

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 T_H17 polarization. T_H17 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 OTH CD4⁺ T cells to secrete IL-17A, IL-17F, IL-21, IL-22, and IFN γ in the presence of ovalbumin. IL-17A, IL-17F, IL-21, and IFN γ production occurred even when the CD4⁺ T cells were treated with dexamethasone (Dex), whereas glucocorticoid treatment abolished 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 inhalation 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.

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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.^{2,6–8} 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,

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Abbreviations: Alum, aluminum hydroxide; Bad, Bcl-2 antagonist of cell death; Bax, Bcl-2-associated x protein; BAL, bronchoalveolar lavage; Bcl-2, B-cell lymphoma; Bcl-XL, BCL2L1 long isoform; Bim, Bcl-2-interacting mediator of cell death; BMDC, bone marrow-derived dendritic cell; Clca3, calcium-dependent chloride channel 3; Dex, dexamethasone; Dusp1, dual specificity phosphatase-1; Glul, glutamine synthetase; glutamine ammonia ligase; GR, glucocorticoid receptor; HSP70, heat shock protein 70; HSP70i, heat shock protein 70 inhibitor (KNK437); IL-1, interleukin-1; IL-4, interleukin-4; IL-5, interleukin-5; IL-6, interleukin-6; IL-13, interleukin-13; IL-17, interleukin-17; IL-21, interleukin-21; IL-22, interleukin-22; IFN γ , interferon gamma; KC, keratinocyte chemoattractant (chemokine (C-X-C motif) ligand 1); LDH, lactate dehydrogenase; Muc5ac, mucin 5 AC; OVA, ovalbumin; SAA, serum amyloid A; Tc22d3, glucocorticoid-induced leucine zipper; TIAP, baculoviral IAP repeat-containing 5 (Birc5); TNF α , tumor necrosis factor alpha; zVAD, Z-Val-Ala-Asp(OMe)-CH₂F

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atherosclerosis, and allergic airway disease.^{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 T_H17 allergic asthma response upon subsequent OVA challenge.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.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 T_H17 CD4⁺ 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⁺ 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.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_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^{-/-} 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^{-/-} 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,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- α , and IL-1 β after 24 h (Figure 2f). Whereas the secretion of IL-6 and TNF- α was inhibited by HSP70i, IL-1 β was markedly increased in the presence of SAA and HSP70i.

BMDC treated with apo-SAA drive a pro-inflammatory CD4⁺ T-cell response that is resistant to dexamethasone.

We have previously demonstrated that BMDC treated with apo-SAA can readily induce OTII CD4⁺ T cells to secrete IL-17 in the presence of OVA.¹⁰ Here, we investigated the OTII CD4⁺ 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⁺ T cells to secrete enhanced amounts of the T_H17 cytokines IL-17A, IL-17F, IL-21, and IL-22, whereas they did not enhance the production of the T_H2 cytokine IL-13, and only marginally increased the levels of the T_H1 cytokine IFN γ (Figure 3). Treatment of the serum-starved BMDC cocultures with the corticosteroid dexamethasone (Dex) at the time of CD4⁺ 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 γ , 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- α -neutralizing antibody to the coculture system had no effect on OVA-induced T-cell cytokine production or the Dex sensitivity of the CD4⁺ 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 Alangari¹⁴) 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,¹⁵ we compared the Dex sensitivity of an Alum/OVA allergic airway disease

