodeling after injury (19). SAA can also promote the development of Th17 responses, which has been demonstrated to be an important mechanism by which segmented filamentous bacteria induce intestinal disease (20). However, the importance of SAA on modulating adaptive immune responses in other tissues and beyond the scope of infection has not yet been demonstrated. Most well known is the role of SAA fibril deposition in severe conditions such as amyloidosis (21). It has recently been demonstrated that β -amyloid fibrils in Alzheimer's disease (22) and islet amyloid polypeptide in type 2 diabetes (2) signal through the NLRP3 inflammasome and drive caspase-1–dependent cleavage of IL-1 β .

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Abbreviations used in this article: Alum, aluminum hydroxide; BAL, bronchoalveolar lavage; BMDC, bone marrow-derived dendritic cell; CAIKK β , constitutively active I κ B kinase β ; DC, dendritic cell; Dox, doxycycline; FPR, formyl-peptide receptor; o.a., oropharyngeal aspiration; SAA, serum amyloid A.

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Asthma is conventionally considered to be a Th2-driven disease associated with wheezing, airway hyperresponsiveness, IgE, eosinophilia, and mucus metaplasia. However, in a substantial percentage of patients, asthma presents as nonatopic, instead manifesting as a neutrophilic and steroid-resistant phenotype that results in increased severity and morbidity of disease (23, 24). Severe allergic asthma is associated with elevated levels of several mediators, including SAA (25–27), IL-1 β (28), and IL-17 (29–36), although a mechanistic link among these molecules has not yet been established. IL-1 β and IL-17A have been demonstrated to upregulate expression of the mucin gene *Muc5ac* (37), and IL-1 β also acts via cyclooxygenase-2 and PGE₂ production to desensitize airway smooth muscle cells to β -adrenergic agonists (38).

Mouse models of allergic asthma have classically exploited the Th2-promoting adjuvant aluminum hydroxide (Alum), delivered as an emulsion with Ag via i.p. injection (39). However, allergic asthma models are evolving to encompass inhalational methods of sensitization to aeroantigens, which promote a mixed Th2/Th17 allergic airway disease phenotype (40). Although the Th17 response in mouse models of allergic airway disease is associated with neutrophilia, tissue destruction, and steroid unresponsiveness, little is known about the endogenous mediators that are critical to this response. Recent reports have implicated IL-1 β , IL-6, and IL-23 in the initiation and expansion of IL-17-producing T cells, three cytokines that are highly induced by SAA (14, 41).

In this study, we report that multiple models of respiratory system exposure that promote mixed Th2/Th17 responses also induce pulmonary *Saa3* expression. SAA signals through TLR2 to induce inflammatory mediators and through the *Nlrp3* inflammasome to induce IL-1 β secretion. In addition, SAA induces dendritic cells (DCs) to undergo maturation and produce soluble mediators, including IL-1 α , IL-1 β , IL-6, PGE₂, and IL-23, that function in an IL-1-dependent manner to promote CD4⁺ T cells to secrete IL-17A upon stimulation. Finally, SAA sensitizes mice to a mixed Th2/Th17 allergic airway disease via an IL-1R-dependent mechanism. Together, these data implicate pulmonary SAA as a proinflammatory mediator capable of promoting Ag-specific pulmonary Th17 responses through the activities of the cytokine mediator IL-1.

Materials and Methods

Mice

C57BL/6 and IL-1R $\alpha^{-/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). TLR2 $^{-/-}$ (42), TLR4 $^{-/-}$ (43), MyD88 $^{-/-}$ (44), NLRP3 $^{-/-}$ (45), ASC $^{-/-}$ (45), caspase-1 $^{-/-}$ (46), and CC10-rtTA \times TetOP-CAIKK β bitransgenic mice on the C57BL/6 background (47), which express a constitutively active I κ B kinase β (CAIKK β) in bronchiolar epithelium following administration of 6 g/kg doxycycline (Dox) in chow (TestDiet, Richmond, IN), and age- and sex-matched transgene-negative littermates were also bred at the University of Vermont. Mice were housed in an American Association for the Accreditation of Laboratory Animal Care-approved facility, maintained on a 12-h light/dark cycle, and were provided food and water ad libitum. All animal studies were approved by the University of Vermont Institutional Animal Care and Use Committee.

Exposures

For acute studies, mice were anesthetized with inhaled isoflurane and received either 50 μ l sterile saline, 100 ng ultra-pure LPS (InvivoGen, San Diego, CA), or 10 μ g apo-SAA (PeproTech, Rocky Hill, NJ) in 50 μ l sterile saline by oropharyngeal aspiration (o.a.), and analyzed 24 h later. Mice were exposed to 15 ppm NO₂ or high-efficiency particulate air-filtered room air for 1 h and analyzed 24 h later as previously described (48). CAIKK β mice and transgene-negative littermates were provided Dox-containing chow for 60 h prior to analysis. For treatment with anakinra (Biovitrum, Stockholm, Sweden), mice were administered 1 mg drug in

200 μ l sterile saline by s.c. injection twice daily beginning 1 d before SAA exposure. For Ag-sensitization studies, mice received either saline or SAA (as above) once on day 0, followed by 30 min of nebulized 1% OVA, Fraction V (Sigma-Aldrich, St. Louis, MO), in saline on days 0, 1, and 2. Mice were then challenged with 30 min of nebulized 1% OVA on days 14, 15, and 16 and analyzed on day 18. Alum/OVA-treated mice were sensitized on day 0 with 100 μ g OVA in Imject Alum (Thermo Scientific, Rockford, IL), challenged with 30 min of nebulized 1% OVA on days 14, 15, and 16, and analyzed on day 18.

Bronchoalveolar lavage collection and lung processing

Lungs were lavaged with 1 ml DPBS (Sigma-Aldrich) from which cells were counted by hemocytometer, and differential analysis was performed by cytospin and H&E stain. After lavage, lungs were flash frozen in liquid nitrogen for RNA analysis.

Macrophages

For the isolation of primary macrophages, C57BL/6, TLR2 $^{-/-}$, TLR4 $^{-/-}$, and MyD88 $^{-/-}$ mice were administered 1 ml 4% thioglycollate by i.p. injection. Ninety-six hours later, mice were euthanized, and peritoneal lavage was performed to collect peritoneal exudate cells. Cells were plated in RPMI 1640 supplemented with 10% FBS, penicillin and streptomycin, L-glutamine, and 2-ME and challenged for 16 h with apo-SAA followed where indicated by 30 min of 5 mM ATP or 8 h of 500 μ g/ml Imject Alum (Thermo Scientific). C57BL/6, NLRP3 $^{-/-}$, ASC $^{-/-}$, and caspase-1 $^{-/-}$ transformed macrophage cell lines (49) were maintained in vitro in RPMI 1640 with 10% FBS, penicillin and streptomycin, L-glutamine, and 2-ME. Following stimulation, cell-free supernatants were flash frozen for later analysis.

