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Interference of the T Cell and Antigen-Presenting Cell Costimulatory Pathway Using CTLA4-Ig (Abatacept) Prevents Staphylococcal Enterotoxin B Pathology

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Staphylococcal enterotoxin B (SEB) is a bacterial superantigen that binds the receptors in the APC/T cell synapse and causes increased proliferation of T cells and a cytokine storm syndrome in vivo. Exposure to the toxin can be lethal and cause significant pathology in humans. The lack of effective therapies for SEB exposure remains an area of concern, particularly in scenarios of acute mass casualties. We hypothesized that blockade of the T cell costimulatory signal by the CTLA4-Ig synthetic protein (abatacept) could prevent SEB-dependent pathology. In this article, we demonstrate mice treated with a single dose of abatacept 8 h post SEB exposure had reduced pathology compared with control SEB-exposed mice. SEB-exposed mice showed significant reductions in body weight between days 4 and 9, whereas mice exposed to SEB and also treated with abatacept showed no weight loss for the duration of the study, suggesting therapeutic mitigation of SEB-induced morbidity. Histopathology and magnetic resonance imaging demonstrated that SEB mediated lung damage and edema, which were absent after treatment with abatacept. Analysis of plasma and lung tissues from SEB-exposed mice treated with abatacept demonstrated significantly lower levels of IL-6 and IFN-γ ($p < 0.0001$), which is likely to have resulted in less pathology. In addition, exposure of human and mouse PBMCs to SEB in vitro showed a significant reduction in levels of IL-2 ($p < 0.0001$) after treatment with abatacept, indicating that T cell proliferation is the main target for intervention. Our findings demonstrate that abatacept is a robust and potentially credible drug to prevent toxic effects from SEB exposure. ***The Journal of Immunology*****, 2017, 198: 3989–3998.**

Staphylococcus aureus is known to produce at least 15 serologically distinct superantigens, including staphylococcal enterotoxin B (SEB) (1, 2). SEB is the superantigen commonly associated with staphylococcal food poisoning, but it can also cause widespread systemic damage and toxic shock syndrome (3, 4). The toxin has been shown to cause lethal pulmonary disease and contribute to pneumonia in vivo (5–7). SEB is stable to aerosolization, and inhalation of small amounts can cause severe lung pathology, shock, and death (4–7).

Aerosolized SEB exposure in nonhuman primates has an estimated LD_{50} of 21.7–44.1 μg/kg; however, no direct data are available for this in humans (2). The relative ease with which stable aerosols of SEB can be produced has resulted in this superantigen featuring on both the U.K. and U.S. biological warfare threat lists (2). Thus, weaponized SEB used in either a bioterrorist or military context can result in a mass casualty scenario (2).

Recent research has looked into the development of effective treatments for SEB exposure and intoxication, including agent-specific medical countermeasures (e.g., antitoxins) and wider-spectrum therapies (e.g., anti-inflammatory agents) to target the resulting immunopathology (8). The development of broad-spectrum therapeutic agents, that is, those that are effective against superantigens in general, is of particular interest.

Several challenges in regard to these therapeutic agents remain. Ideally, an effective treatment for superantigen exposure should only require a single dose to minimize the logistic constraints in a mass casualty situation and facilitate management of milder forms of the disease, such as food poisoning (9, 10). Treatment of superantigen exposure will need to occur within a manageable postexposure window, allowing the time for detection and diagnosis required for effective treatment. Thus, interventions that offer a broad spectrum of activity against multiple superantigens and are effective when administered as a postexposure treatment are credible therapeutic candidates (11).

Staphylococcal enterotoxins are extremely potent activators of T cells (12, 13). These toxins bind directly to the MHC class II molecules on APCs and the variable β-chains of the TCR, and activate the endogenous pathways dependent upon immune

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Abbreviations used in this article: CI, confidence interval; i.n., intranasal; MRI, magnetic resonance imaging; NIBSC, National Institute for Biological Standards and Control; SEB, staphylococcal enterotoxin B; TSST, toxic shock syndrome toxin.

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synapse formation (12, 13). CD28, a costimulatory receptor on T cells, has been recently identified as a superantigen receptor (14). Concerted interaction of the superantigen with all three receptors (CD28, MHC class II, and TCR) allows stable synapse formation resulting in exceptionally robust T cell responses, particularly Th1 cytokine induction, and lethality (14). Via this mechanism, SEB activates ~20% of the T cell population, whereas exposure to normal Ags activates <0.01% of T cells (14, 15). CTLA4 plays an important role in controlling excessive T cell activation (16). This receptor specifically binds the B7 receptors (CD80/CD86) on APCs and acts as a negative costimulatory receptor by preventing CD28 interaction with B7 receptors (16). The inhibitory effect of native CTLA4 is through the active removal of the B7 receptor from the surface of APC (16). The resultant removal of the B7 receptor prevents interaction with CD28. Furthermore, expression of CTLA4 has been shown to set T cell activation thresholds through intracellular tyrosine phosphatase regulation (17). A synthetic version of CTLA4 comprising a fusion protein with the Fc region of the human Ig (IgG1) and the extracellular domain of the receptor, termed abatacept (CTLA4-Ig), has been developed to mitigate T cell activation in reactive arthritis (18, 19).

We hypothesized that a single dose of abatacept, given post exposure, could mitigate T cell and APC activation through immune synapse formation, and thereby reduce the pathology induced by SEB in mice and in human PBMCs.

Materials and Methods

Toxin and therapeutics

SEB toxin (1 endotoxin unit/50 μg) was obtained from Public Health England (Porton Down, Wiltshire, U.K.). Abatacept was obtained from Bristol-Myers Squibb (Uxbridge, Middlesex, U.K.). Ipilimumab (Yervoy) was obtained from Idis (Weybridge, U.K.). Con A and LPS were from Sigma-Aldrich (Poole, Dorset, U.K.). All reagents were initially prepared in PBS (Life Technologies, Paisley, U.K.) with working dilutions in sterile RPMI 1640 media containing 15% (v/v) FCS (Sigma-Aldrich), 100 U/ml penicillin, 0.1 mg/ml streptomycin solution (Sigma-Aldrich), and 2 mM L-glutamine (Sigma-Aldrich).

Splenocyte preparation

Fifteen male BALB/C mice (6–7 wk old; Charles River Laboratories, Margate, Kent, U.K.) were killed by cervical dislocation, and their spleens were removed aseptically. For each experiment spleens from three mice were passed through a 10-μm cell strainer. Each strained spleen preparation was resuspended in 30 ml of RPMI 1640 medium, supplemented as described earlier. Each spleen cell suspension was centrifuged for 10 min at 350 × *g*, and the cell pellet was lysed with 3 ml of RBC lysing buffer (Sigma-Aldrich) for 1 min. Splenocytes were washed and resuspended in RPMI 1640 medium. Cell counts were determined using a Neubauer hemocytometer and adjusted with supplemented RPMI 1640 medium to give a final live cell count of 2.0×10^7 cells/ml.

