Serum amyloid A inhibits dendritic cell apoptosis to induce glucocorticoid resistance in CD4⁺ T cells

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Mediators produced by the airway epithelium control the activation, recruitment, and survival of pulmonary dendritic cells (DC) that present antigen to CD4⁺ T cells during the genesis and exacerbation of allergic asthma. The epithelial-derived acute phase protein, serum amyloid A (SAA), induces DC maturation and Th17 polarization. Th17 responses are associated with severe forms of allergic asthma that are poorly controlled by corticosteroids. We sought to determine whether SAA would enhance the survival of DC during serum starvation and could then contribute to the development of a glucocorticoid-resistant phenotype in CD4⁺ T cells. Bone marrow-derived dendritic cells (BMDC) that were serum starved in the presence of SAA were protected from activation of caspase-3 and released less lactate dehydrogenase. In comparison with untreated serum-starved BMDC, treatment with SAA downregulated mRNA expression of the pro-apoptotic molecule Bim, increased production of the pro-survival heat shock protein 70 (HSP70), and induced secretion of pro-inflammatory cytokines. SAA-treated BMDC that were serum starved for 48 h remained capable of presenting antigen and induced OTII CD4⁺ T cells to secrete IL-17, IL-17F, IL-21, IL-22, and IFNγ in the presence of ovalbumin. IL-17A, IL-17F, IL-21, and IFNγ production occurred even when the CD4⁺ T cells were treated with dexamethasone (Dex), whereas glucocorticoid treatment ablated cytokine secretion by T cells cocultured with untreated serum-starved BMDC. Measurement of Dex-responsive gene expression demonstrated CD4⁺ T cells as the target of glucocorticoid hyperresponsiveness manifest as a consequence of BMDC stimulation by SAA. Finally, allergic airway disease induced by SAA and antigen inhibition was unresponsive to Dex treatment. Our results indicate that apo-SAA affects DC to both prolong their viability and increase their inflammatory potential under apoptosis-inducing conditions. These findings reveal mechanisms through which SAA enhances the CD4⁺ T-cell-stimulating capacity of antigen-presenting cells that may actively participate in the pathogenicity of glucocorticoid-resistant lung disease.

Subject Category: Immunity

Dendritic cells (DC) function both as innate responders that take up antigen and secrete acute inflammatory mediators, and as modulators of the adaptive response, directly affecting the phenotype of effector and helper T cells.¹⁻³ Under normal conditions, a naive DC that encounters a harmless antigen will not mature, and will instead undergo apoptosis; likewise, mature DC treated with Toll-like receptor (TLR) agonists possess a ‘molecular timer’ that limits their lifespan and, subsequently, their ability to present antigen to T cells.⁴ DC that presented both antigen and the apoptotic trigger Fas ligand (FasL) to T cells were able to induce T-cell hypo-responsiveness and ameliorate the development of allergic airway disease,⁵ suggesting that interference with the normal apoptotic pathway during DC–T cell interactions could lead to inappropriate and prolonged antigen presentation and an exacerbation of disease. Dysregulation in DC apoptosis, whether through over-expression of pro-survival Bcl-2 proteins or loss of the pro-apoptotic protein, Bcl-2-interacting mediator of cell death (Bim), can trigger autoimmune disease, tumorigenesis, and prolonged immune responses.⁶⁻⁸ Bim⁻/- mice also exhibit defective T regulatory (Treg) cells that ineffectively suppress IL-17 secretion from effector T cells.⁹

A variety of stimuli, from microbial TLR ligands to endogenous cytokines, can stimulate DC to mature and present antigen to T cells. The acute phase protein serum amyloid A (SAA) is produced by a variety of cells in response to inflammatory insult and has been linked to a number of diseases, including Alzheimer’s disease, rheumatoid arthritis,
atherosclerosis, and allergic airway disease.\textsuperscript{10–12} We have previously demonstrated that recombinant human apo-SAA is sufficient to cause BMDC to upregulate inflammatory genes, induce cytokine secretion, and augment the surface expression of MHC II and the co-stimulatory molecules CD80 and CD86. Furthermore, when administered to the lungs of mice along with OVA, apo-SAA is sufficient to sensitize mice to OVA and promote a Th17 allergic asthma response upon subsequent OVA challenge.\textsuperscript{10}