Cytokine analysis

Cytokines from bronchoalveolar lavage (BAL) and cell supernatants were analyzed by ELISA for IL-1 β and TNF- α (BD Biosciences, San Jose, CA), as well as IL-23 (R&D Systems, Minneapolis, MN). PGE₂ was assessed by enzyme immunoassay (Cayman Chemical, Ann Arbor, MI). Customized Bio-Plex (Bio-Rad, Hercules, CA) and Milliplex assays (Millipore, Billerica, MA) were used to measure IL-5, IL-13, and IL-17, as well as IL-1 α , IL-1 β , TNF- α , IL-6, GM-CSF, G-CSF, keratinocyte-derived chemokine, MIP-1 α , MIP-1 β , MCP-1, IL-12p40, and IL-12p70.

Quantitative RT-PCR

Total RNA was extracted from frozen whole lungs or transformed macrophages using the PrepEase RNA Isolation kit (USB, Cleveland, OH) and reversed transcribed to cDNA using the iScript kit from Bio-Rad. Primers were designed for mouse *Saa1*, *Saa2*, *Saa3*, and *Il1b*, and RT-PCR was performed using SYBR Green Supermix (Bio-Rad) and normalized to *Gapdh* or *Actb* using the $\Delta\Delta C_T$ method, as previously described (47).

Splenocyte restimulation

Splenocytes from experimental mice and C57BL/6 control mice were isolated using Lymphocyte Separation Media (MP Biomedicals, Solon, OH) as previously described (48). A total of 4×10^6 cells/ml were cultured in RPMI 1640 supplemented with 10% FBS, penicillin/streptomycin, L-glutamine, and 2-ME and were activated with 100 μ g/ml OVA in 48-well plates. Following 96 h of stimulation, supernatants were collected for analysis by Milliplex (Millipore).

Bone marrow-derived DCs

Bone marrow was flushed from the femurs and tibiae and cultured on 24-well plates at 1×10^6 cells/well (1 ml/well) in RPMI 1640 containing 10% serum and 10% conditioned media from X63-GM-CSF myeloma cells transfected with murine GM-CSF cDNA (kindly provided by Dr. Brent Berwin, Dartmouth College). Media was replaced on days 2 and 4, and the adherent and lightly adherent bone marrow-derived DCs (BMDCs), predominantly CD11b⁺CD11c⁺ by FACS, were collected on day 6. BMDCs were treated with SAA for 16 h. For flow cytometry, BMDCs were detached using versene and gentle scraping, washed in FACS buffer (DPBS with 5% FBS and 0.1% sodium azide), and 1×10^6 cells were incubated with Fc block (2.5 μ g/ml anti-CD16/CD32) (BD Pharmingen) for 30 min at 4°C, washed in FACS buffer, and then stained for 30 min at 4°C in 100 μ l Ab solution at the optimal concentration. Cells were stained with: anti-CD80-PE (BD Pharmingen), anti-CD86-Alexa 647 (Caltag Laboratories, Carlsbad, CA), anti-MHC class II-PerCP/Cy5.5 (BD Pharmingen), and biotinylated anti-OX40L (BD Pharmingen). Biotinylated Abs were detected using streptavidin-PE (BD Pharmingen). Following staining, all

cells were washed and fixed in DPBS with 5% FBS and 1% paraformaldehyde. Cells were analyzed on an LSR II FACS flow cytometer (BD Biosciences) equipped to distinguish as many as seven fluorophores 1–3 d following staining. Dead cells were excluded from analysis by forward light scatter and side scatter gating. Data were analyzed using FlowJo (Tree Star, Ashland, OR).

SAA contaminant analysis

BMDCs were treated with SAA for 16 h in the presence or absence of polymyxin B (Sigma-Aldrich) at 25 and 1 $\mu\text{g/ml}$. Proteinase K (Sigma-Aldrich) at 25 $\mu\text{g/ml}$ and 1 $\mu\text{g/ml}$ (or absent) was incubated with apo-SAA at 37°C for 1 h, heated to 100°C for 5 min to deactivate the enzyme, and allowed to cool to room temperature before addition to cells. Cells were treated for 16 h, and cell-free supernatants were flash frozen prior to further analysis.

CD4⁺ T cell culture with BMDC-conditioned media

Cell-free conditioned media from unstimulated or 24 h SAA-exposed BMDCs were incubated with 1×10^6 splenic CD4⁺ T cells from naive mice that were stimulated in the presence of 5 $\mu\text{g/ml}$ immobilized anti-CD3 and 1 $\mu\text{g/ml}$ soluble anti-CD28 for 96 h. Alternatively, CD4⁺ T cells were polyclonally stimulated in media alone or in the presence of 1 $\mu\text{g/ml}$ apo-SAA.

Splenocyte cultures

Splenocytes from C57BL/6, TLR2^{-/-}, NLRP3^{-/-}, ASC^{-/-}, and caspase-1^{-/-} mice were cultured at 4×10^6 cells/ml in RPMI 1640 containing 10% FBS, penicillin/streptomycin, L-glutamine, and 2-ME on plates coated with 5 $\mu\text{g/ml}$ anti-CD3 and treated with 1 $\mu\text{g/ml}$ soluble anti-CD28,

10 ng/ml anakinra, and 1 $\mu\text{g/ml}$ SAA. Cell-free supernatants were analyzed 96 h after stimulation.

Statistics

Data were analyzed by two-tailed unpaired *t* test or one-way ANOVA and Bonferroni post hoc test using GraphPad Prism 4 for Windows (GraphPad). A *p* value <0.05 was considered statistically significant.

Results

SAA3 expression in mouse lung

Our studies have employed multiple mechanisms of allergic sensitization, including NO₂ exposure, oropharyngeal administration of LPS, and airway epithelial-specific NF- κ B activation, each of which can promote mixed Th2/Th17 responses (40, 47, 48). Following exposure to these stimuli, lungs of C57BL/6 mice exhibited a preferential mRNA induction of *Saa3* over *Saa1* or *Saa2* (Fig. 1A). Mice exposed to 15 ppm NO₂ for 1 h and analyzed 24 h later showed a 6-fold induction of *Saa3* in the lung, very similar to the response in transgenic mice that inducibly express CAIKK β in the airway epithelium following 48 h of Dox administration (Fig. 1A). Oropharyngeal administration of 100 ng LPS, a low dose used in models of inhalational allergic sensitization (40, 50), induced high mRNA levels of *Saa3* 24 h post-challenge (Fig. 1A). These results demonstrate expression of *Saa3* in the lung under conditions that facilitate mixed Th2/Th17 polarization.