Cell proliferation (MTT) assay

The ability of abatacept to inhibit the proliferative effect of SEB was measured using a modified cell proliferation technique (20). Splenocytes were seeded into 96-well flat-bottom Corning Costar cell culture plates (Sigma-Aldrich) at a cell density of 1×10^6 cells per well. To the wells we added 100 μl of PBS (negative control), 0.5 μg SEB/ml (positive control), or a mixture of 0.5 μg SEB/ml and abatacept (2-fold dilution ranging from 0.3 to 10 μg/ml); Con A was included as a positive control.

After 48 h, cell proliferation was measured using an MTT assay with minor modifications from the manufacturer's instructions (Promega, Madison, WI). Cell proliferation was determined by measuring absorbance at 570 nm (A_{570}) using a plate reader (Thermo Fisher Scientific, Basingstoke, U.K.), and the result was normalized to the negative control (0% baseline) and the positive control (100% SEB).

Cell cytotoxicity assay

A baseline cytotoxicity assay was conducted on a 48-h culture of murine splenocytes cultured to establish the relative toxicities of SEB and abatacept, individually and in combination. Splenocytes were seeded into 96-well flat-bottom cell culture plates (B. E. Thompson Supplies, Andover, U.K.) at a cell density of 5×10^4 cells per well and incubated under the following conditions: 1) splenocytes in RPMI 1640 alone; 2) splenocytes in RPMI 1640 with abatacept; 3) splenocytes in RPMI 1640 with 0.5 μg SEB/ml; and 4) splenocytes in RPMI 1640 supplemented with SEB 0.5 μg/ml and abatacept at 0.625 to 10 μg/ml.

After 48 h, cell death was determined using a Promega Multi-Fluor cytotoxicity assay (Promega), in accordance with the manufacturer's instructions. Dead cell numbers were determined by fluorescence at 485Ex/535Em 3 h after the addition of 5× Multi-Fluor reagent. Results were expressed as mean fluorescence intensity and SEM for each group.

SEB stimulation of human cells in vitro

Human whole blood was donated by consenting employees of National Institute for Biological Standards and Control (NIBSC; Potters Bar, U.K.). PBMCs were isolated from heparinized whole blood (10 IU/ml; Wockhardt, Wrexham, U.K.) by density gradient centrifugation using Lymphoprep (Axis-Shield Diagnostics, Dundee, U.K.) layered beneath whole blood diluted 1:2 with PBS. PBMCs were resuspended at 1×10^6/ml in complete RPMI 1640 (RPMI 1640, 10% FCS, L-glutamine, penicillin, and streptomycin) and 200 μl of cells was seeded per well in 96-well cell culture cluster plates (Corning, New York, NY). SEB (10 ng/ml), abatacept (1 and 10 μg/ml), and ipilimumab (1 and 10 μg/ml) were added to the wells.

For measurement of cytokine release by ELISA, cells were incubated at 37°C under 5% CO_2 for 24 h. Each condition was tested in triplicate. The cells were centrifuged, and the supernatants were collected and stored at −20°C until analysis.

Where intracellular cytokine staining was measured, 10 μg of brefeldin A/ml (secretion inhibitor) was added to the cell cultures after 1-h stimulation with the different treatments to block the secretion of cytokines (21). Cells were incubated at 37°C under 5% CO_2 overnight. Each condition was tested in triplicate. Cytokine responses to therapeutic mAbs were assessed after overnight incubation using intracellular staining and FACS analysis.

Human cytokine responses to in vitro treatments

Concentrations of TNF-α, IL-2, and IFN-γ in culture supernatants were measured by ELISA. In brief, Nunc MaxiSorp plates (Thermo Fisher Scientific) were coated overnight at 4°C with respective capture Abs (anti–TNF-α capture Ab mAb clone 357-101-4), anti–IL-2 capture Ab (R&D MAB602; R&D Systems, U.K.), or anti–IFN-γ capture Ab (BD 551221; BD Biosciences, Oxford, U.K.). Plates were blocked with 1% BSA; then 50 μl of the culture supernatant or the relevant WHO International Standards (Second WHO Internal Standard for TNF-α NIBSC 88/786, IL-2 standard NIBSC 86/564, IFN-γ standard NIBSC 88/606) was added to the plates together with 50 μl of biotinylated anti–TNF-α (clone H91; NIBSC), or biotinylated anti–IL-2 (R&D BAF202) or biotinylated anti–IFN-γ (BD 554550; BD Biosciences). Plates were incubated overnight at 4°C and developed using streptavidin-HRP conjugate (Jackson) diluted 1/30,000 and *o*-phenylenediamine dihydrochloride substrate (Sigma-Aldrich). The reaction was then stopped with 1M H_2SO_4, and colorimetric measurements were performed at 490 nm using SpectraMax plate reader (Molecular Devices).

Polychromatic intracellular cytokine staining was performed using the manufacturer-recommended amounts of the following anti-human fluorochrome conjugates for surface staining for 30 min at 4°C: CD3-FITC (clone UCHT1; BioLegend, San Diego, CA), CD4-allophycocyanin-Cy7 (clone RPA-T4; BioLegend), and CD8-PerCP-Cy5.5 (clone RPA-T8; BioLegend). After surface staining, cells were washed, fixed, and permeabilized using Fix&Perm Cell Permeabilization Kit (GAS004; Thermo Fisher Scientific). For intracellular cytokine staining, combinations of the following anticytokine fluorochrome conjugates were used at the manufacturer-recommended amounts for 30 min at 4°C: TNF-α–PE (clone Mab11; BD Biosciences), IL-2–allophycocyanin (clone MQ1-17H12; BioLegend), and IFN-γ Pacific blue (clone 4S.B3; BioLegend). The cells were washed and resuspended in PBS 0.5–1% FCS for analysis. Acquisition of 10,000 gated PBMC events per well was performed using a FACSCanto II flow cytometer equipped with a high-throughput screening plate reader (BD Biosciences) and analyzed using FlowJo v10 software. Percentage of PBMCs positive for the different cytokines was quantified.

In vitro expression of IL-1β, IL-2, and CCL2 in murine splenocytes

Murine splenocytes were cultured in RPMI 1640 with PBS (negative control), 0.5 μg SEB/ml (positive control), 3.1 μg Con A/ml (proliferation control), 5 μg LPS/ml (positive control), and 0.5 μg SEB/ml plus abatacept at 0.3 to 10 μg/ml for 48 h in a final volume of 1 ml/well. Supernatants were prepared

by centrifugation of cultures at 1000 × *g* for 10 min at room temperature. Quantitative ELISAs for IL-1β, IL-2, and CCL2 were performed according to manufacturer's instructions (Quantikine; R&D Systems, Minneapolis, MN). Supernatants recovered from splenocytes exposed to SEB and treated with abatacept were compared with supernatants from the negative control and positive controls (cells exposed to either SEB, Con A, or LPS).