In the present study, we investigated the effect of apo-SAA on BMDC under conditions of serum starvation, which would normally induce apoptosis mediated by mitochondrial outer membrane permeabilization and caspase-3 activation.\textsuperscript{6} Our results demonstrate that apo-SAA treatment interferes with the induction of Bim, inhibits caspase-3 activation, and induces expression of the chaperone protein and cytokine, heat shock protein 70 (HSP70). In addition, the Th17 CD4\textsuperscript{+} T-cell response generated from apo-SAA-treated BMDC is resistant to steroid treatment, and this effect depends in part upon HSP70 expression. Therefore, SAA represents an endogenous mediator of DC lifespan and function that both quantitatively and qualitatively dictates the CD4\textsuperscript{+} T-cell response.

**Results**

**BMDC treated with apo-SAA are resistant to serum starvation-induced apoptosis.** To recapitulate the conditions encountered under homeostatic conditions, BMDC were cultured in serum-free media for up to 72 h. Starved, untreated cells released lactate dehydrogenase (LDH) into the supernatant in increasing amounts over time (Figure 1a). In contrast, LDH secretion was reduced in serum-starved BMDC treated with apo-SAA (Figure 1a). Visualization of the cells revealed a marked difference in cellular morphology, with the apo-SAA-treated cells exhibiting more dendritic processes, whereas the untreated cells were more rounded (Figure 1b). Furthermore, caspase-3 activity, an early marker of apoptosis, was significantly reduced in apo-SAA-treated cells compared with untreated controls (Figure 1c).

**apo-SAA treatment downregulates expression of the pro-apoptotic protein Bim.** Nutrient deprivation-induced BMDC apoptosis relies on the pro-apoptotic protein Bim.\textsuperscript{6} BMDC were serum starved for up to 72 h and analyzed for mRNA abundance of a panel of pro- and anti-apoptotic genes. No differences were observed in the expression of the anti-apoptotic genes Bcl-2, Bcl-X\textsubscript{L}, and TIAP or the pro-apoptotic genes Bad and Bax as a consequence of apo-SAA stimulation (data not shown). However, untreated serum-starved controls upregulated Bim expression over time, whereas apo-SAA-treated BMDC displayed marked Bim downregulation (Figure 1d). Western blot analysis at 24 h confirmed the lack of Bim protein in Bim\textsuperscript{-/-} BMDC (Figure 1e) as well as in apo-SAA-treated wild type BMDC (Figure 1f). Caspase-3 activity was also absent in BMDC from Bim\textsuperscript{-/-} mice, both under conditions of serum starvation or when serum starved and treated with apo-SAA (Figure 1g). The absence of caspase-3 cleavage in serum-starved Bim-deficient BMDC is reminiscent of the effects of serum starvation and apo-SAA treatment of wild type BMDC.

**HSP70 expression is critical for apo-SAA-induced caspase-3 inactivation.** As the pro-survival protein HSP70 causes dysfunction in apoptosis downstream of cytochrome c release from the mitochondria,\textsuperscript{13} we analyzed HSP70 mRNA expression and HSP70 protein in serum-starved BMDC. HSP70 was upregulated at 8 and 24 h post apo-SAA treatment (Figure 2a), as was HSP70 protein (Figure 2b). Addition of an HSP70 inhibitor (HSP70i), blocked mRNA expression of HSP70 both in control and in apo-SAA-treated cells (Figure 2c) and also dose-dependently restored caspase-3 activation in serum-starved, apo-SAA-treated BMDC (Figure 2d). Inhibition of HSP70 also increased TUNEL staining in apo-SAA-treated cells (Figure 2e). We next examined whether HSP70 modulated the capabilities of apo-SAA to induce pro-inflammatory cytokine production. BMDC that were serum starved in the presence of apo-SAA showed a strong secretion of IL-6, TNF-\textalpha, and IL-1\beta after 24 h (Figure 2f). Whereas the secretion of IL-6 and TNF-\textalpha was inhibited by HSP70i, IL-1\beta was markedly increased in the presence of SAA and HSP70i. BMDC treated with apo-SAA drive a pro-inflammatory CD4\textsuperscript{+} T-cell response that is resistant to dexamethasone.