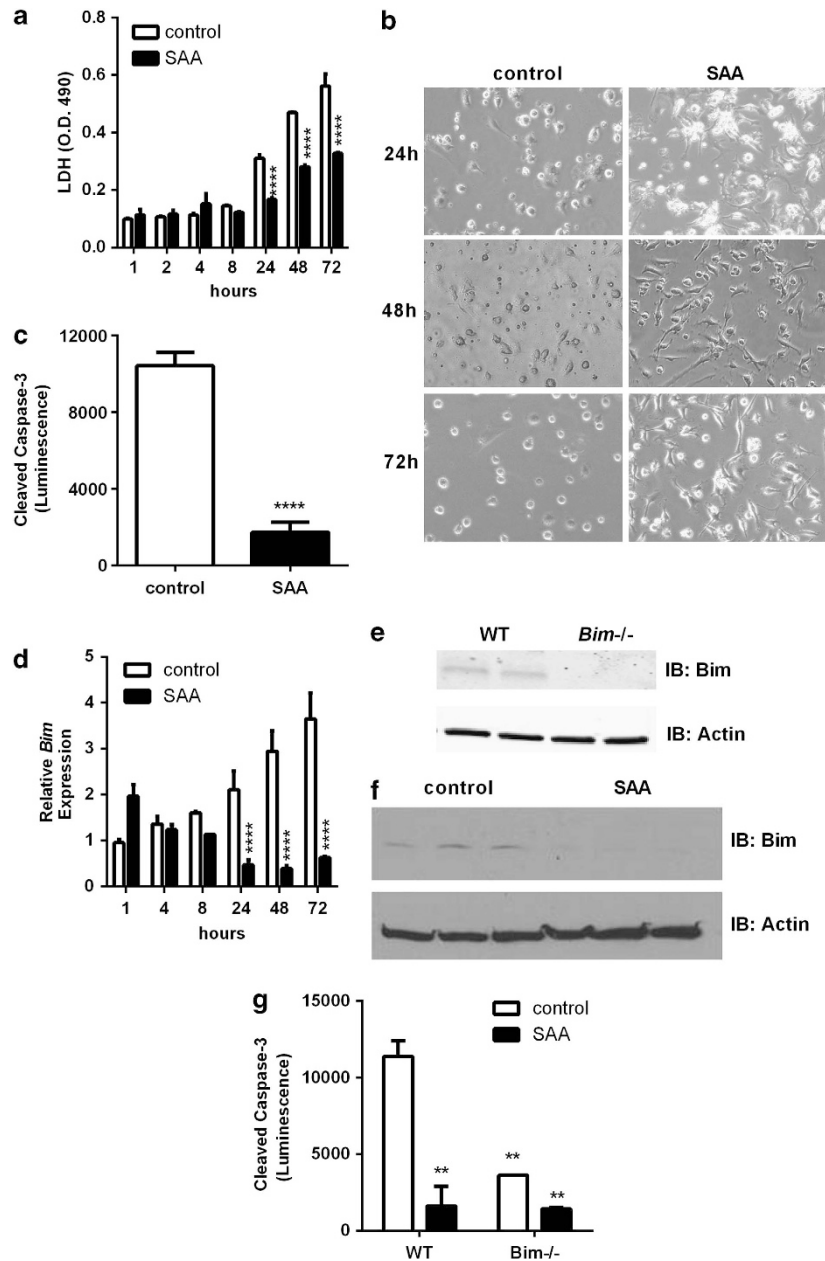


Figure 1 apo-SAA inhibits *Bim* expression and protects BMDC from serum starvation-induced apoptosis. (a) LDH levels in supernatant from BMDC serum starved in the presence (SAA) or absence (control) of 1 μ g/ml apo-SAA for the indicated times. (b) Light photomicrographs of BMDC in 12-well plates at 24, 48, and 72 h post serum starvation in the absence or presence of apo-SAA. (c) Caspase-3 activity in BMDC serum starved for 6 h in the presence or absence of apo-SAA. (d) Time course of *Bim* expression in serum-starved BMDC in the presence or absence of 1 μ g/ml apo-SAA. (e) Immunoblot (IB) for *Bim* and β -actin from whole cell lysate from wild type (WT) and *Bim*^{-/-} BMDC that were serum starved for 24 h. (f) IB for *Bim* and β -actin from 30 μ g of whole cell lysate from BMDC that were serum starved for 24 h in the presence or absence of apo-SAA. (g) Caspase-3 activity in WT and *Bim*^{-/-} BMDC that were serum starved for 6 h in the presence or absence of apo-SAA. *n* = 3–5 replicates per condition. ***P* < 0.005, *****P* < 0.0001 compared with control cells (or WT control, g) at the same timepoint

model to our apo-SAA/OVA allergic sensitization model.¹⁰ 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