Animal husbandry

Age-matched male BALB/c mice (6–7 wk old; Charles River Laboratories) were used in all in vivo studies. On receipt, animals acclimatized to the facility before being studied. All investigations involving animals conformed to the Animal (Scientific Procedures) Act 1986 and were carried out according to the Home Office Project License, adhering to the clearly defined humane end points of no >30% weight loss and reduced mobility in the presence of severe clinical signs of intoxication, which include piloerection, limping, hunched posture, and dull or sluggish movement.

Mice were housed in rooms maintained at 21 ± 2°C on a 12/12 h dawn/dusk cycle. Humidity was maintained at 55 ± 10% with air flow of 15–18 changes/h, and mice were given food and water ab libitum. Mice were fed a standard pelleted Teklad 19% protein irradiated diet (Harlan Teklad, Bicester, U.K.).

Development of an in vivo model for intranasal exposure to SEB

A murine model for intranasal (i.n.) exposure to SEB was developed. The model eschewed the use of adjuncts such as LPS, normally used to sensitize the mice, because these agents may cause systemic inflammation and liver damage (22). Mice (six per group) were randomly assigned to control or treatment groups and were weighed on day 0. Intranasal instillation of SEB or PBS was performed under light anesthesia (halothane 5% in oxygen) with recovery. Either SEB was prepared in 50 μl of sterile PBS (Life Technologies) or the total dose was divided between the two nares. Control animals received 50 μl of PBS divided between the two nares. After exposure, the mice were returned to their home cages and allowed to recover, then subsequently received 100 μl of i.v. PBS at 3 or 8 h post i.n. administration of SEB. All animals were assessed daily for 14 d or until they met the criteria for humane cull. To determine the optimal sublethal i.n. dose of SEB, we exposed mice to SEB 0.5 and 0.25 μg/g body weight (Supplemental Fig. 2).

Abatacept in vivo efficacy study: 3 and 8 h post exposure

In subsequent studies, mice were exposed i.n. to SEB at 0.25 μg/g body weight and abatacept given i.v. at 10 μg/g body weight. The efficacy of abatacept was determined at 3 h post exposure: mice received an i.n. administration of SEB or PBS followed by an i.v. injection of either abatacept or PBS.

The efficacy of abatacept against SEB was also determined at 8 h post exposure. In this case mice were divided in three groups: group 1, i.n. PBS followed 8 h later by i.v. PBS; group 2, i.n. SEB followed 8 h later by i.v. PBS; and group 3, i.n. SEB followed 8 h later by i.v. abatacept. Each group consisted of six mice, which were monitored for 14 d post i.v. administration.

In addition, three experimental replicates were performed to study the efficacy of abatacept against SEB at 8 h post exposure with a final total of 42 mice per group (two experimental replicates of 18 mice and one experimental replicate of 6 mice for magnetic resonance imaging [MRI]). On days 3, 6, and 14 post SEB exposure, a total of six mice from each group were culled and analyzed for histopathology, gross pathology, and cytokine analysis. At days 3 and 6, three mice from each group were also culled for MRI analysis of lung edema. Mice were weighed and observed for signs of distress daily.

Behavioral observations and signs of intoxication

Mice were assessed daily for 14 d post SEB exposure for changes in body weight and signs of SEB intoxication. The appearance of each mouse was noted and graded according to severity guidelines and the requirements of the Home Office license. For piloerection, mice were scored 0–3, with 0 representing a normal mouse and 3 representing a mouse showing severe piloerection. For abdominal pinching, a code of mild, medium, or severe was recorded in each case. This study was repeated on three occasions to ensure robust data were collected.

Gross pathology and histopathology

Eighteen mice were treated to study the efficacy of abatacept given 8 h post exposure. On days 3, 6, and 14, six mice were culled and tissues taken for gross pathology. Lungs, liver, and spleen were visually scored 0–4 according to in-house guidelines ranging from normal (0), mild (1), moderate (2), substantial (3), to severe tissue damage (4), and their weights as a percentage of the animal's body weight were calculated.

Lung tissue from each animal was snap frozen in liquid N_2, cryosectioned at 5 μm after a postmortem examination, and stained with H&E.

Sections were examined using a Zeiss Axioplan MRc5 microscope (Zeiss, Oberkochen, Germany) and camera using the 10× (0.30) objective to determine tissue damage seen from SEB intoxication. Severity of lung inflammatory cell infiltrate and necrosis was assessed and scored 0–4 as percentage area of tissue damage: 0% (0), <25% (1), 26–50% (2), 51–75% (3), or 76–100% (4).

Cytokine and chemokine analysis

On days 3, 6, and 14, whole blood in EDTA was taken from six mice in the 8-h postexposure treatment experiment for cytokine analysis. Blood samples were rolled for 5 min at room temperature before being centrifuged at 1000 × *g* for 15 min at 4°C. Plasma supernatant was subsequently removed, frozen, and stored before analysis using a Bio-Rad Bio-Plex analyzer 200 (Bio-Rad, Hercules, CA). Samples were diluted 1:4, according to manufacturer's instructions, before analysis with Luminex performance assay for murine CCL2, GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL10, IL-12p70, IL-17A, CXCL1, CXCL2, TNF-α, and VEGF (R&D Systems).

Magnetic resonance imaging

Three mice from each group were killed on days 3 and 6 for immediate postmortem MRI. Mice were imaged within 10 min of sacrifice to mitigate postmortem changes. All MRI was performed at 9.4 T using a Bruker AVIII microimaging system operating at 400.16 MHz for ^{1}H and Bruker ParaVision5.1 for data acquisition (Bruker, Coventry, U.K.). A transmit/receive ^{1}H Quad 40-mm birdcage resonator was used in all experiments. Data were acquired using the microimaging Bruker FLASH method using a gradient echo and an echo time of 2.075 ms. A total of 16 averages were acquired with a slice thickness of 0.5 mm, a 30° flip angle, and a repetition time of 1 s. All images were acquired as a 256 × 256 matrix resulting in a scan time of ~1 h 8 min. Images were processed using OriginPro 9.1 software.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6. Normality of values was determined using a Kolmogorov–Smirnov normality test and, where appropriate, the data were log transformed to normality. For the proliferation, in vitro mouse cytokine and cytotoxicity assays matched analysis was performed using a one-way ANOVA with a Dunnett posttest. A linear mixed model was developed to compare the weight changes in the in vivo studies. Survival analysis was performed using Kaplan–Meier survival curves; statistical comparisons were made with log-rank (Mantel–Cox) test. For cytometric bead array cytokine analysis, five-parameter logistic curves were used to fit standard curves. Lung visual pathology scores, lung inflammatory cell infiltrate, lung body to organ weight, and plasma cytokine/chemokine levels were compared using a Kruskal–Wallis with a Dunn posttest comparing the SEB-control with the PBS-control and abatacept treatment group. Cytokine/chemokine levels in the lungs were analyzed using a one-way ANOVA with Dunnett posttest. For human in vitro assays, statistical analysis was performed using two-way repeated-measures ANOVA with Dunnett posttest.