We have previously demonstrated that BMDC treated with apo-SAA can readily induce OTII CD4\textsuperscript{+} T cells to secrete IL-17 in the presence of OVA.\textsuperscript{10} Here, we investigated the OTII CD4\textsuperscript{+} T-cell responses to BMDC that had been serum starved for 48 h in the presence or absence of apo-SAA. apo-SAA-treated BMDC induced CD4\textsuperscript{+} T cells to secrete enhanced amounts of the Th17 cytokines IL-17A, IL-17F, IL-21, and IL-22, whereas they did not enhance the production of the Th1 cytokine IL-13, and only marginally increased the levels of the Th1 cytokine IFN\gamma (Figure 3). Treatment of the serum-starved BMDC cocultures with the corticosteroid dexamethasone (Dex) at the time of CD4\textsuperscript{+} cell stimulation decreased the production of nearly all cytokines measured (Figure 3). However, pretreatment of the BMDC with apo-SAA blocked steroid responsiveness; apo-SAA was still able to induce secretion of IFN\gamma, IL-17A, IL-17F, and IL-21 (Figure 3). Only the production of IL-13 and IL-22 remained sensitive to Dex treatment. Dex did not diminish control levels of IL-21, and in fact enhanced its secretion in the presence of apo-SAA. Addition of a TNF-\textalpha-neutralizing antibody to the coculture system had no effect on OVA-induced T-cell cytokine production or the Dex sensitivity of the CD4\textsuperscript{+} T cells (data not shown).

**Allergic sensitization in mice induced by apo-SAA is resistant to Dex treatment.** To translate the in vitro findings that apo-SAA modulates steroid responsiveness, we utilized an in vivo allergic sensitization and antigen challenge model. Glucocorticoids are a primary therapy for asthma (reviewed in Alangari\textsuperscript{14}) and in preclinical models of the disease. As allergic sensitization induced by aluminum-containing adjuvants is responsive to Dex treatment, inhibiting airway inflammation following antigen challenge,\textsuperscript{15} we compared the Dex-sensitivity of an Alum/OVA allergic airway disease...
model to our apo-SAA/OVA allergic sensitization model. In comparison to unsensitized mice that were OVA challenged (sal/OVA), mice sensitized by i.p. administration of Alum/OVA (Alum/OVA) demonstrated robust eosinophil recruitment into the bronchoalveolar lavage (BAL), along with elevated numbers of neutrophils and lymphocytes (Figure 4a) following antigen challenge. However, when treated with Dex during antigen challenge, BAL cell recruitment was substantially reduced (Figure 4a). Mice sensitized by apo-SAA/OVA administration also recruited eosinophils, neutrophils, and lymphocytes into the BAL (Figure 4a), but in contrast to the Alum/OVA model, inflammatory cell recruitment persisted in the SAA/OVA mice in spite of Dex treatment (Figure 4a). Concurrent with these findings, the
induction of the mucin genes Clca3 (Gob5) and Muc5ac were significantly reduced by Dex treatment in Alum/OVA-sensitized mice, whereas expression of these genes remained upregulated in SAA/OVA-sensitized mice that had been treated with Dex (Figure 4b). In addition, SAA/OVA-sensitized mice maintained upregulation of the neutrophil-recruiting cytokine KC, even in the presence of Dex (Figure 4b).

An apo-SAA-induced soluble mediator from BMDC decreases Dex sensitivity in CD4⁺ T cells. To determine the relative Dex sensitivity of the BMDC and CD4⁺ T cells in our coculture system, CD4⁺ T cells from OTII mice were plated and polyclonally stimulated with plate-bound anti-CD3 and soluble anti-CD28, in the presence or absence of apo-SAA and Dex. After 24 h, IL-17A and IFNγ were measured from cell-free supernatants. As demonstrated in Figure 5a (and as we have previously published¹⁰), apo-SAA treatment did not increase IL-17A or IFNγ in CD4⁺ T cells (black bars). Additionally, Dex effectively inhibited production of IL-17A and IFNγ, regardless of apo-SAA treatment (Figure 5a, white bars).