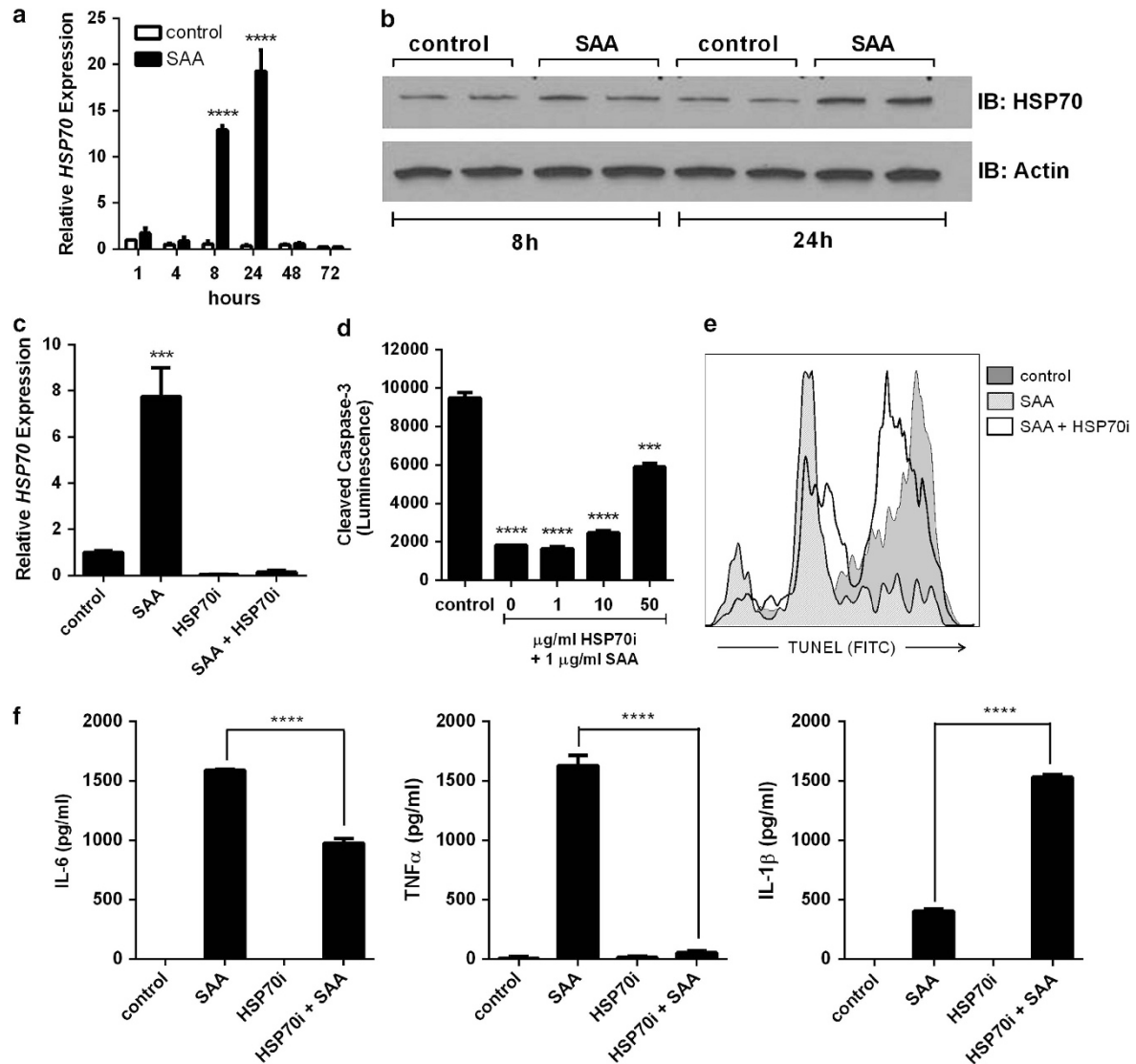


Figure 2 apo-SAA-induced HSP70 modulates caspase-3 activity and is required for cytokine secretion. (a) Time course of HSP70 expression in BMDC that were serum starved in the presence or absence of 1 μ g/ml apo-SAA (SAA). (b) Immunoblot (IB) for HSP70 and β -actin from 30 μ g of whole cell lysate from 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 μ g/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, \pm 0, 1, 10, or 50 μ g/ml HSP70i. (e) Assessment of DNA strand breaks by TUNEL assay in serum starved BMDC in the presence or absence of apo-SAA, \pm 25 μ g/ml HSP70i after 72 h. (f) IL-6, TNF- α , and IL-1 β levels from supernatants of BMDC that were serum starved for 24 h, \pm apo-SAA, \pm HSP70i. $n = 3-6$ replicates per condition. *** $P < 0.005$, **** $P < 0.0001$ compared with control (or compared with SAA in f)

induction of the mucin genes *C1ca3* (*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 IFN γ 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 IFN γ in CD4⁺ T cells (black bars). Additionally, Dex effectively inhibited production of IL-17A and IFN γ , 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