Results

Abatacept reduces SEB-induced proliferation and proinflammatory cytokine and chemokine production of murine splenocytes in vitro

SEB is known to induce T cell proliferation and proinflammatory cytokine production (8). In the MTT assay, treatment of cells exposed to SEB with abatacept resulted in a significant dose-dependent reduction in proliferation by 40% at 0.3 μg abatacept/ml and by 60% at 10 μg abatacept/ml ($p = 0.0002$; Fig. 1A). No significant cytotoxicity was observed after treatment of splenocytes with 10 μg abatacept/ml either alone or in combination with SEB 0.5 μg/ml (Supplemental Fig. 1). The production of IL-1β, IL-2, and CCL2 of cells exposed to SEB increased significantly when compared with PBS (all cytokine levels were $p < 0.05$; see Fig. 1B–D). Treatment of SEB-stimulated splenocytes with abatacept significantly reduced levels of IL-1β ($p < 0.01$; Fig. 1B), IL-2 ($p < 0.001$; Fig. 1C), and CCL2 ($p < 0.05$; Fig. 1D) compared with untreated controls.

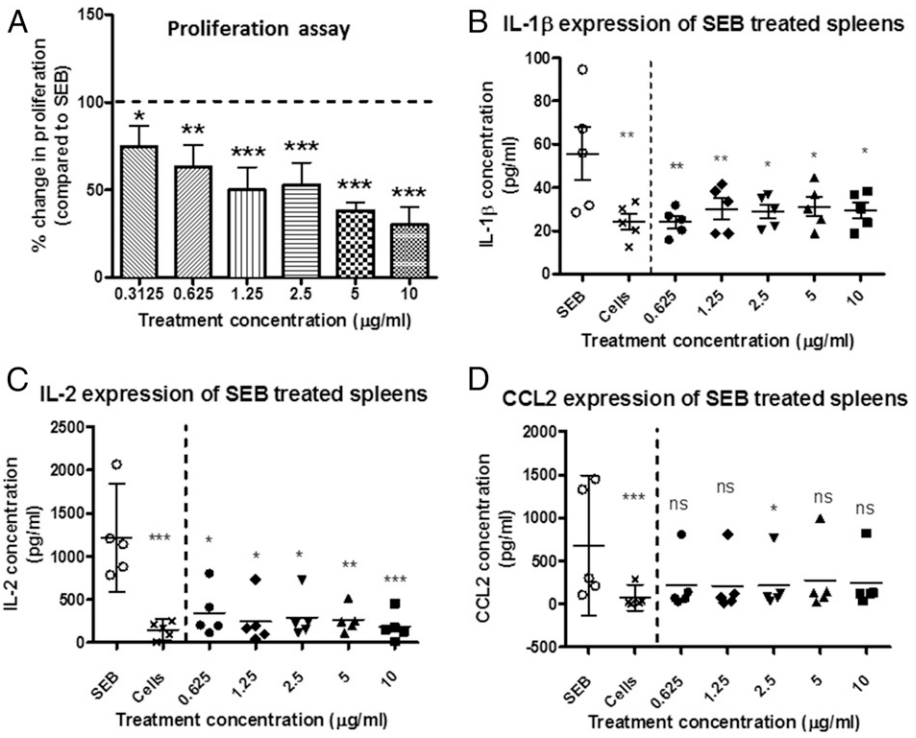

FIGURE 1. Murine splenocyte proliferation and cytokine assay. Isolated splenocytes were treated with PBS (negative control), SEB (positive control), or SEB and abatacept ($n = 5$). Proliferation assays were performed. Abatacept-treated cells were normalized against the negative control and the positive SEB control (**A**) (0 and 100%, respectively). Supernatants were analyzed for IL-1β (**B**), IL-2 (**C**), and CCL2 (**D**) production. Repeated-measures ANOVA showed that the effect of treatment was significant for proliferation, $F(6, 24) = 7.141$, $p = 0.0002$; IL-1β, $F(6, 24) = 3.948$, $p = 0.0069$; IL-2, $F(6, 24) = 5.831$, $p = 0.0007$; and CCL2, $F(6, 24) = 3.988$, $p = 0.0065$. Means of five replicates and SEM are represented. Post hoc analysis using Dunnett multiple comparison are indicated. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. ns, $p > 0.05$.

Abatacept therapy after i.n. administration of SEB after 3 and 8 h prevents weight loss and reduces clinical signs of morbidity

To assess the efficacy of abatacept to prevent SEB-induced pathology, we used an i.n. murine model of SEB intoxication. Mice were given either SEB 0.5 or 0.25 μg/g body weight via i.n. instillation followed by subsequent i.v. administration of PBS. At a dose of SEB 0.5 μg/g, two of six of mice survived, whereas SEB 0.25 μg/g resulted in four of six mice surviving (Fig. 2A). Significant differences in survival were observed between the control PBS group and mice receiving SEB 0.5 μg/g ($p = 0.0004$) or 0.25 μg/g ($p = 0.0188$). Therefore, a dose of SEB 0.25 μg/g body weight was subsequently used to evaluate the efficacy of abatacept. Loss in body weight of SEB-exposed mice was most severe at day 5, with a reduction in mean body weight of 25.5% (95% confidence interval [CI] ± 5.0%; Fig. 2C).

The mitigating effect on SEB was determined 3 and 8 h post exposure (Fig. 2B, 2C). Mice, treated with SEB or PBS i.n., received abatacept or PBS via i.v. injection. Mice were weighed and scored for clinical signs daily until day 14 (Fig. 2D–F).

Mice exposed to SEB showed a significant reduction (as determined by mixed linear modeling and 95% CI) in body weight between days 4 and 9 (Fig. 2B, 2C). Treatment with abatacept at 3 or 8 h prevented weight loss in SEB-exposed mice at these time points (Fig. 2B, 2C).

Behavioral observations and intoxication scoring supported the observed changes in weight. By day 3, 50% of the SEB-exposed mice showed signs of hunching and piloerection, with recovery by day 10 (Fig. 2E). By contrast, mice exposed to SEB and treated with abatacept showed few signs of intoxication (Fig. 2F) and appeared similar to the PBS-control group (Fig. 2D).