We next examined CD4⁺ T cells that were polyclonally stimulated in the presence of cell-free conditioned media (CM) from BMDC that had been serum starved for 48 h without

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Figure 2  apo-SAA-induced HSP70 modulates caspase-3 activity and is required for cytokine secretion. (a) Time course of HSP70 expression in BMDC that were serum starved in the presence or absence of 1 μg/ml apo-SAA (SAA). (b) Immunoblot (IB) for HSP70 and β-actin from 30 μg of whole cell lysate from BMDC serum starved for 8 or 24 h in the presence (SAA) or absence (control) of apo-SAA. (c) mRNA expression of HSP70 in cells serum starved for 8 h after treatment with apo-SAA (SAA), 25 μg/ml HSP70 inhibitor (HSP70i), or both. (d) Caspase-3 activity in BMDC that were serum starved for 6 h in the presence or absence of apo-SAA, ± 0, 1, 10, or 50 μg/ml HSP70i. (e) Assessment of DNA strand breaks by TUNEL assay in serum starved BMDC in the presence or absence of apo-SAA, ± 25 μg/ml HSP70i after 72 h. (f) IL-6, TNF-α, and IL-1β levels from supernatants of BMDC that were serum starved for 24 h, ± apo-SAA, ± HSP70i. n = 3–6 replicates per condition. ***P < 0.005, ****P < 0.0001 compared with control (or compared with SAA in f).
The CM from apo-SAA-treated BMDC induced an increase in IL-17A (and to a lesser extent IFN-γ) production from CD4+ T cells compared with control CM (Figure 5b, black bars). Furthermore, Dex treatment did not effectively eliminate either IL-17A or IFN-γ production from CD4+ T cells stimulated in the BMDC+ SAA CM (Figure 5b, white bars). These results implicate the CD4+ T cells as the primary Dex-desensitized cell type in the BMDC/CD4+ T-cell coculture system.

To examine whether there were differences in the initial Dex responsiveness of the BMDC and CD4+ T cells, we measured the mRNA expression of genes documented to be induced by Dex: Glul, Tc22d3, and Dusp1. Analysis of Dex-induced gene expression in BMDC versus CD4+ T cells from separate cultures indicated that Dex effectively induced Glul, Tc22d3, and Dusp1 expression in BMDC, regardless of apo-SAA treatment (Figure 6a). Dex also significantly induced expression of these genes in CD4+ T cells polyclonally stimulated in the presence of control CM from BMDC (Figure 6b, BMDC CM, white bar). However, gene expression was significantly diminished in the Dex-treated CD4+ T cells that received apo-SAA-conditioned BMDC media (Figure 6b, BMDC + SAA CM, white bars). These results further indicate that the CD4+ T cells are the primary Dex-desensitized cell type in the BMDC/CD4+ T-cell coculture system.

Caspase-3 inhibition is sufficient to induce IL-17A, IL-21, and IL-22 production in CD4+ T cells. It has been proposed that caspase-3, rather than controlling cell fate in apoptosis, is responsible for modifying endogenous cell proteins to limit the inflammatory capacity of damage-associated molecular patterns (DAMPs) upon release from the dying cell. As apo-SAA caused marked diminution of caspase-3 activation, which could lead to an increase in the inflammatory potential of cell DAMPs, we sought to determine whether caspase-3 inhibition itself would be sufficient to enhance CD4+ T-cell activation and induce corticosteroid resistance. However, Bim deficiency in DC itself was not sufficient to induce corticosteroid resistance in CD4+ T cells (Figure 7a) and serum-starved Bim−/− cells did not produce IL-1/β or TNF-α without stimulation (data not shown). Wild type BMDC were serum starved for 48 h in the presence or absence of the pan-caspase inhibitor zVAD, prior to coculture with OTII CD4+ T cells and OVA. zVAD-treated cells upregulated IL-17A (trend only), IL-21, and IL-22 (Figure 7b). While the overall levels of IL-17A induced by zVAD (1729.7 ± 348.5 pg/ml) were not as high as those induced by SAA treatment (5038.0 ± 501.0 pg/ml, Figure 3), the fold changes in IL-17A production compared to controls were similar. zVAD treatment induced a 3.7-fold increase in IL-17A and SAA induced a 2.3-fold increase in IL-17A. zVAD also induced a 3.2-fold increase in IL-22 compared with the 10.4-fold increase induced by apo-SAA treatment. However, zVAD treatment was not sufficient to induce corticosteroid insensitivity; Dex substantially inhibited the production of all cytokines measured, except for IL-21 (Figure 7b). These results indicate that blockade of caspase-3 activation alone in BMDC is insufficient to induce corticosteroid resistance from CD4+ T cells. Figure 7b also demonstrates an overall additive effect of...
SAA and zVAD treatment together for IL-13, IL-17A, IL-17F, and IL-21 production.