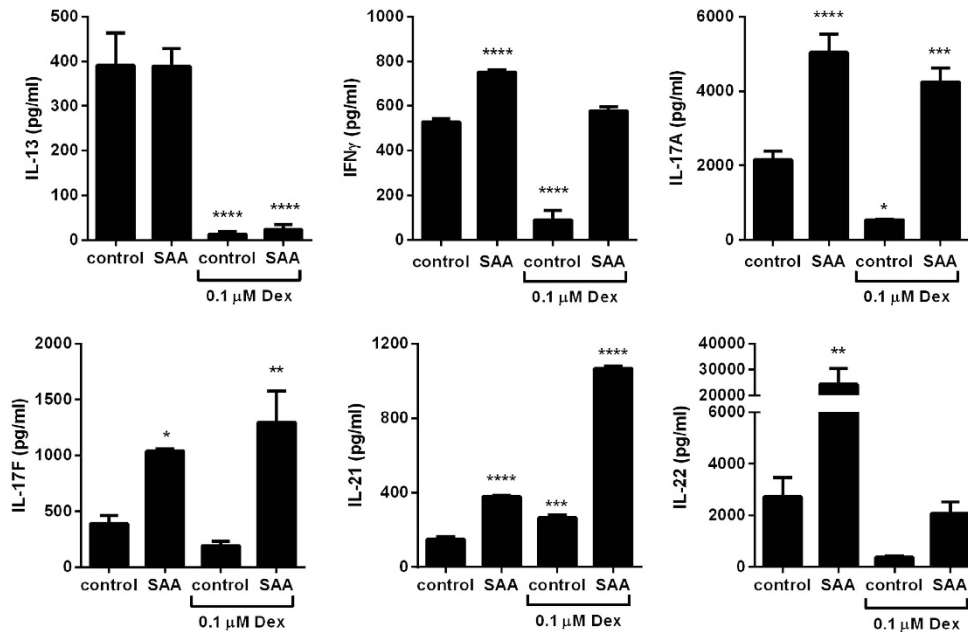


Figure 3 BMDC serum starved in the presence of apo-SAA can induce T_H17 cytokine secretion from OTII CD4⁺ T cells that is resistant to Dex. BMDC were serum starved for 48 h in the presence (SAA) or absence (control) of 1 μ g/ml apo-SAA prior to coculture with OTII CD4⁺ T cells and OVA, \pm 0.1 μ M Dex. Supernatants from cocultures were collected 72 h later and analyzed for IL-13, IFN γ , IL-17A, IL-17F, IL-21, and IL-22. (IL-4 and IL-5 were undetectable in supernatants.) $n = 3$ –5 replicates per condition. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.0001$ compared with control

(BMDC CM) or with apo-SAA (BMDC + SAA CM). 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*,¹⁶ *Tc22d3*,¹⁷ and *Dusp1*.¹⁸ 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.¹⁹ 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 \pm 348.5 pg/ml) were not as high as those induced by SAA treatment (5038.0 \pm 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

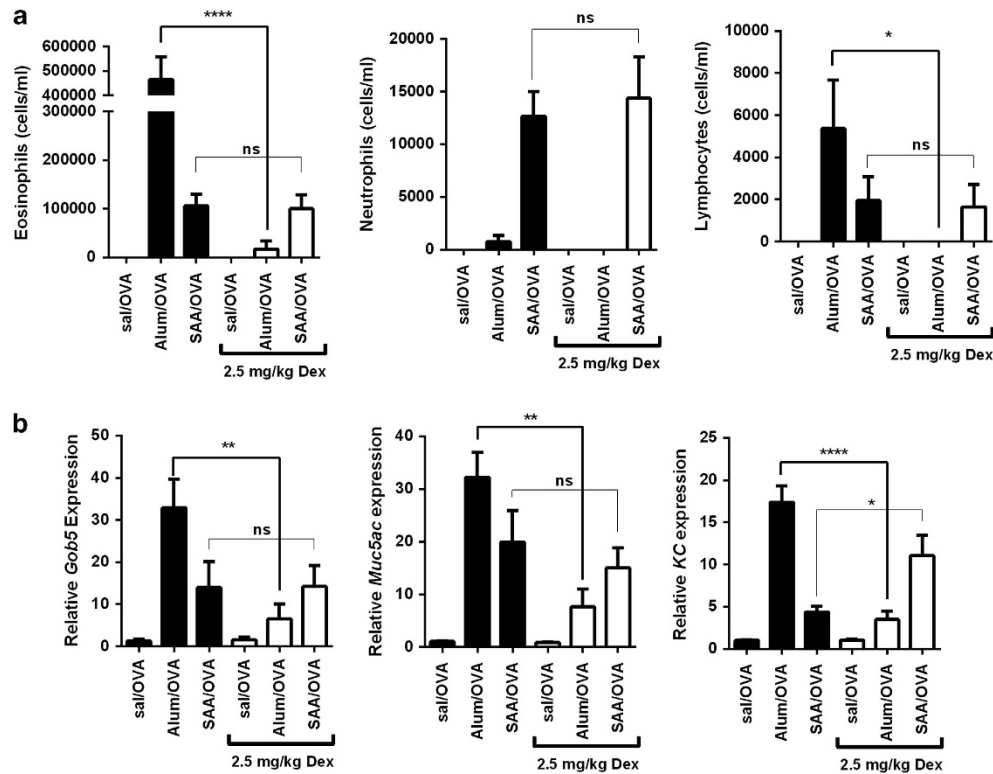


Figure 4 Inflammatory cell recruitment in apo-SAA-induced allergic airway disease is resistant to Dex treatment. Mice were sensitized to ovalbumin with either saline (sal/OVA), i.p. injection of aluminum hydroxide (Alum/OVA), or 10 μ g o.a. apo-SAA. Some groups received Dex two weeks later on the first and third day of OVA challenge. (a) Cell counts from BAL 48 h after the final challenge. (b) Whole lung gene expression from mice 48 h challenge. $n = 4$ mice per group. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$ compared with control

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.²⁰ In addition, HSP70 comprises part of the chaperone protein complex that governs the folding and cellular localization of the glucocorticoid receptor (GR).^{21–23} 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 IFN γ , IL-17F, IL-21, and IL-22 compared with control, and blocked apo-SAA-induced secretion of IL-13 and IFN γ (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.