FIGURE 1. SAA is expressed in the lungs during mixed Th2/Th17 allergic sensitization regimens and induces pulmonary inflammation upon inhalational exposure. Quantitative PCR of whole lung for SAA isoforms in mice exposed to NO₂, LPS, or in which NF- κ B has been activated in the airway epithelium (A). C57BL/6 mice were administered 10 μg SAA by o.a. and analyzed 4 and 24 h later. BAL total cell counts were performed by hemocytometer, and differential analysis was by cytopsin (B). BAL fluid was analyzed by Milliplex assay (Millipore) for IL-1 β (C), TNF- α (D), IL-6 (E), GM-CSF (F), G-CSF (G), keratinocyte-derived chemokine (H), MIP-1 α (I), MIP-1 β (J), MCP-1 (K), IL-12p40 (L), and IL-12p70 (M). Data are representative of three independent experiments. C57BL/6 mice were administered saline or 1 mg anakinra ($n = 3$ per group) by s.c. injection twice daily beginning one day prior to o.a. of 10 μg SAA. At 24 h, BAL total cell counts were performed by hemocytometer and differential analysis was by cytopsin (N). * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$ compared with control exposures (A) or saline controls (B–N).

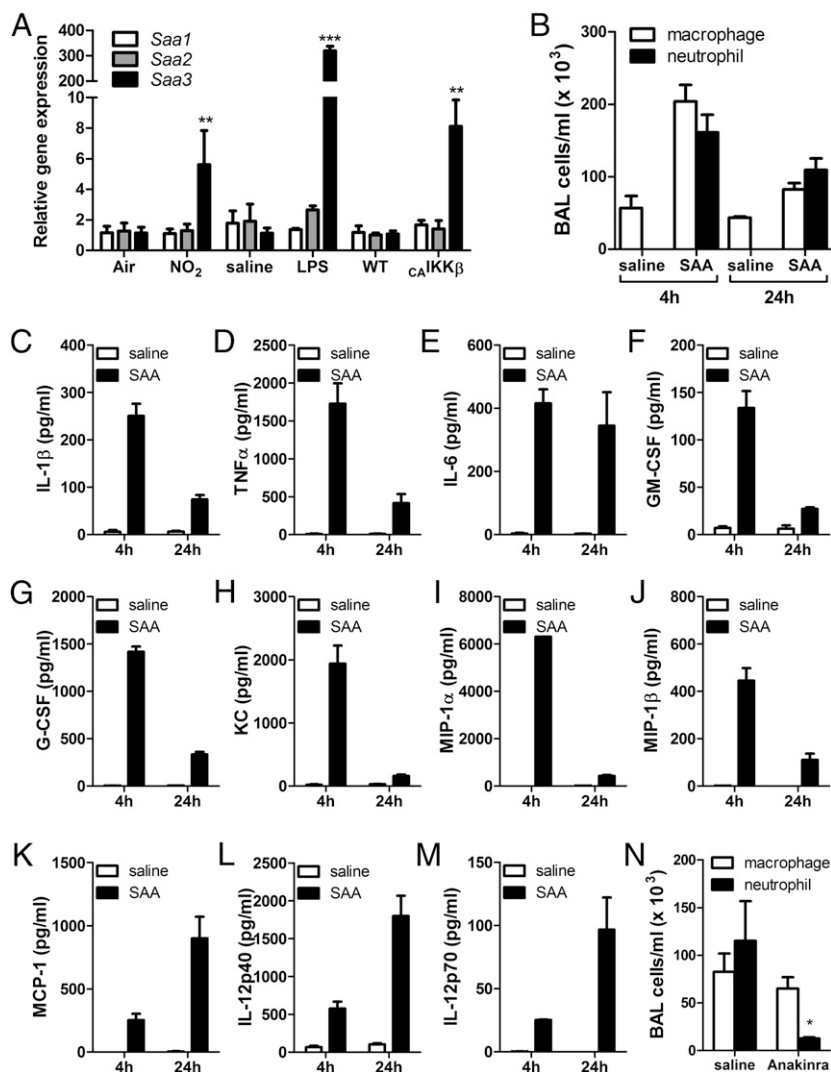
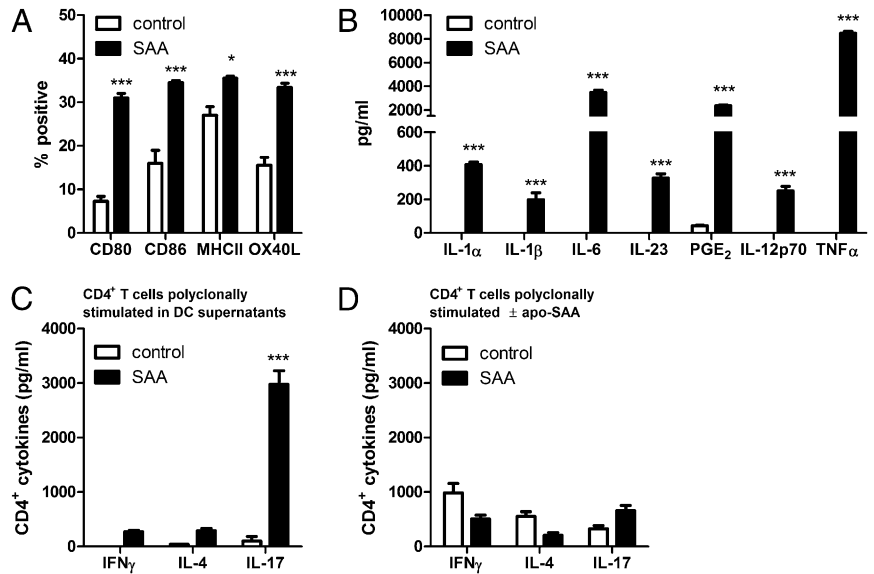


FIGURE 2. SAA elicits inflammatory mediator production and DC maturation in vitro. BMDCs were treated with 1 $\mu\text{g/ml}$ SAA for 16 h and analyzed for surface markers of maturation (A) and secretion of Th17-polarizing mediators (B). The conditioned media from control or SAA-exposed BMDCs (C) or fresh media with or without SAA (D) was transferred to CD4⁺ T cells that were polyclonally stimulated with anti-CD3 and anti-CD28. After 96 h, IFN- γ , IL-4, and IL-17A were measured in supernatants. Data are representative of three independent experiments. * $p < 0.05$, *** $p < 0.001$ compared with control exposures.