**SAA and zVAD treatment together for IL-13, IL-17A, IL-17F, and IL-21 production.**

HSP70 expression is not necessary for SAA-induced production of IL-17A and IL-17F from OTII CD4⁺ T cells, but is required for corticosteroid resistance. HSPs can function as DAMPs to exert cytokine-like effects on DC and encourage autoimmune disease. In addition, HSP70 comprises part of the chaperone protein complex that governs the folding and cellular localization of the glucocorticoid receptor (GR). As apo-SAA potently induced the upregulation of HSP70, we explored the possibility that this protein had a role in cytokine release and steroid insensitivity in our coculture system. Therefore, BMDC were serum starved for 48 h in the presence or absence of apo-SAA, alone or with HSP70i. Inhibition of HSP70 blocked production of IFNγ, IL-17F, IL-21, and IL-22 compared with control, and blocked apo-SAA-induced secretion of IL-13 and IFNγ (Figure 8). IL-17A and IL-17F were still significantly induced by apo-SAA in the presence of HSP70i, suggesting a differential regulation of these cytokines. However, when the experiment was conducted in the presence of Dex, the corticosteroid insensitivity induced by apo-SAA treatment disappeared across the board (Figure 8, SAA + HSP70i, white bars), suggesting that HSP70 was indeed required for CD4⁺ T-cell steroid resistance in this model.

**HSP70 expression is not necessary for SAA-induced production of IL-17A and IL-17F from OTII CD4⁺ T cells, but is required for corticosteroid resistance.**

**Discussion**

Recent studies have highlighted the importance of apoptosis not only in the clearance of dying cells, but also in the removal of cellular proteins such as HSPs, HMGB1, and S-100 proteins that can function extracellularly as DAMPs. Apoptotic processes active under homeostatic conditions protect the organism from endogenous inflammatory stimuli and also assist in the resolution of the inflammatory response. In a previous publication, we have explored the inflammatory potential of recombinant apo-SAA in vitro and in a mouse model of allergic airway disease, implicating SAA as a DAMP that induces NLPR3 inflammasome activation, IL-1β production, and asthma-like disease with a mixed T H2/TH17 response in mice. Here, we have more closely explored the effect of apo-SAA specifically on DC, and found that it can increase DC lifespan, downregulate Bim expression and caspase-3 activity while upregulating HSP70, and that this unique intracellular DC milieu induces antigen-specific CD4⁺ T cells to secrete T H17 cytokines that are resistant to corticosteroid treatment. As a consequence, apo-SAA renders a glucocorticoid-unresponsive allergic airway disease phenotype *in vivo.*