SEB-induced lung pathology is prevented after abatacept therapy

To determine the efficacy of abatacept to prevent SEB lung pathology, we analyzed lung tissue from mice exposed to SEB, PBS, and a mixture of SEB and abatacept for edema and tissue damage. At days 3, 6, and 14, mice were sacrificed and lung-to-body weight ratio, inflammatory cell infiltrate by histology, and visual evaluation of lung necropsy were determined. Mice treated with PBS showed no signs of tissue damage during the experiment with a mean pathology and inflammatory cell infiltrate score of 0.08 and 0.08, respectively (Fig. 3A, 3B). At day 3 post exposure to SEB, no significant difference was observed in mean lung pathology scores or lung-to-body ratio for abatacept-treated mice compared with SEB-control mice (0.4 versus 1.9, $p > 0.05$, Fig. 3A, and 1.317 versus 1.460, $p > 0.05$, Fig. 3C, respectively). However, a significant decrease in inflammatory cell infiltrate was observed between abatacept-treated mice and SEB-control mice (2.3 versus 3.9, respectively, $p < 0.05$; Fig. 3B). At day 6, a significant difference in abatacept-treated mice compared with SEB-treated mice was observed for lung pathology scores (0.1 versus 3.6, $p < 0.001$; Fig. 3A), lung inflammatory cell infiltrate (1.1 versus 2.6, $p < 0.05$; Fig. 3B), and lung-to-body ratio (0.864 versus 1.669, $p < 0.01$; Fig. 3C). Thus, abatacept treatment reduced lung pathology from day 3 to day 6. This finding was supported by representative H&E staining of lung tissue at day 6 (Fig. 4A–F). No tissue damage or inflammatory infiltration was observed in PBS-exposed mice, whereas SEB-exposed mice showed granulocyte infiltration into the lung with constriction and infiltration of the alveolar sacs with WBCs (Fig. 4D, 4E). In contrast, SEB-exposed mice treated with abatacept had no obvious WBC infiltration or tissue damage (Fig. 4F). We further evaluated the lungs for edema by MRI of the lungs. PBS-treated mice showed no signs of edema by MRI. SEB mice had visible fluid buildup in the lungs at day 6, whereas abatacept-treated and PBS-control mice did not have any observable fluid in the lungs (Fig. 4G–I, Supplemental Fig. 4).

Abatacept treatment of SEB-exposed mice reduces local and systemic release of proinflammatory cytokines and chemokines

To determine the ability of abatacept to reduce SEB-induced inflammation, we measured plasma cytokine and chemokine levels at 3, 6, and 14 d using Luminex assays. At day 3, several proinflammatory cytokines and chemokines were significantly raised in either plasma and/or lung tissue compared with mice receiving PBS

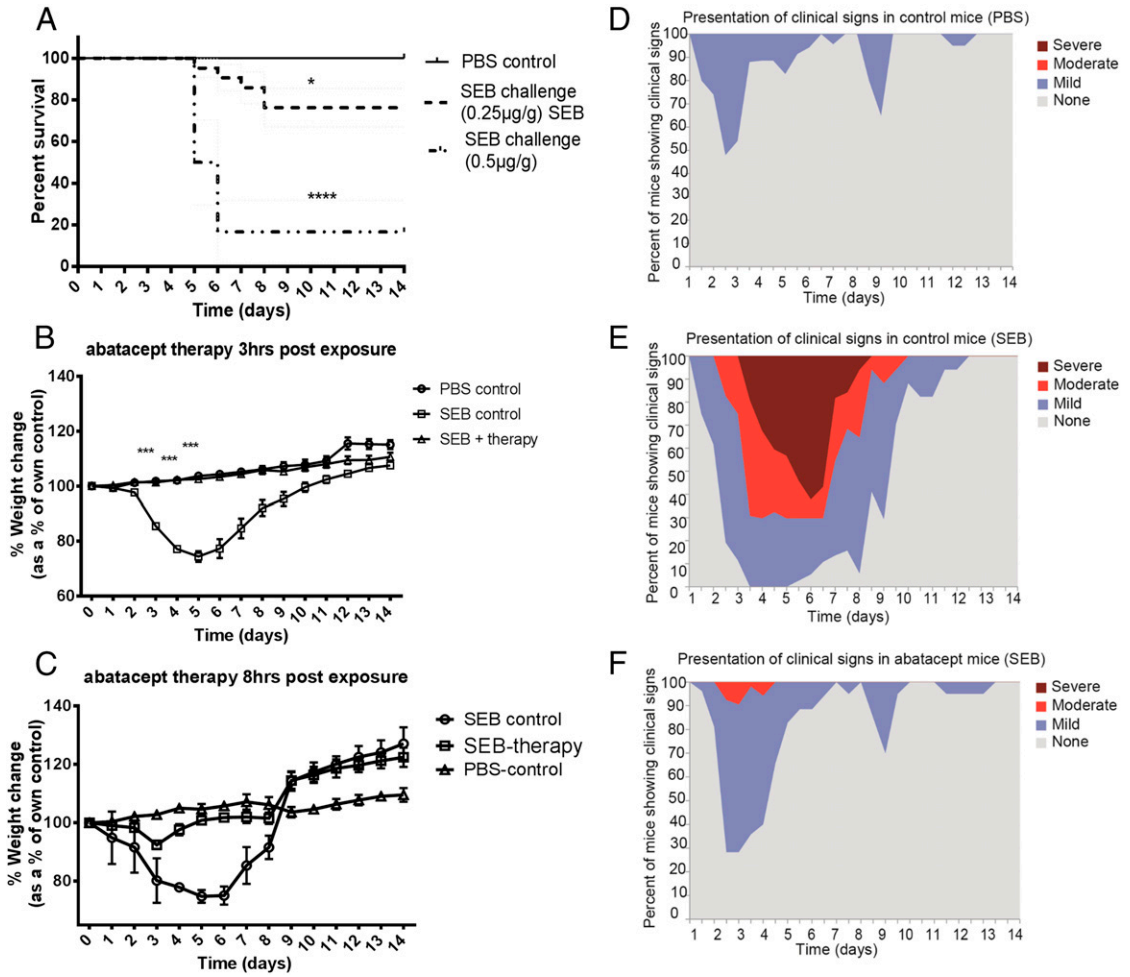

FIGURE 2. Murine model of SEB with abatacept treatment. Kaplan–Meier plot of SEB mouse survival exposed to 0.25 and 0.5 μg SEB per gram of mouse weight (**A**) and weight change after abatacept treatment at 3 (**B**) or 8 h (**C**) postexposure. Significant differences in survival were observed between the control PBS group and mice receiving 0.5 μg/g SEB ($\chi_2 = 24.24$, $p < 0.0001$) or 0.25 μg/g SEB ($\chi_2 = 5.52$, $p = 0.0188$). For weight change, graphs represent mean with error bars for 95% CI. Clinical signs of mice receiving PBS (**D**), SEB (**E**), or SEB followed by abatacept treatment (**F**) at 8 h are shown. Graphs represent the percentage of mice with severe, moderate, mild, or no clinical signs plotted for each time point. Statistical differences are summarized as: $*p < 0.05$, $****p < 0.0001$.

i.n. Notably, CXCL1, IFN-γ, IL-1β, IL-6, IL-5, CCL2, and TNF-α were raised significantly ($p < 0.05$; Figs. 5, 6, Supplemental Fig. 2).

Treatment with abatacept after SEB exposure reduced plasma levels of IFN-γ, IL-1β, and IL-6 significantly compared with SEB at day 3 ($p < 0.001$; Fig. 6). Furthermore, lung cytokine and chemokine levels of CCL2, IFN-γ, IL-5, and IL-6 were significantly lower in the abatacept-treated group compared with the SEB-exposed mice, indicating that abatacept therapy reduces inflammatory cytokine and chemokine levels at the site of SEB exposure ($p < 0.01$ for all cytokines; Fig. 6, Supplemental Fig. 3). Surprisingly, systemic levels of TNF-α remained elevated after abatacept treatment compared with PBS-control mice (Fig. 5). Furthermore, TNF-α was statistically higher in the treatment group compared with the PBS-control group at 3 d post exposure in plasma ($p < 0.0001$) and lung tissue ($p < 0.001$) (Figs. 5, 6), thus indicating that effective therapeutic treatment of SEB is not dependent upon the reduction in systemic levels of TNF-α at day 3 in this model. Although this result was surprising, it should be noted that earlier time points were not investigated, and thus an earlier systemic reduction of TNF-α may have been missed. Furthermore, lung tissue levels of TNF-α were significantly reduced at day 3 in the abatacept-treated group compared with the SEB-exposed mice (Fig. 6). IL-2 levels were also significantly raised in the SEB-exposed mice compared with the PBS-control mice (Fig. 5). In the treated group there was a trend in reduction of IL-2, although this reduction did not reach significance (Fig. 5).