SAA elicits robust IL-1-dependent pulmonary inflammation

Recombinant human apo-SAA (with functional similarity to mouse SAA3) is available commercially from PeprOtech and reported to contain < 1 EU/ μg endotoxin. Our own *Limulus* amebocyte lysate assay confirmed that this was indeed true (data not shown). To determine the effects of SAA in the lung, mice were administered 10 μg apo-SAA or saline by o.a. and analyzed at 4 and 24 h. In contrast to saline, SAA induced robust airway neutrophilia (Fig. 1B) and the production of inflammatory cytokines, as measured from BAL fluid (Fig. 1C–M). Because IL-1 β was present at elevated concentrations in the BAL fluid (Fig. 1C), we administered

an IL-1R antagonist (anakinra) or saline to mice prior to apo-SAA aspiration. In the anakinra-treated mice, airway neutrophilia was significantly reduced compared with apo-SAA-exposed mice treated with saline vehicle (Fig. 1N). These results implicate an important function of IL-1 in SAA-promoted inflammation.

SAA activates DCs that promote IL-17A production from CD4⁺ T cells

To determine the effects of SAA on APCs, which are critical for initiation of CD4⁺ T cell responses, BMDCs from C57BL/6 mice were challenged with apo-SAA and analyzed 16 h later. We

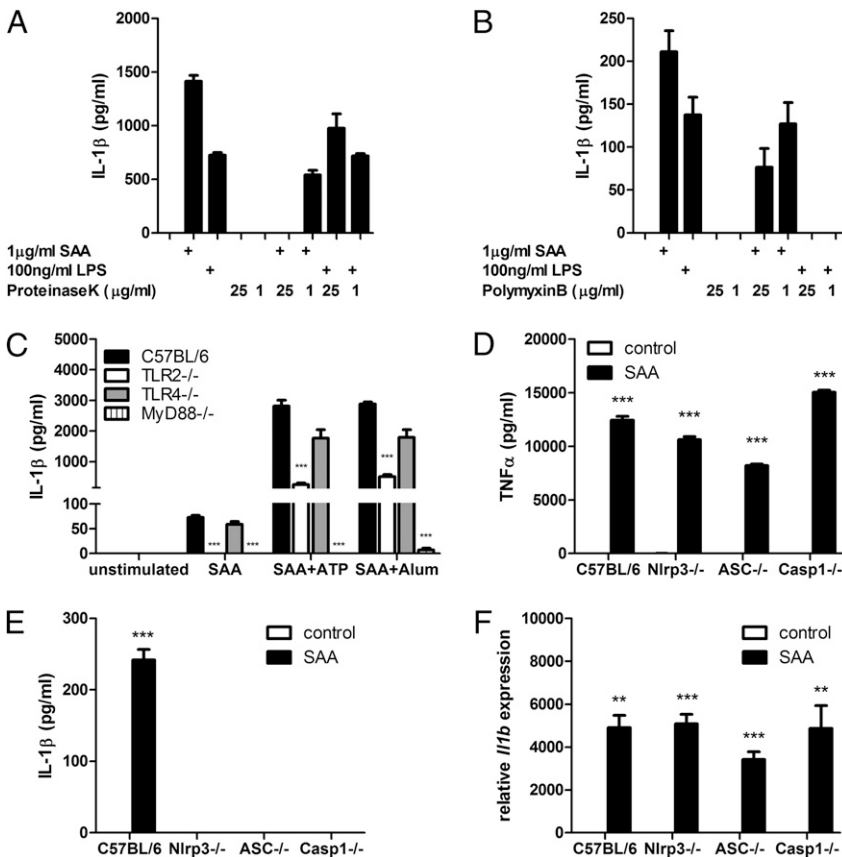


FIGURE 3. SAA-induced IL-1 β production requires TLR2, MyD88, and the NLRP3 inflammasome. Cell-free supernatants from BMDCs treated with SAA or LPS in the presence or absence of proteinase K (A) or polymyxin B (B) were analyzed by ELISA after 24 h. Peritoneal exudate macrophages from wild-type (C57BL/6), TLR2^{-/-}, TLR4^{-/-}, and MyD88^{-/-} mice were unstimulated or primed overnight with 1 $\mu\text{g/ml}$ SAA alone or followed by 30 min of 5 mM ATP or 8 h of 500 $\mu\text{g/ml}$ Alum, and supernatants were analyzed for IL-1 β secretion (C). Transformed macrophages from wild-type (C57BL/6), Nlrp3^{-/-}, ASC^{-/-}, and caspase-1^{-/-} mice were treated for 24 h with 1 $\mu\text{g/ml}$ SAA, and supernatants were analyzed for TNF- α (D) and IL-1 β (E) secretion. *Il1b* expression was measured from wild-type (C57BL/6), Nlrp3^{-/-}, ASC^{-/-}, and caspase-1^{-/-} transformed macrophages that had been untreated or exposed for 4 h to 1 $\mu\text{g/ml}$ SAA (F). ** $p < 0.005$, *** $p < 0.001$ compared with wild-type (C) or control exposures (D–F).

observed increases in surface markers of DC maturation, including CD80, CD86, MHC class II, and OX40L (Fig. 2A), upon exposure of BMDCs to apo-SAA. In addition, BMDCs secreted IL-1 α , IL-1 β , IL-6, and IL-23 (Fig. 2B), cytokines that participate in Th17 polarization and maintenance (10, 23, 51). Furthermore, treatment with SAA caused BMDCs to secrete a significant amount of PGE₂ (Fig. 2B), which has recently been shown to induce DCs to preferentially secrete IL-23 and thus contribute to the development of a Th17 response (52, 53). DCs exposed to apo-SAA also secreted small amounts of the Th1-polarizing cytokine, IL-12p70, and substantial amounts of proinflammatory TNF- α (Fig. 2B). When the cell-free supernatants from these SAA-treated BMDCs were provided to polyclonally stimulated naive CD4⁺ T cells, a significant production of IL-17 was induced compared with very small amounts of IFN- γ and IL-4 (Fig. 2C). Importantly, treatment of the polyclonally stimulated naive CD4⁺ T cells with SAA resulted in no significant production of any of these cytokines (Fig. 2D), indicating that the effect of SAA is directly on DCs, which in turn drive the polarization of the CD4⁺ T cells through the secretion of soluble mediators, including IL-1 β .