T cells undergo apoptosis in a Bim-dependent manner upon treatment with corticosteroids such as Dex. Glucocorticoids pass through the cell membrane in order to bind to the GR, which resides in the cytosol in the company of a chaperone complex.
protein complex that includes HSPs. These molecular chaperones are shed from the receptor once ligand binding occurs, and this reveals the nuclear localization sequence that allows the GR to migrate to the nucleus and bind to glucocorticoid response elements (GREs) on DNA, thereby modulating gene function directly.22,25 Our in vitro coculture system is intended to model interactions between DC and CD4⁺ T cells as they occur in vivo, a situation in which both cell types are exposed to administered corticosteroids. The experiments presented in Figures 5 and 6 attempt to distinguish between the effects of apo-SAA on the Dex responsiveness of CD4⁺ T cells and BMDC. Direct apo-SAA treatment of the CD4⁺ T cells did not augments cytokine secretion from these cells compared with controls (Figure 5a), and neither did direct apo-SAA treatment alter the Dex responsiveness of these cells (Figure 5a). However, use of cell-free CM from BMDC that had received apo-SAA treatment allowed for cytokine secretion by polyclonally stimulated CD4⁺ T cells that was enhanced by Dex treatment (Figure 3), induced by caspase-3 inhibition alone (Figure 4b) and blocked by inhibition of HSP70 (Figure 5). IL-21 promotes the differentiation of T H17 cells and appears to be involved in autoimmune pathologies.33–35 Previous studies have also implicated IL-21 as a D-existant cytokine.36 The role of HSP70 in IL-21 induction has not previously been published, although it has been demonstrated that HSP70 can activate transcription factors such as NF-κB and stimulate the release of other cytokines such as IL-6, IL-1β, and TNF-α. Our current study agrees that HSP70 has a role in the modulation of these cytokines in response to apo-SAA treatment of BMDC (Figure 2e). Previously, we have demonstrated that HSP70 is released into the lavageable airspaces of mice exposed to the pollutant nitrogen dioxide (NO₂)37 and may contribute to the ability of NO₂ to induce DC maturation38 and allergic sensitization.39 It is possible that HSP70 executes multiple functions in our system: as a pro-survival and pro-inflammatory cytokine as well as a GR chaperone.

The studies presented herein reveal that an endogenous protein, SAA, can induce antigen-presenting cells to create a production observed in our experimental conditions. Nonetheless, the capacity for SAA to induce a DC phenotype that permits CD4⁺ T-cell cytokine production, even in the presence of inhibitory concentrations of Dex, remains a significant finding. Alterations in metabolism and the cell surface molecules expressed, as well as the mediators, including gases such as reactive oxygen and nitrogen species, lipids such as PGEs₂, and cytokines released by apo-SAA-activated BMDC,10,26 are all candidates for affecting corticosteroid responsiveness of CD4⁺ T cells. In addition, it is of special interest that BMDC-induced HSP70 appears to have a role in this process, as it was clearly shown to be critical in inducing corticosteroid resistance in our model.

Our model demonstrates that apo-SAA treatment of BMDC/CD4⁺ T-cell cocultures induced the robust secretion of IL-17A and IL-17F from CD4⁺ T cells. In mouse models, IL-17A is capable of promoting neutrophilic asthma and exacerbating allergic airway disease.27 Mice unable to respond to IL-17A or IL-17F do not develop allergic airway disease in several models,27,28 and adoptive transfer of in vitro-polarized CD4⁺ T cells secreting IL-17A induced corticosteroid-insensitive allergic airway disease following antigen challenge.29 As presented in Figure 4, mice that were allergically sensitized with apo-SAA and OVA were resistant to Dex treatment, in comparison to the Alum/OVA sensitization model in which Dex ameliorated inflammatory responses in the lung. BMDC that were pretreated with apo-SAA were able to induce staggering amounts of IL-22 from CD4⁺ T cells, to an extent not seen in our other models. T cells from HIV-1-resistant patients produced both large amounts of IL-22 and an acute SAA cleavage product that downregulated cell surface expression of CCR5 and rendered cells more resistant to HIV-1 viral infection.30 Other reports have revealed that IL-22 is a critical instigator of lung damage, reducing pulmonary function in Aspergillus fumigatus models of allergic airway disease,31 and that IL-22, IL-17A, and IL-17F, can each induce proliferation of human airway smooth muscle cells.32 Our findings revealed that IL-21 secretion appeared to be differentially regulated from the T₁₇ cytokines measured. IL-21 production was enhanced by Dex treatment (Figure 3), induced by caspase-3 inhibition alone (Figure 4b) and blocked by inhibition of HSP70 (Figure 5). IL-21 promotes the differentiation of T₁₇ CD4⁺ T cells and appears to be involved in autoimmune pathologies.33–35 Previous studies have also implicated IL-21 as a D-existant cytokine.36 The role of HSP70 in IL-21 induction has not previously been published, although it has been demonstrated that HSP70 can activate transcription factors such as NF-κB and stimulate the release of other cytokines such as IL-6, IL-1β, and TNF-α. Our current study agrees that HSP70 has a role in the modulation of these cytokines in response to apo-SAA treatment of BMDC (Figure 2e). Previously, we have demonstrated that HSP70 is released into the lavageable airspaces of mice exposed to the pollutant nitrogen dioxide (NO₂)37 and may contribute to the ability of NO₂ to induce DC maturation38 and allergic sensitization.39 It is possible that HSP70 executes multiple functions in our system: as a pro-survival and pro-inflammatory cytokine as well as a GR chaperone.