Abatacept treatment of SEB-exposed human PBMCs reduces release of proinflammatory cytokines

The effects of SEB, abatacept, and CTLA4 antagonist ipilimumab were tested in vitro on human PBMCs. Ipilimumab was used as a positive control and also to sensitize human PBMCs to the effects of SEB. After treatments, IFN-γ, IL-2, and TNF-α levels were quantified by flow cytometry and ELISA; median baseline values of unstimulated PBMCs were 2.19, 1.00, and 1.04 pg/ml, respectively. No significant changes in intracellular cytokine levels were observed among the three treatment groups (Fig. 7B). However, when PBMCs exposed to SEB were treated with ipilimumab at 10 μg/ml, levels of TNF-α and IL-2 in the culture medium were significantly raised compared with cells exposed to SEB only (median values 6200 versus 4100 and 4400 versus 3300 pg/ml, respectively, for TNF-α and IL-2; $p < 0.0001$; Fig. 7C). IL-2 production in cultured human PBMCs exposed to SEB was significantly reduced by abatacept treatment when compared with controls (median values 3300 versus 1900 pg/ml, respectively; $p < 0.0001$).

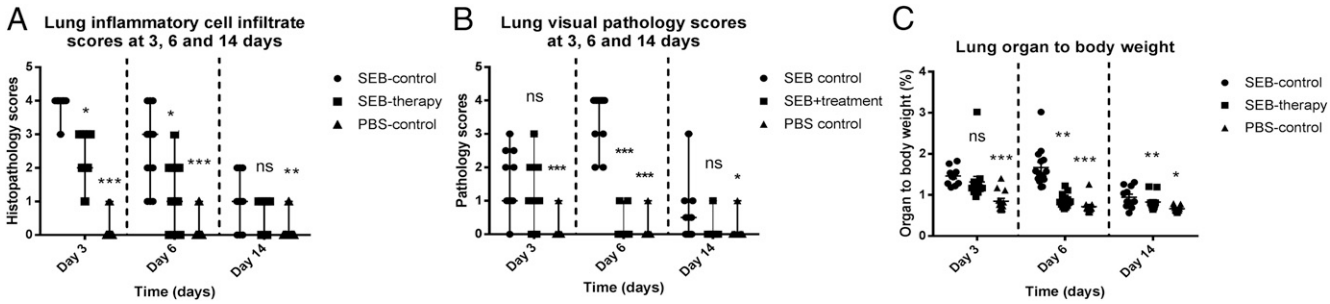

FIGURE 3. Lung pathology scores and organ to body percent weight of lung. Lungs were removed and scored for visible signs of damage (**A**) and histopathology scores (**B**), and subsequently weighed to determine the lung-to-body weight ratio (**C**). Scores ranged from 1 (normal) to 4 (very severe lung pathology/75–100% inflammatory cell infiltrate), as defined in the *Materials and Methods*. Lung visual pathology, cell infiltrate scores, and lung-to-body weight ratio were analyzed by Kruskal–Wallis test with post hoc Dunn multiple comparison test. Visual pathology scores significance for treatment were: day 3, $p = 0.0002$; day 6, $p < 0.0001$; and day 14, $p = 0.0169$. Cell infiltrate scores significance for treatment were: day 3, $p < 0.0001$; day 6, $p < 0.0001$; and day 14, $p = 0.0029$. Lung-to-body weight ratio had significant differences in treatment: day 3, $p < 0.0001$; day 6, $p < 0.0001$; and day 14, $p = 0.0059$. Graphs for lung pathology, histopathology scores, and lung-to-body weight ratio represent median and range. Post hoc analysis using Dunnett multiple comparison are indicated. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. ns, $p > 0.05$.

Discussion

Our findings show that after an i.n. challenge with SEB, a single dose of abatacept is effective at preventing morbidity and lung injury in mice. Abatacept treatment also reduces the production of the proinflammatory cytokines INF-γ and IL-6, both systemically and in lung tissue. The severity of pathology after SEB exposure is heavily dependent upon the route of exposure. SEB is more toxic via inhalation, compared with ingestion, and severe lung damage after exposure to SEB aerosols has been demonstrated (23). Numerous animal models of SEB intoxication have been developed that use survival as a primary outcome for the efficacy of medical countermeasures (24, 25). However, weaponized SEB has potential incapacitating properties for exposed military personnel because of the ED_{50} (the median effective dose of incapacitation) being ~50 times lower than the LD_{50} (26, 27). We therefore chose to develop a sublethal model of i.n. SEB