SAA-induced IL-1 β secretion requires TLR2 and the NLRP3 inflammasome

Because IL-1 β has been implicated as a Th17-polarizing and -priming factor, we determined cell receptors required for SAA-induced secretion of this cytokine. To ensure that the effects of SAA required the recombinant SAA protein and were not due to endotoxin contamination, we cultured BMDCs for 16 h with apo-SAA or LPS that had been treated with proteinase K or left untreated. All samples were then boiled to inactivate the proteinase K. Under these conditions, we observed a dose-dependent reduction in the response to apo-SAA, but no effect on the LPS-induced IL-1 β response (Fig. 3A). In contrast, exposure of BMDCs to LPS in the presence of polymyxin B completely

blocked IL-1 β production, whereas the presence of polymyxin B during apo-SAA treatment still allowed for substantial amounts of IL-1 β to be produced (Fig. 3B). Having demonstrated the requirement for SAA protein and the minimal contribution of contaminating endotoxin in the effects of apo-SAA, we exposed peritoneal exudate macrophages from TLR2^{-/-}, TLR4^{-/-}, and MyD88^{-/-} mice to SAA for 16 h. In these cells, IL-1 β secretion is primarily dependent upon TLR2 and MyD88, both in the absence or presence of ATP or aluminum crystals (Fig. 3C), potent inducers of IL-1 β secretion. TLR4^{-/-} peritoneal exudate cells showed no significant reduction in their ability to secrete IL-1 β following SAA treatment, further ruling out the contribution of endotoxin contamination of apo-SAA to its biological effects in these experimental systems. The response to SAA was also examined in transformed macrophage cell lines from wild-type, NLRP3^{-/-}, ASC^{-/-}, and caspase-1^{-/-} mice. Whereas apo-SAA-induced levels of TNF- α , an inflammasome-independent cytokine, were highly induced in all cells (Fig. 3D), complete abrogation of IL-1 β secretion was observed in response to apo-SAA in NLRP3-, ASC-, and caspase-1-deficient macrophages (Fig. 3E). Indicative of the roles these molecules play in processing the pro-IL-1 β protein, levels of *Il1b* message expression were robustly induced in the wild-type, NLRP3-, ASC-, and caspase-1-deficient macrophages (Fig. 3F).

SAA-induced pulmonary inflammation requires TLR2 and involves the NLRP3 inflammasome

Having demonstrated the important functions of TLR2, NLRP3, and caspase-1 for SAA-induced IL-1 β secretion in vitro, we next performed additional pulmonary SAA exposures to determine the contribution of these molecules to inflammation in vivo. Oropharyngeal administration of 10 μ g apo-SAA elicited a robust influx of neutrophils into the lung after 24 h in C57BL/6 mice, a response that was diminished in TLR2^{-/-}, but not NLRP3^{-/-} or

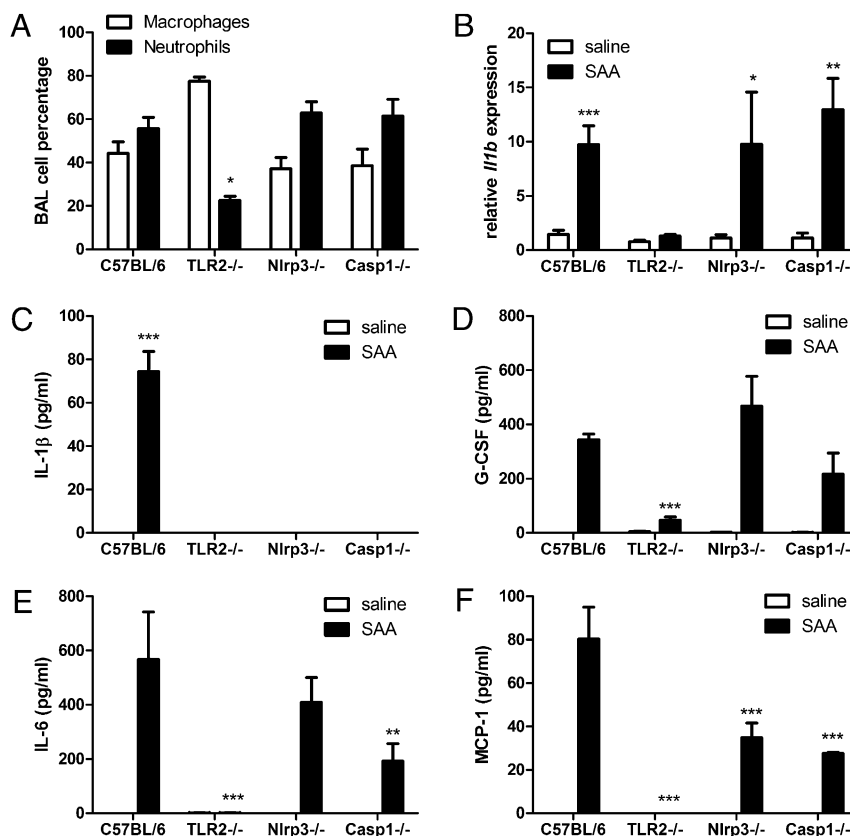


FIGURE 4. SAA-induced pulmonary inflammation requires TLR2 and involves the NLRP3 inflammasome. Wild-type (C57BL/6), TLR2^{-/-}, Nlrp3^{-/-}, and caspase-1^{-/-} mice were administered 10 μ g SAA by o.a. and analyzed 24 h later. BAL total cell counts were performed by hemocytometer and differential analysis was by cyto-spin (A). *Il1b* expression was measured from whole lung by quantitative RT-PCR (B). BAL fluid was analyzed by Milliplex assay (Millipore) for IL-1 β (C), G-CSF (D), IL-6 (E), and MCP-1 (F). Data are representative of three independent experiments. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$ compared with wild-type (A) or saline (B–F).

caspase-1^{-/-} mice (Fig. 4A). Measurement of *Il1b* gene expression in the lung following SAA aspiration revealed increased mRNA abundance in wild-type, NLRP3^{-/-}, and caspase-1^{-/-} mice, but not in TLR2^{-/-} mice (Fig. 4B). Analysis of the BAL fluid from these mice demonstrated that TLR2 signaling was also required for IL-1β, G-CSF, IL-6, and MCP-1 production, whereas NLRP3 and caspase-1 were necessary only for the production of IL-1β (Fig. 4C–F). The NLRP3^{-/-} and caspase-1^{-/-} mice also displayed reduced levels of IL-6 and MCP-1, two cytokines that can be induced by IL-1β (54, 55) and are perhaps diminished as a consequence of lost IL-1β signaling.