The studies presented herein reveal that an endogenous protein, SAA, can induce antigen-presenting cells to create a
pro-inflammatory environment that is resistant to apoptosis, and therefore, resistant to resolution of the inflammatory state. This in turn drives production of Th17 cytokines from CD4^+ T cells in response to antigen, a response that is insensitive in vitro and in vivo to corticosteroids. Although further studies are required to define the precise mechanism of glucocorticoid insensitivity in CD4^+ T cells, the chaperokine HSP70 appears to be an important participant, and modulation of this protein may provide a method by which to circumvent corticosteroid resistance in allergic, autoimmune, and inflammatory diseases.

Materials and Methods

Mice. B6^−/− mice on the C57BL/6J background were obtained from Dr. Karen Fortner and were generated as previously described. C57BL/6J mice and OTII TCR transgenic mice (C57BL/6-Tg(TcraTcrb)425Cbn), which produce CD4^+ T cells responsive to the peptide ova 323–339, an immunodominant MHC II antigenic epitope from the protein ovalbumin, were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and bred at the University of Vermont. Mice were housed in an AAALAC-approved facility, maintained on a 12-h light/dark cycle, and provided food and water ad libitum. All animal studies were approved by the University of Vermont Institutional Animal Care and Use Committee.

Allergic sensitization studies. C57BL/6 mice were sensitized either by i.p. injection of 100 μg OVA in 100 μl of 50% Imject Alum (Thermo Fisher Scientific, Rockford, IL, USA) in one i.p. injection, or by oropharyngeal administration of 10 μg apo-SAA or saline followed by 30 min of aerosolized OVA (1% w/v in sterile saline) inhalation, on day 0. Additional 30-min OVA nebulizations were provided on days 1 and 2. All mice were challenged on days 14, 15, and 16 by 30 min of aerosolized OVA (1% w/v) inhalation. Mice that received Dex did so via i.p. injection of 2.5 mg/kg Dex (Sigma-Aldrich, St. Louis, MO, USA) on days 14 and 16. Mice were analyzed 48 h after the final challenge, on day 18. Bronchoalveolar lavage (BAL) was collected in 1 ml of DPBS, and whole lungs were flash frozen for RNA analysis.

Bone marrow-derived dendritic cells. Bone marrow was flushed from the femurs and tibiae of C57BL/6 mice and cultured on six-well plates at 1 x 10^6 cells/well (3 ml/well) in RPMI-1640 containing 10% serum and 5% CM from X63-GMCSF 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 BMDC, predominantly CD11b^+ CD11c^+ by FACS, were collected on day 6. For serum starvation, BMDC were plated at 1 x 10^5 cells/ml, washed with DPBS, and maintained in RPMI-1640 without serum, in the presence or absence of 1 μg/ml apo-SAA (Peprotech, Rocky Hill, NJ, USA). As indicated, BMDC were visualized on tissue culture plates by light microscopy using a 20x objective on a Nikon Eclipse TS100 inverted microscope and images were acquired using a Nikon/Leica 38 mm Iso Port camera (Micro Video Instruments, Avon, MA, USA).
Flow cytometric analysis of apoptosis. Cells were labeled for DNA breaks and assessed by flow cytometry using the In Situ Cell Death Detection Fluorescein kit (Roche Diagnostics, Indianapolis, IN, USA). Cells were analyzed on an LSR II FACS flow cytometer (BD Biosciences, San Jose, CA, USA) equipped to distinguish as many as seven fluorophores 1–3 days following staining, and data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Enzymatic activity assessment. Cell-free supernatants from BMDC were analyzed for the presence of LDH using the Cytotox 96 Non-Radioactive Cytotoxicity Kit (Promega, Madison, WI, USA). Cell lysates were collected in NP-40 buffer, and 50 μg of total protein was used to analyze the presence of cleaved caspase-3/7, utilizing the Caspase-Glo 3/7 Assay (Promega).