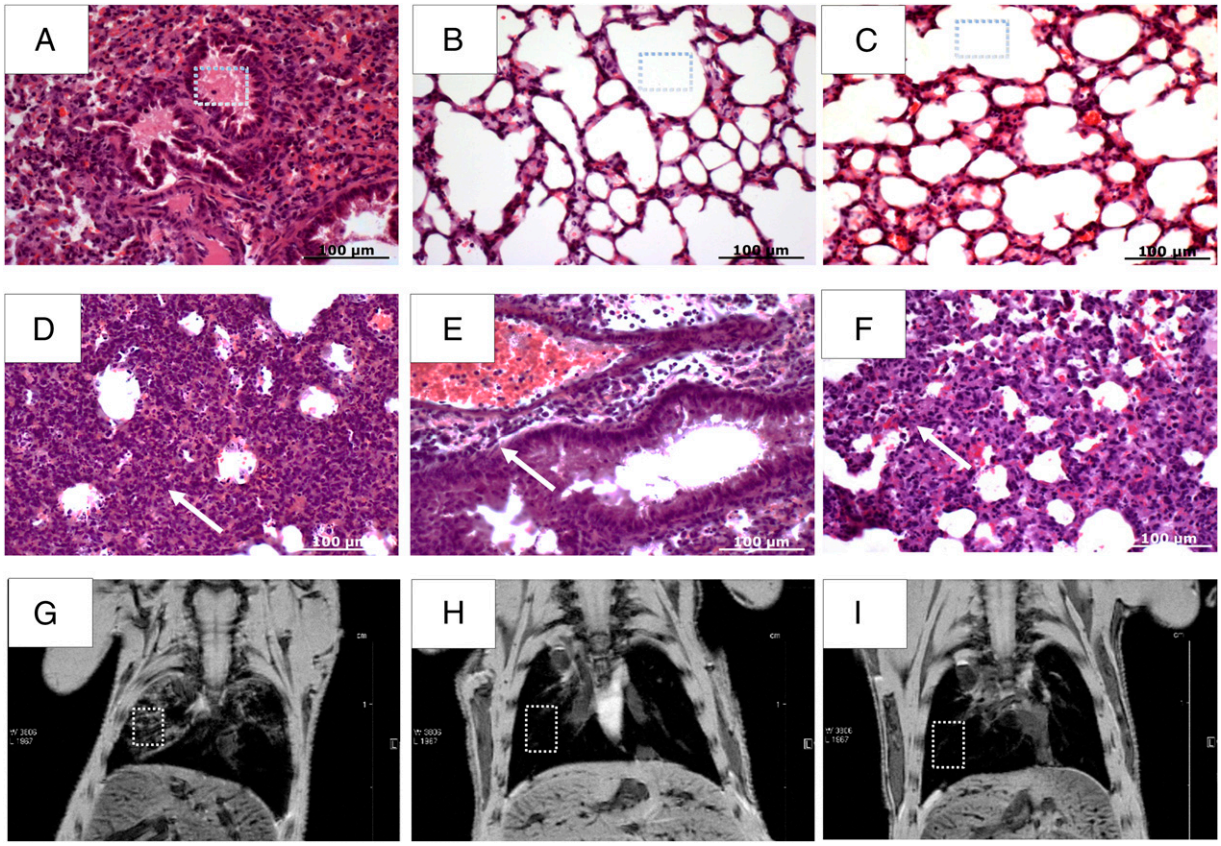

FIGURE 4. Histopathology and MRI of lungs after SEB intoxication in mice. Tissue sections were stained using H&E, at a nominal 5-μm thickness before assessment [SEB treated (**A**, **D**, and **E**); PBS-control (**B**); and SEB after abatacept therapy (**C** and **F**)]. White boxes highlight air space within the lung, and white arrows indicate inflammatory cell infiltrate; SEB lung pathology is predominantly characterized by an acute alveolar inflammatory cell infiltrate. Scale bar, 100 μm. Mice were sacrificed on days 3, and whole-body imagery was performed on SEB-treated (**G**), PBS-control (**H**), or SEB mice treated with abatacept at 8 h (**I**). White boxes indicate lung cavity, whereas gray shading within white boxes indicates the presence of fluid.

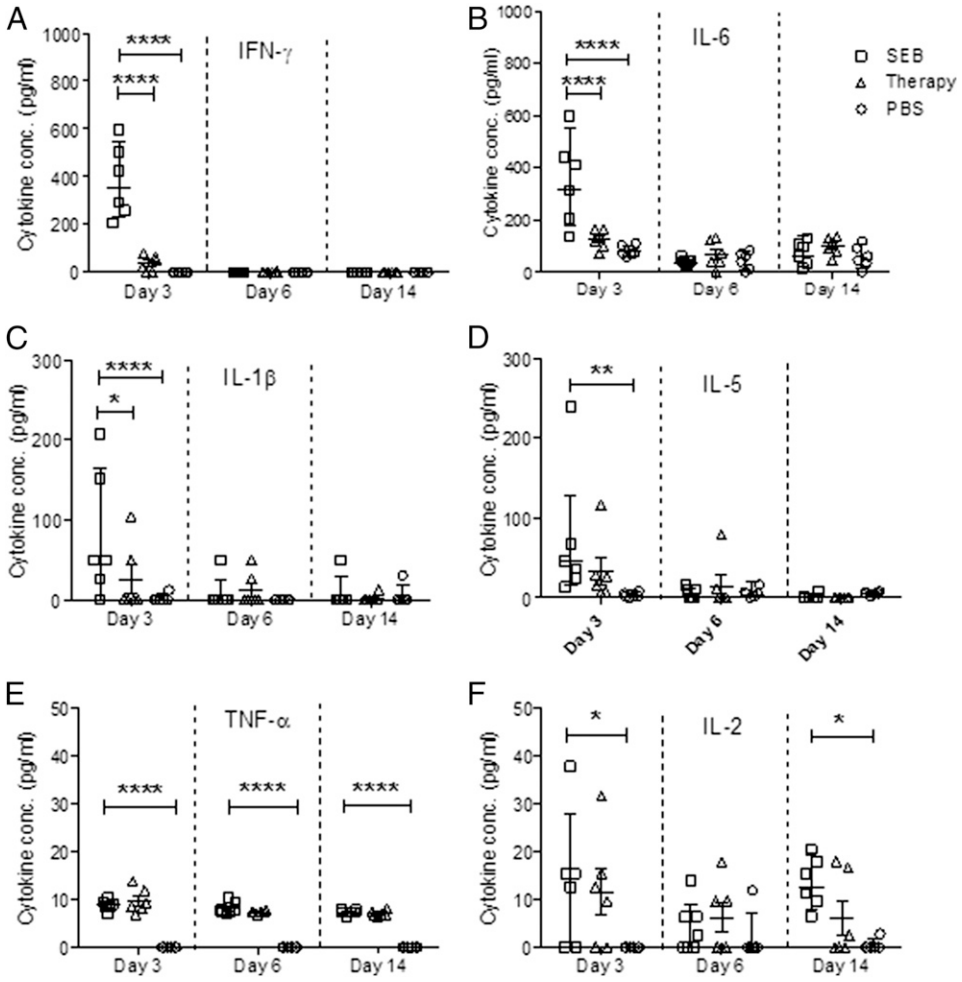

FIGURE 5. Plasma concentrations of cytokines. Plasma concentrations of IFN-γ (**A**), IL-6 (**B**), IL-1β (**C**), IL-5 (**D**), TNF-α (**E**), and IL-2 (**F**) were measured for six animals in the SEB negative control, PBS positive control, and SEB-treated groups at 3, 6, and 14 d post SEB exposure. Two-way ANOVA analysis was performed for cytokines with post hoc Bonferroni multiple comparison test. Significance for treatment was: IFN-γ, $F(2, 46) = 34.75$, $p < 0.0001$; IL-6, $F(2, 46) = 9.61$, $p = 0.0003$; IL-1β, $F(2, 46) = 3.88$, $p = 0.0277$; IL-5, $F(2, 46) = 1.87$, $p = 0.1652$; TNF-α, $F(2, 46) = 346.15$, $p < 0.0001$; and IL-2, $F(2, 46) = 7.66$, $p = 0.0013$. Significance for time was: IFN-γ, $F(2, 46) = 44.37$, $p < 0.0001$; IL-6, $F(2, 46) = 22.23$, $p < 0.0001$; IL-1β, $F(2, 46) = 5.19$, $p = 0.0093$; IL-5, $F(2, 46) = 5.32$, $p = 0.0084$; TNF-α, $F(2, 46) = 8.28$, $p = 0.0008$; and IL-2, $F(2, 46) = 1.41$, $p = 0.254$. Graphs depict mean cytokine values with SEM. Post hoc Bonferroni multiple comparison tests are summarized as: $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$.