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 proteins¹⁹ that can function extracellularly as DAMPs.²⁴ 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 NLRP3 inflammasome activation, IL-1 β production, and asthma-like disease with a mixed T_H2/T_H17 response in mice.¹⁰ 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.²⁵ Glucocorticoids pass through the cell membrane in order to bind to the GR, which resides in the cytosol in the company of a chaperone

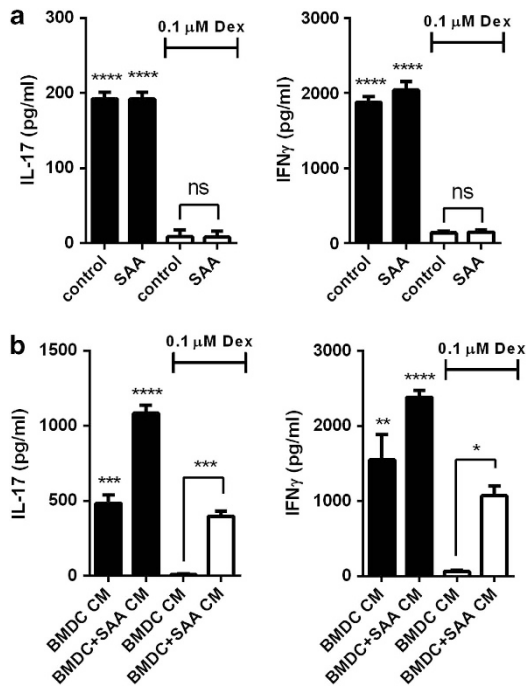


Figure 5 An apo-SAA-induced soluble mediator from BMDC decreases Dex sensitivity in CD4⁺ T cells. (a) CD4⁺ T cells from OTII mice were plated and polyclonally stimulated with plate-bound anti-CD3 (5 μ g/ml) and soluble anti-CD28 (2 μ g/ml) \pm 1 μ g/ml apo-SAA and \pm 0.1 μ M Dex for 24 h. IL-17A and IFN γ were measured from cell-free supernatants by ELISA. (b) CD4⁺ T cells from OTII mice were plated and polyclonally stimulated with plate-bound anti-CD3 (5 μ g/ml) and soluble anti-CD28 (4 μ g/ml), and treated with CM from serum-starved BMDC that were untreated (BMDC CM) or treated with apo-SAA (BMDC + SAA CM), in the absence (black bars) or presence (white bars) of 0.1 μ M Dex for 24 h. Cell-free supernatants were analyzed for IL-17A and IFN γ by ELISA. $n = 3-4$ replicates per condition. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.0001$

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 augment 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 from polyclonally stimulated CD4⁺ T cells despite glucocorticoid treatment (Figure 5b), and also diminished the expression of Dex-responsive genes in CD4⁺ T cells (Figure 6b). Taken together, these data demonstrate that apo-SAA treatment of BMDC induces release of a soluble mediator that modulates the steroid sensitivity of CD4⁺ T cells.

As T-cell viability may be affected by Dex, reduced numbers of live cells could account for the decreases in cytokine

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 PGE₂, 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 T_H17 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 T_H17 CD4⁺ T cells and appears to be involved in autoimmune pathologies.³³⁻³⁵ Previous studies have also implicated IL-21 as a Dex-resistant 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- κ B and stimulate the release of other cytokines such as IL-6, IL-1 β , 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