SAA-promoted allergic sensitization favors a Th17 response that requires IL-1Rα signaling

Having demonstrated evidence for DC maturation and Th17 polarization in response to apo-SAA in vitro, we sought to compare a conventional Th2 allergic sensitization protocol, a well-characterized Alum/OVA model (56, 57), with an experimental model of apo-SAA-promoted Ag sensitization (Fig. 5A). A very distinct profile of SAA gene expression occurred in the lung 24 h following Ag sensitization in the two models (Fig. 5B). Intraperitoneal injection of the adjuvant Alum elicited little SAA expression in the lung (Fig. 5B; 3.4-fold induction of *Saa1*, 6.9-fold induction of *Saa2*, and 3.0-fold induction of *Saa3*), but in-

duced strong expression of *Saa1* (781-fold induction) and *Saa2* (199-fold induction) in the liver, with only 4.2-fold induction of *Saa3* (data not shown). This is consistent with the reported roles of liver SAA1 and SAA2 in the systemic inflammatory response. In contrast, pulmonary administration of apo-SAA by o.a. selectively induced mRNA expression of *Saa3* in the lungs (Fig. 5B), with no systemic (liver) production of any other isotypes (data not shown).

The disparate induction of *Saa* expression in liver and lung led us to speculate that differences in route of sensitization and adjuvant used modulate local effects that may contribute to distinct responses in the Alum- and SAA-promoted allergic sensitization models. Therefore, we examined the BAL cell profiles from challenged mice subjected to the two models of Ag sensitization. Mice sensitized i.p. with Alum/OVA robustly recruited eosinophils into the lung compared with unsensitized saline/OVA controls, whereas the SAA/OVA sensitized mice showed eosinophilia on the order of 10–20%, which is more representative of that typically present in the BAL fluid in an asthmatic patient (Fig. 5C). Splenocytes from sensitized and control mice were cultured and restimulated with OVA for 96 h. The Alum/OVA mice, as expected, responded by producing copious amounts of the Th2 cytokines IL-5 and IL-13, whereas SAA/OVA mice produced modest but elevated levels of these cytokines compared with the saline control mice (Fig. 5D, 5E). However, SAA/OVA-sensitized

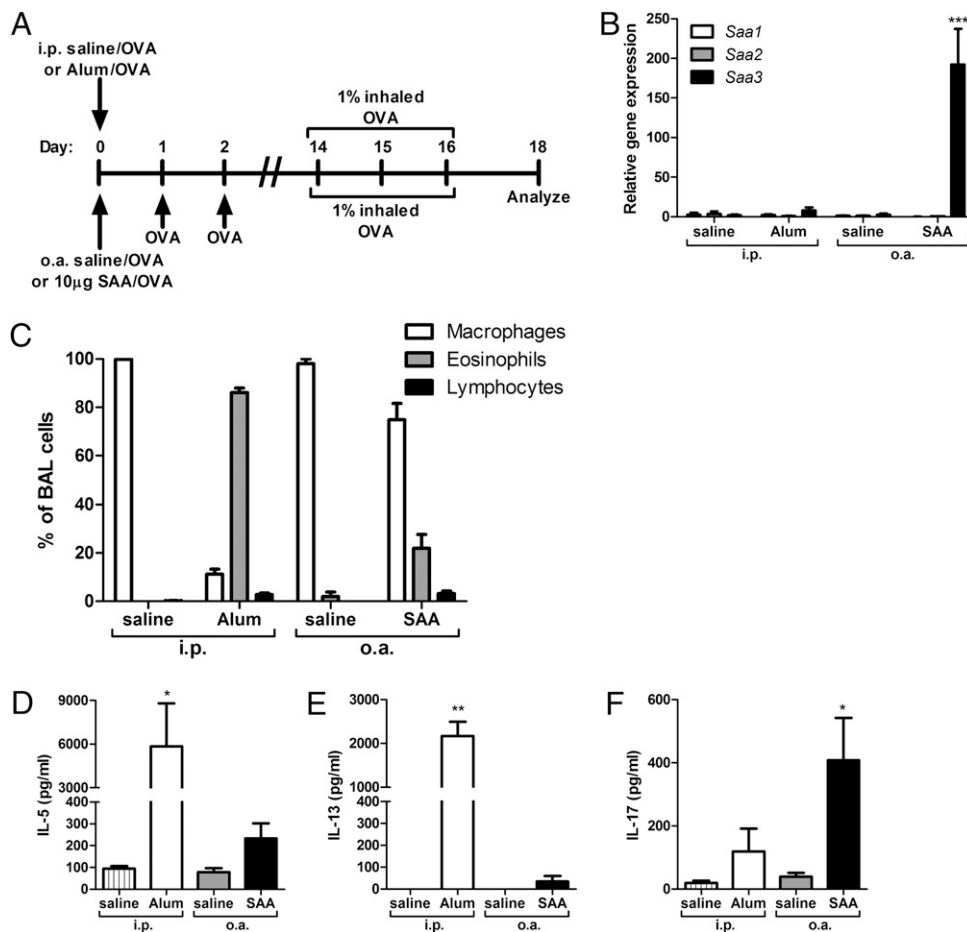


FIGURE 5. SAA inhalation promotes Th17 allergic sensitization. Mice underwent Ag sensitization via o.a. with either saline and OVA (saline/OVA) or SAA and OVA (SAA/OVA) or via i.p. injection with either saline and OVA (saline/OVA) or Alum and OVA (Alum/OVA), according to the schema (A). *Saa1*, *Saa2*, and *Saa3* gene expression in whole lung was measured on day 1, 24 h after i.p. injection with saline or Alum or 24 h after o.a. administration of saline or SAA (B). On day 18, total cell counts from BAL fluid were performed by hemocytometer, and differential analysis was by cytopsin (C). On day 18, splenocytes from i.p. saline, i.p. Alum, o.a. saline, and o.a. SAA mice were restimulated in vitro with OVA for 96 h, and IL-5 (D), IL-13 (E), and IL-17A (F) levels in culture media were measured. Data are representative of three independent experiments. **p* < 0.05, ***p* < 0.005, ****p* < 0.001 compared with saline controls (B, D–F).

mice, unlike Alum/OVA mice, displayed significant production of IL-17 in response to Ag (Fig. 5F), recapitulating our in vitro findings from Fig. 2C that the SAA-induced inflammatory response can polarize T cells to secrete primarily IL-17A.