RT-qPCR. RNA from whole lung and from BMDC was isolated using the PrepEase RNA Spin Kit (Affymetrix, Santa Clara, CA, USA) and reversed transcribed to cDNA using the Script kit (Bio-Rad, Hercules, CA, USA). Primers were designed for mouse Bim (forward: CTACAGACAGAAGCCGAG; reverse: CCTGAGACTGTCGTATGGAAG), HSP70 (forward: ATCACCATCACCAACGACAAGG; reverse: TGCCCAAGCAGCTATCAAGTGC), 40 Glutamine synthetase; glutamine ammonia ligase (forward: TTATGGGAACAGACGGCCAC; reverse: AAAGTCTTCGCACACCCGAT), Tc22d3: glucocorticoid-induced leucine zipper (forward: GGAGCCGGTTTACCTGAAGT; reverse: CCGAAAGTTGCTCACAGAGG), and Dusp1: dual specificity phosphatase-1 (forward: GAGCTGTGCAGCAAACAGTC; reverse: CGAGAAGCGTGATAGGCACT), Gob5 (forward: AAGCAAAACCACTCCCATGAC; reverse: TGCCGAAGCATACAAGAC), Muc5ac (forward: CCAATGCAGAGTCCTCAGAACAA; reverse: TTACTGGAAAGGCCCAAGCA), and KC (forward: GCTGGGATTCACCTCAAGAA; reverse: TGGGGAACCTTTAGCATC) and quantitative PCR was performed on cDNA using iQ SYBR Green (Bio-Rad). To normalize cycle threshold (C_T) values, Gapdh was analyzed using an Assay-On-Demand primers and probe cocktail (Applied Biosystems, Foster City, CA, USA) and iQ Supermix (Bio-Rad), and calculations were made using the ∆∆CT method, as previously described.37

Western blotting. Cell lysates were collected in NP-40 buffer, total protein was quantitated using the Bradford method (Bio-Rad), and 30 μg of total protein was loaded onto 4–20% gradient Tris-Glycine precast gel (Bio-Rad). Gels were transferred to nitrocellulose membranes using the iBlot system (Life Technologies, Carlsbad, CA, USA). Blots were probed with anti-HSP70 (Enzo Life Sciences, Farmingdale, NY, USA), anti-Bim (Thermo Scientific,
Rockford, IL, USA) and anti-β-actin (Sigma-Aldrich) primary antibodies and either HRP-conjugated secondary antibodies (Thermo Scientific) or infra-red-conjugated secondary antibodies (LI-COR, Lincoln, NE, USA). Bands were visualized using enhanced chemiluminescence (Thermo Scientific) and conjugated secondary antibodies (LI-COR, Lincoln, NE, USA). Bands were further analyzed by Bonferroni post-hoc analysis (where indicated). A P-value < 0.05 was considered statistically significant.

Figure 8 HSP70 is required for Dex resistance of apo-SAA-induced T helper 17 cytokine secretion. BMDC were serum starved for 48 h in the presence (SAA) or absence (control) of 1 μg/ml apo-SAA, ± 25 μg/ml HSP70i, prior to coculture with OTII CD4\(^+\) T cells and OVA, ± 0.1 μM Dex. Supernatants from cocultures were collected 72 h later and analyzed for IL-13, IFN\(\gamma\), IL-17A, IL-17F, IL-21, and IL-22. (IL-4 and IL-5 were undetectable in supernatants.) n = 3–5 replicates per condition. *P < 0.05, **P < 0.01, ***P < 0.001 compared with control without Dex.

**Conflict of Interest**
The authors declare no conflict of interest.

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Statistical analysis. Data were analyzed by two-tailed unpaired Student’s t-test, a one-way ANOVA, or a two-way ANOVA using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). Statistically significant results by ANOVA were further analyzed by Bonferroni post-hoc analysis (where indicated). A P-value < 0.05 was considered statistically significant.
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