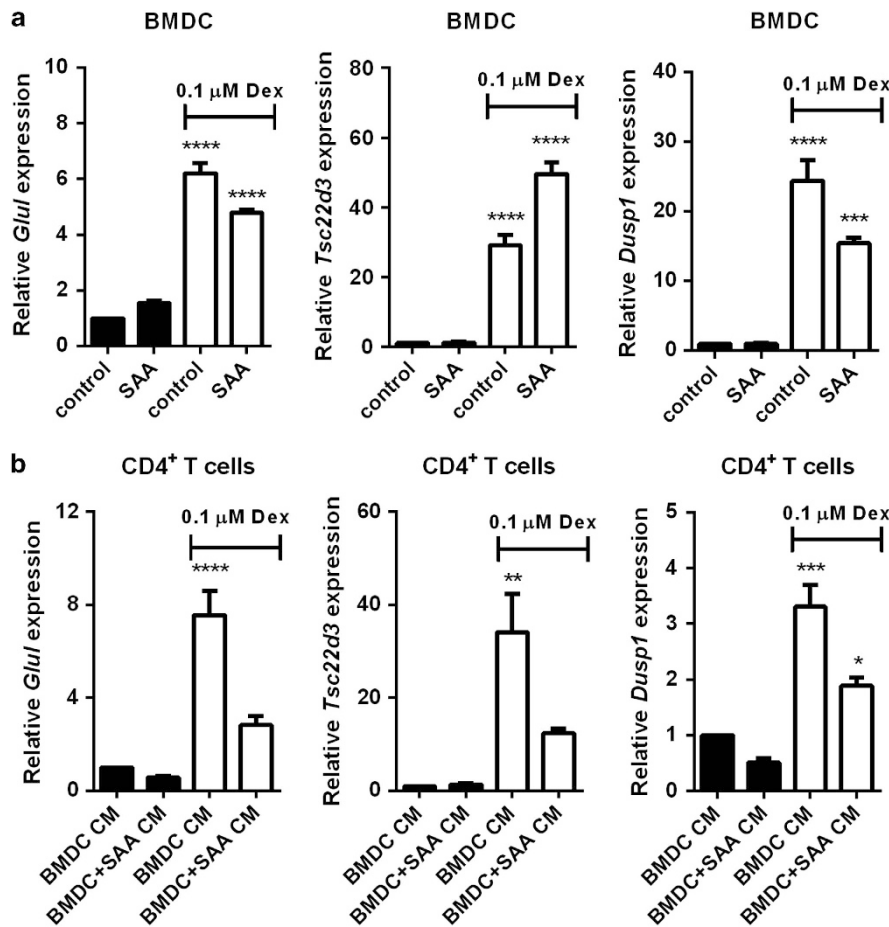


Figure 6 apo-SAA treatment of BMDC substantially diminishes the expression of Dex-responsive genes in CD4⁺ T cells. (a) BMDC were serum starved for 48 h ± 1 μg/ml apo-SAA and ± 0.1 μM Dex. Cell lysates were collected and cDNA was analyzed by quantitative PCR and statistically compared with control, no Dex samples. (b) CD4⁺ T cells from OTII mice were plated and polyclonally stimulated with plate-bound anti-CD3 (5 μg/ml) and soluble anti-CD28 (4 μg/ml) and treated with CM from serum-starved BMDC that were untreated (BMDC CM) or treated with apo-SAA (BMDC + SAA CM) in the absence (black bars) or presence (white bars) of 0.1 μM Dex for 24 h. Cell lysates were collected and cDNA was analyzed by quantitative PCR. *n* = 3–4 replicates per condition. **P* < 0.05, ***P* < 0.01, ****P* < 0.005, *****P* < 0.0001 compared with control without Dex

pro-inflammatory environment that is resistant to apoptosis, and therefore, resistant to resolution of the inflammatory state. This in turn drives production of T_H17 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 chaperone 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. Bim^{-/-} mice on the C57BL/6J background were obtained from Dr. Karen Fortner and were generated as previously described.⁸ C57BL/6J mice and OTII TCR transgenic mice (C57BL/6-Tg(TcrαCrb)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 American Association for the Accreditation of Laboratory Animal Care (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% Inject 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 × 10⁶ cells/well (3 ml/well) in RPMI-1640 containing 10% serum and 5% CM 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 BMDC, predominantly CD11b⁺CD11c⁺ by FACS, were collected on day 6. For serum starvation, BMDC were plated at 1 × 10⁶ 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 20 × 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).