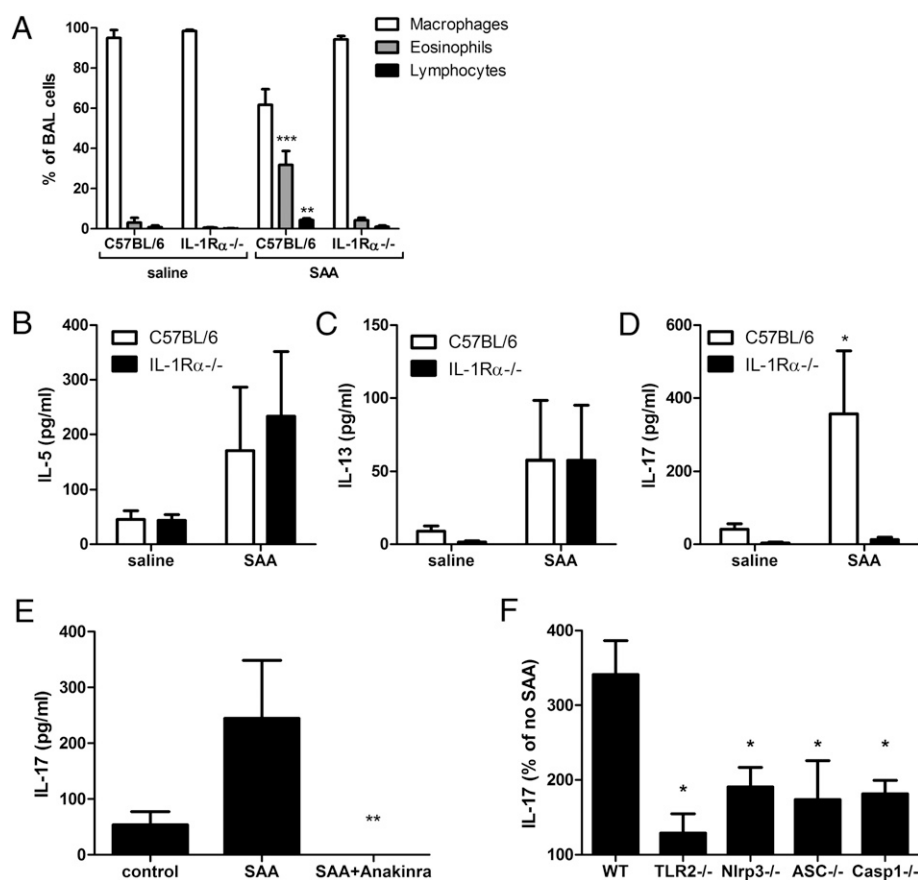
As we have demonstrated, the capacity of Ag-restimulated splenocytes to produce IL-17 may rely on the microenvironmental cytokine milieu that is generated by DCs and other APCs in response to SAA. Because SAA promotes IL-1-dependent pulmonary inflammation, we repeated our SAA/OVA sensitization model (Fig. 5A) using IL-1R α knockout mice to determine whether IL-1 signaling played a critical role in the CD4⁺ T cell priming and polarization process. Following Ag challenge, the cellular BAL profile revealed that IL-1R α ^{-/-} mice exposed to SAA/OVA recruited fewer eosinophils and lymphocytes to the lung than did wild-type mice (Fig. 6A). Furthermore, when splenocytes from these sensitized and challenged mice were restimulated in vitro with OVA, wild-type and knockouts showed a similar induction of IL-5 and IL-13, but the IL-17A production was absent in the IL-1R α ^{-/-} mice (Fig. 6B–D). To show that an IL-1R α ligand was required for SAA to induce IL-17A production and that the IL-17A deficiency seen in IL-1R α ^{-/-} mice was not due to a developmental defect, splenocytes from wild-type mice were polyclonally stimulated in the presence of SAA, with or without anakinra. The addition of anakinra, even at a relatively low dose of 10 ng/ml, completely abrogated IL-17A production (Fig. 6E). Finally, to investigate the requirement for TLR2 and NLRP3 inflammasome components for SAA-promoted IL-17A production, splenocytes from C57BL/6, TLR2^{-/-}, NLRP3^{-/-}, ASC^{-/-}, and caspase-1^{-/-} mice were plated and polyclonally stimulated in the presence or absence of apo-SAA. The TLR2^{-/-} splenocytes showed a nearly complete inability of SAA to augment IL-17A production after 96 h of stimulation (Fig. 6F). IL-17

production was also impaired in splenocytes that lacked components of the NLRP3 inflammasome complex, although the abrogation was more moderate (Fig. 6F). Clearly, IL-1 signaling plays a critical role in the induction of SAA-promoted allergic airway disease, specifically in mediating the production of IL-17A.

Discussion

Our results reported in this study implicate a causal role and a molecular mechanism for SAA in the pathogenesis of allergic asthma. Several of our previous studies have focused on inhalational Ag sensitization via exogenous insults, predominantly the pollutant NO₂. Inhalation of NO₂ activates airway epithelial NF- κ B and promotes a mixed Th2/Th17 response (48, 58), which can be recapitulated through the activation of NF- κ B in airway epithelium and inhalation of Ag (47). Within the lung, SAA3 is induced as a consequence of both NO₂ inhalation and airway epithelial NF- κ B activation. SAA isotypes are differentially expressed in distinct tissues (13). SAA1 and -2 in mice are expressed predominantly in the liver, most commonly found bound to high-density lipoproteins in the circulation, and acutely upregulated in systemic disease states (13, 59). In contrast, SAA3 in mice has been shown to be expressed in a wide variety of cells and tissues, including leukocytes and epithelium, and has never been identified bound to high-density lipoproteins (60–62). In humans, SAA1 and -2 are expressed in the liver and in the lung and have been associated locally with the TLR2-dependent development of sarcoidosis (63). The rapid and robust induction of SAA in response to a panel of inhalational stimuli (Fig. 1A) indicates a possible role for SAA as a mediator in both allergic sensitization and during Ag challenge (exacerbation). Based on our results, we speculate that local production of SAA, rather than the particular isoform expressed, is capable of influencing local

FIGURE 6. SAA-promoted allergic sensitization and Th17 polarization require IL-1R. C57BL/6 and IL-1R α ^{-/-} mice were Ag-sensitized with saline and OVA (saline) or SAA and OVA (SAA) by o.a., according to the timeline in Fig. 5A. On day 18, total and differential cell counts from BAL fluid were performed (A). Splenocytes were restimulated in vitro with OVA for 96 h, and IL-5 (B), IL-13 (C), and IL-17 (D) levels in culture media were measured. Splenocytes from C57BL/6 mice in the presence or absence of SAA and 10 ng/ml anakinra (E) and splenocytes from C57BL/6, TLR2^{-/-}, NLRP3^{-/-}, ASC^{-/-}, and caspase-1^{-/-} (F) mice were polyclonally stimulated for 96 h with anti-CD3 and anti-CD28 in the presence or absence of 1 μ g/ml SAA, and IL-17A was measured by ELISA (F). Data are representative of two independent experiments. **p* < 0.05, ***p* < 0.005, ****p* < 0.001 compared with saline controls (A–D), SAA (E), or wild-type (F).



innate and adaptive immune responses. Therefore, whereas SAA3 is the predominant form of SAA expressed in mouse lungs, SAA1 or SAA2 in human lungs may exert effects similar to those we report in this study.