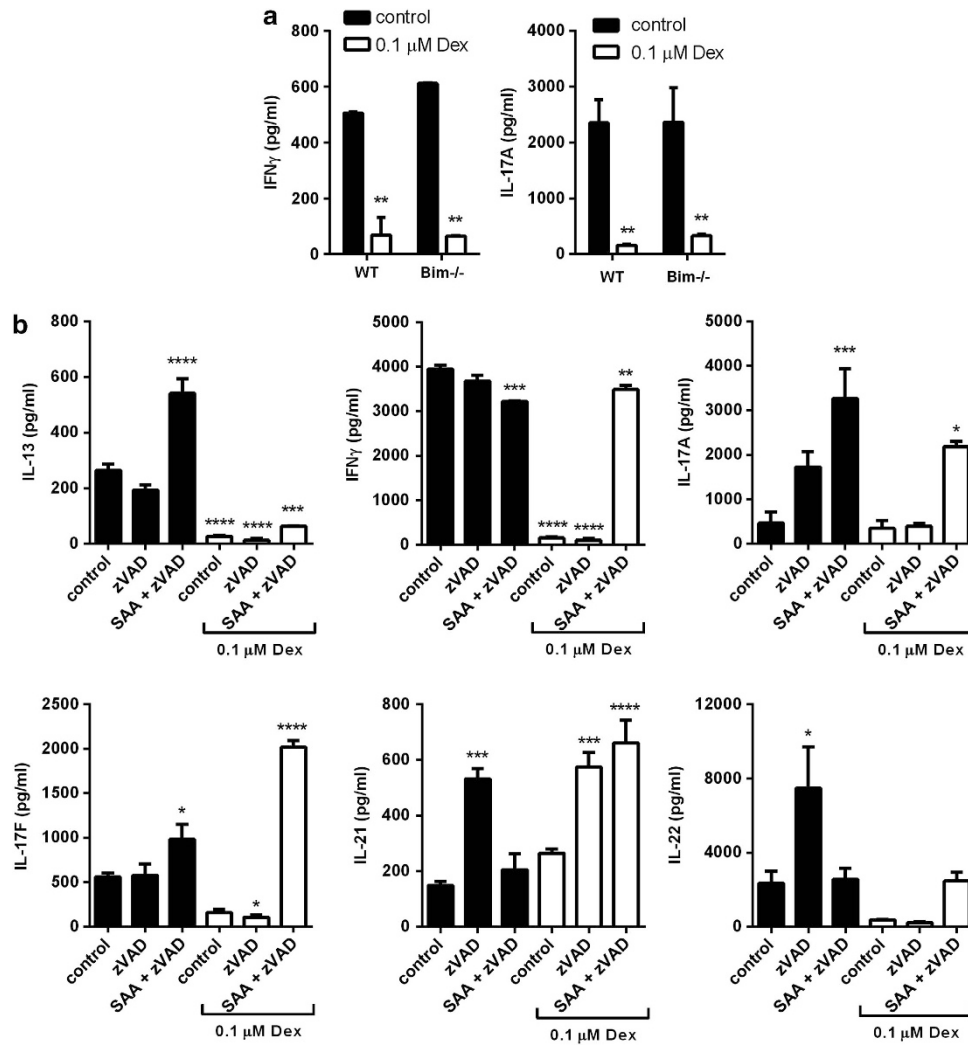


Figure 7 Caspase-3 inhibition is not sufficient to induce Dex resistance. (a) BMDC from WT or Bim^{-/-} mice were serum starved for 48 h prior to coculture with OTII CD4⁺ T cells and OVA, \pm 0.1 μ M Dex. Supernatants from cocultures were collected 72 h later and analyzed for IFN γ and IL-17A. (b) BMDC from WT mice were serum starved for 48 h in the presence or absence of 20 μ M zVAD prior to coculture with OTII CD4⁺ T cells and OVA, \pm 0.1 μ M Dex. Supernatants from cocultures were collected 72 h later and analyzed for IL-13, IFN γ , IL-17A, IL-17F, IL-21, and IL-22. (IL-4 and IL-5 were undetectable in supernatants.) $n = 3-5$ replicates per condition. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.0001$ compared with control without Dex

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 μ g 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 iScript kit (Bio-Rad, Hercules, CA, USA). Primers were designed for mouse *Bim* (forward: CTACAGACAGAACC GCAAGGT; reverse: CCTGAGACTGTCGTATGGAAG), *HSP70* (forward: ATCACCATCAC CAACGACAAGG; reverse: TGCCCAAGCAGCTATCAAGTGC),⁴⁰ *Glul*: glutamine

synthetase; glutamine ammonia ligase (forward: TTATGGGAACAGCGGCCAC; reverse: AAAGTCTTCGCACACCCGAT), *Tc2d3*: glucocorticoid-induced leucine zipper (forward: GGAGCCGGTTTACCTGAAGT; reverse: CCGAAAGTTGCTCAC GAAGG), and *Dusp1*: dual specificity phosphatase-1 (forward: GAGCTGTGCAG CAAACAGTC; reverse: CGAGAAGCGTGATAGGCACT), *Gob5* (forward: AAGC AAACCACTCCCATGAC; reverse: TGC GAAAGCATCAACAAGAC). *Muc5ac* (forward: CCATGCAGAGTCTCAGAACAA; reverse: TTAGTGGAAAGGCC CAAGCA), and *KC* (forward: GCTGGGATTACCTCAAGAA; reverse: TGGGGA CACCTTTTAGCATC) and quantitative PCR was performed on cDNA using iQ SYBR Green Supermix (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 $\Delta\Delta C_T$ 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 μ g 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,

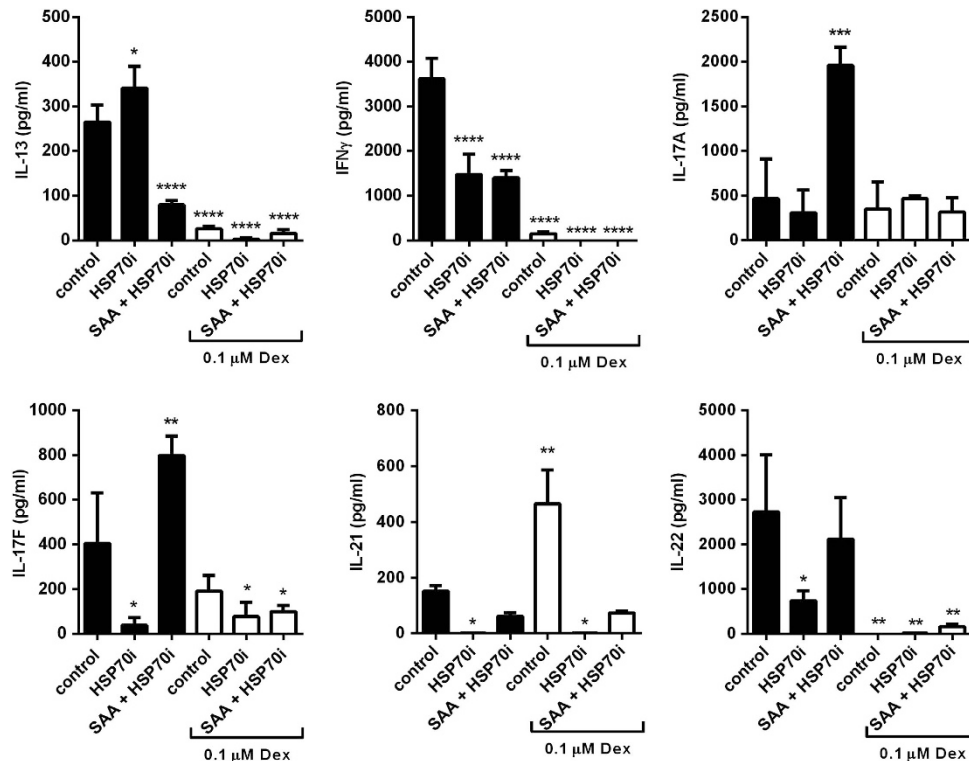


Figure 8 HSP70 is required for Dex resistance of apo-SAA-induced T_H17 cytokine secretion. BMDC were serum starved for 48 h in the presence (SAA) or absence (control) of 1 μ g/ml apo-SAA, \pm 25 μ g/ml HSP70i, prior to coculture with OTII CD4⁺ T cells and OVA, \pm 0.1 μ M Dex. Supernatants from cocultures were collected 72 h later and analyzed for IL-13, IFN γ , IL-17A, IL-17F, IL-21, and IL-22. (IL-4 and IL-5 were undetectable in supernatants.) $n = 3$ –5 replicates per condition. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$ compared with control without Dex

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 exposure of blots to X-ray film, or by LI-COR Odyssey CLx Imaging System (LI-COR).

Cytokine analysis. Cytokines from cell supernatants were analyzed by ELISA for IL-1 β and TNF- α (BD Biosciences), IL-6 (R&D Systems, Minneapolis, MN, USA), and SAA3 (Millipore, Billerica, MA, USA). A customized Milliplex assay was used to measure IL-4, IL-5, IL-13, IL-17A, IL-17F, IL-21, IL-22, and IFN γ (Millipore).

OTII CD4⁺ T-cell coculture studies. CD4⁺ T cells from OTII transgenic mice were isolated from spleen and peripheral lymph nodes by magnetic negative selection (Stem Cell Technologies, Vancouver, BC, Canada) and were cocultured at 1×10^6 cells/ml in a 96-well plate with adherent BMDC that had been plated and serum starved for 48 h in the presence or absence of 1 μ g/ml apo-SAA. At the time of CD4⁺ T-cell addition, cells were also treated with whole OVA (100 μ g/ml, Sigma-Aldrich), and those receiving corticosteroid treatment were supplemented with 0.1 μ M Dex (Sigma-Aldrich). Cells were cultured together for 72 h, at which time supernatants were collected, centrifuged, and snap frozen for later analysis. In separate experiments, BMDC received either 20 μ M Z-Val-Ala-Asp(OMe)-CH₂F (zVAD) (Millipore) or the HSP70 inhibitor (KNK437, an inhibitor of the transcription factor, Heat Shock Factor-1, which regulates expression of the *Hsp70* gene)⁴¹ (Millipore) or 10 μ g/ml anti-TNF- α -neutralizing antibody (BD Biosciences) at the beginning of serum starvation.

In separate experiments, CD4⁺ OTII T cells were polyclonally stimulated with plate-bound anti-CD3 (BD Biosciences) at 5 μ g/ml and soluble anti-CD28 (BD Biosciences) at 2 μ g/ml. These cultures received either treatment with apo-SAA at 1 μ g/ml, or treatment with CM from BMDC cultures that had been serum starved for 48 h in the presence or absence of 1 μ g/ml apo-SAA.

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.

Conflict of Interest

The authors declare no conflict of interest.

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