The inflammatory cytokine milieu surrounding naive DCs is key to their maturation and to the polarization of the CD4⁺ T cell response, as we have previously demonstrated using a model of inducible airway epithelial NF- κ B activation (47). In addition, the work of Ivanov et al. (20) has implicated SAA as an important mediator for Th17 polarization in the gut in response to colonization with segmented filamentous bacteria. We have shown a profound effect of apo-SAA on BMDCs that includes APC maturation and the secretion of the Th17-polarizing mediators IL-1 α , IL-1 β , IL-6, IL-23, and PGE₂. From the studies presented in this article using IL-1R antagonism with anakinra, it is clear that IL-1 α and IL-1 β are potential mediators induced by SAA that are capable of eliciting predominantly IL-17A production from naive, polyclonally stimulated CD4⁺ T cells.

IL-1 β requires cleavage via caspase-1 for proper secretion, which is facilitated as a consequence of inflammasome assembly and activation. The NLRP3 inflammasome has emerged as a critical cytosolic sensor for a number of endogenous mediators, including amyloid proteins (2, 22), that are capable of promoting IL-1 β secretion. Our studies demonstrate that regulation of IL-1 β production in response to SAA occurs as two distinct levels. At the transcriptional level, SAA-induced IL-1 β requires TLR2, a finding first reported in human ThP-1 cells by Cheng et al. (14). At the posttranslational level, use of transgenic mice that are deficient in proteins of the NLRP3 complex demonstrate a clear role for the NLRP3 inflammasome in SAA-induced IL-1 β secretion. TLR2^{-/-} mice exhibit severe impairment in the inflammatory response to oropharyngeal administration of apo-SAA, including a diminution in neutrophil recruitment and decreased secretion of inflammatory cytokines in the BAL. The remaining neutrophilia in these mice is likely due to the capacity of apo-SAA to stimulate neutrophil chemotaxis via FPRL1/FPRL2 (17, 18), which remains intact in all of the mice we studied. Taken together, our *in vitro* and *in vivo* results indicate that proper production and secretion of IL-1 β in response to SAA is regulated both at the transcriptional level by TLR2 and at the level of secretion by the NLRP3 inflammasome. Furthermore, they suggest that IL-1 β not only participates in the inflammatory cascade, but also amplifies the response through the augmented production of select inflammatory cytokines. Nevertheless, our *in vivo* data using anakinra, an antagonist of the IL-1R that blocks the effects of both IL-1 α and IL-1 β , demonstrate a causal role for IL-1 in SAA-promoted pulmonary neutrophilia, whereas the *in vivo* data from the NLRP3- and caspase-1-deficient mice reveal very modest reductions in inflammatory cytokines (aside from IL-1 β) and pulmonary neutrophilia following SAA aspiration. Taken together, these findings suggest that there may be an additional role for IL-1 α release in response to SAA that requires further investigation.

Models of *in vitro* and *in vivo* Th17 polarization require the presence of IL-1, IL-23, and IL-6 (23, 64–67) and repression of Th1- and Th2-polarizing cytokines to establish a unique environment that challenge with apo-SAA appears to replicate. The Th2-polarizing Alum/OVA model has long been criticized for the magnitude of the response, whereas inhalational models of allergic sensitization (using different adjuvants such as LPS or cigarette smoke) tend to induce a modest eosinophilia that is more representative of human asthma (68). The more moderate response seen in our SAA/OVA sensitization model could represent a different aspect of the asthma syndrome, one that is less Th2 in nature and more Th17. It has recently been shown that IL-17

production promoted by IL-1 β involves conversion of Foxp3⁺ T regulatory cells to retinoic acid-related orphan receptor γ t-expressing Th17 cells (10, 11). In addition, in the human disease and in mouse models of severe allergic asthma, CD4⁺ T cell production of IL-17 can originate from a population of CRTh2⁺ effector cells/memory cells that are capable of secreting Th2 cytokines and coexpressing the transcription factors GATA3 and retinoic acid-related orphan receptor γ t, a population that can be induced upon stimulation with proinflammatory cytokines, including IL-1 β (69). Our studies have not identified the precursor CD4⁺ T cell population that develops the capacity to produce IL-17A in response to SAA, nor have we yet thoroughly investigated the spectrum of Th17-related cytokines produced by these IL-17A-producing CD4⁺ T cells. Regardless of the mechanism of CD4⁺ T cell conversion, the role that SAA may play in the process as an endogenous mediator has far-reaching implications for the pathogenesis of IL-17-producing CD4⁺ T cells in severe allergic asthma.

The conclusions of the studies described in this study are 3-fold. First, it is clear that the acute-phase SAA proteins are more than simply biomarkers of disease severity. Instead, they function as biological mediators through the stimulation of TLR2 and the NLRP3 inflammasome to regulate pulmonary cytokine production and neutrophilia. Second, although the properties of SAA that enable activation of these pathways remain to be determined, the capacity for both to induce IL-1 β gene expression and allow for IL-1 β secretion make SAA distinct from other endogenous amyloid peptides and proteins that function solely in the later step of IL-1 β release (2, 22). Third, SAA is sufficient to function as an adjuvant to promote allergy to an innocuous inhaled Ag in a manner that is dependent upon IL-1R signaling to stimulate the capacity of CD4⁺ T cells to produce IL-17A. Whether SAA is necessary for Th17 development in response to inhalational Ag sensitization and at what threshold concentration endogenous SAA manifests TLR2/NLRP3 stimulation remain to be determined. These results are the first, to our knowledge, to link pulmonary SAA, IL-1 β , and IL-17A in a manner that explains their interrelationships in allergic asthma and their mechanisms of action. Based upon our findings, it is evident that novel models of SAA/IL-1-mediated allergic airway disease may provide new insight into the endogenous mechanisms behind the inappropriate or maladaptive immune responses at play in the complex phenotypes of allergic asthma.

Disclosures

The authors have no financial conflicts of interest.

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