administration. The current murine model for SEB intoxication was optimized to maximize severity, but with minimal lethality: SEB exposure induced weight loss close to the 30% limit, at which animals are required to be sacrificed. Although our murine model does not measure the most extreme scenario of SEB exposure leading to mortality, it does reflect the most likely level of disease that would occur in the majority of exposed humans. Another advantage of our murine model is that no additional sensitizing agent is required in conjunction with SEB exposure. Other murine models require potentiating agents such as LPS, actinomycin D, or D-galactosamine to be given in conjunction with SEB because mice are less sensitive to SEB than humans (13, 23). These potentiating agents may mask the efficacy of otherwise effective medical countermeasures (28). D-Galactosamine has been shown to cause liver damage, and LPS is known to induce inflammatory pathways through TLR4 rather than through the APC/T cell immunological synapse, as occurs after exposure of SEB (29, 30). When investigating novel medical countermeasures that work by modulating the host response to SEB, either a previously published model utilizing a humanized MHC class II or a model that uses a high dose of SEB given i.n. is preferred. Safety considerations limit the use of such high concentrations of SEB. Therefore, we chose to develop a challenge model with a dose of SEB leading to a primary end point of severe (30%) weight loss. It should be noted that the rate of SEB absorption via i.n. administration compared with aerosolized exposures is likely to be slower. Additional survival studies in nonhuman primates using previously characterized aerosolization models is required to further determine abatacept efficacy for treatment after SEB exposure (31).

Our findings demonstrate that SEB-dependent morbidity was significantly reduced by the administration of a single dose of abatacept at 8 h post SEB exposure. Previous studies have demonstrated that efficacious therapies for SEB exposure such as lovastatin and SEB antitoxin increased survival of treated mice by 70% if both compounds were given in conjunction with SEB rather than being administered postexposure (32). Prophylactic treatment of mice with abatacept reduced mortality after exposure to the superantigen toxic shock syndrome toxin (TSST) by 75% (33). Treatment of SEB exposure by dexamethasone and rapamycin also reduced mortality (10, 34). However, rapamycin and dexamethasone treatment was given 5 h after exposure and required additional administration at 24 h timepoints for 4 d after intoxication.

By contrast, our study showed that a medical countermeasure is available that can be given as a single dose administered up to 8 h after SEB exposure. SEB exposure in vivo resulted in significant increases in proinflammatory cytokines IL-5, IFN-γ, and IL-6 in plasma and lungs. We show that abatacept treatment significantly reduced the levels of these cytokines that are indicators of systemic inflammation, which regulates T cell responses and the proinflammatory response of eosinophils (35, 36). Pathology induced by SEB is dependent upon T cell and eosinophil activation, and subsequently will lead to a systemic inflammatory response (37). We also observed that SEB-dependent IL-2 expression in human and murine cell cultures was prevented by abatacept. However, this phenomenon was not observed in vivo. The canonical role of IL-2 is to promote T cell expansion (38, 39). This suggests that IL-2 induction in vivo by

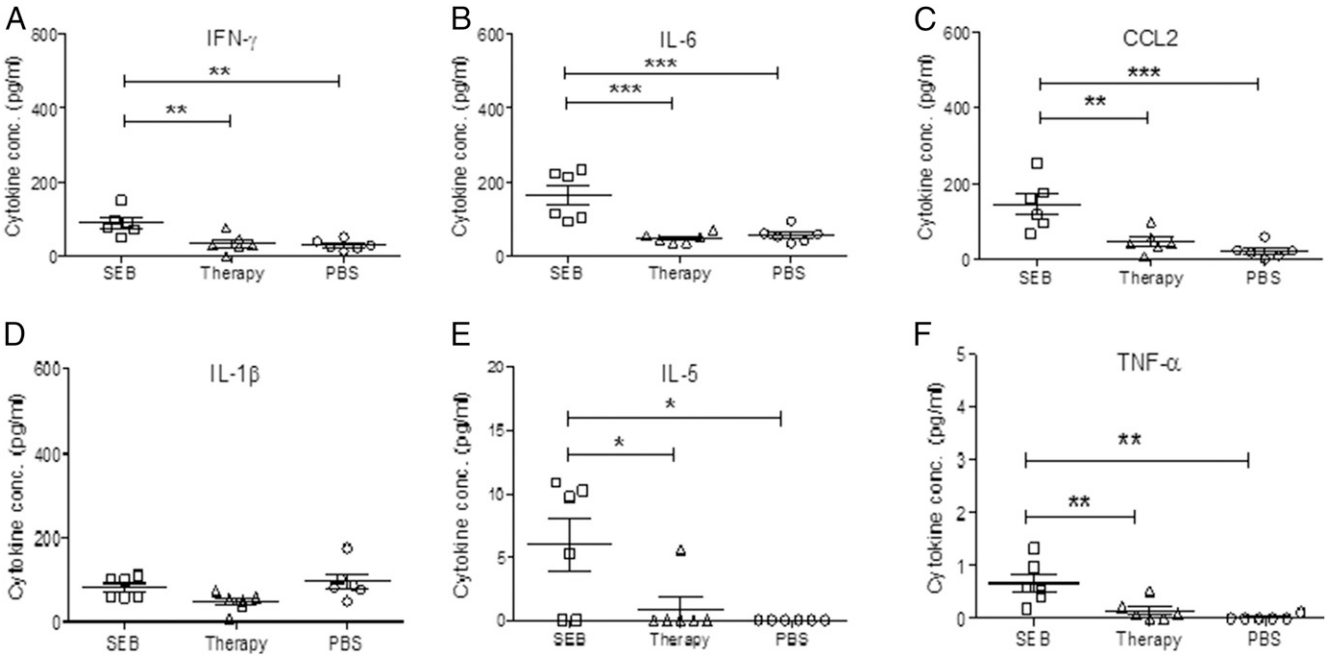

FIGURE 6. Lung tissue extract concentrations of IFN-γ, IL-6, CCL2, IL-1β, IL-5, and TNF-α. Lung tissue extract concentrations of IFN-γ (**A**), IL-6 (**B**), CCL2 (**C**), IL-1β (**D**), IL-5 (**E**), and TNF-α (**F**) were measured. One-way ANOVA analysis was performed for cytokines with post hoc Dunnett multiple comparison test. Significance for treatment was: IFN-γ, $F(2, 15) = 10.04$, $p = 0.0017$; IL-6, $F(2, 15) = 14.95$, $p = 0.0003$; CCL2, $F(2, 15) = 13.16$, $p = 0.0005$; IL-1β, $F(2, 15) = 3.729$, $p = 0.0485$; IL-5, $F(2, 15) = 6.120$, $p = 0.0114$; and TNF-α, $F(2, 15) = 10.29$, $p = 0.0015$. Significant difference graphs depict mean cytokine values with SEM. Post hoc Dunnett multiple comparison test are summarized as: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

SEB has peaked before day 3, and indeed Krakauer et al. (28) demonstrated a significant increase in IL-2 expression up to 24 h post exposure, and this may have been missed in sera collected on day 3. Previous studies have demonstrated that IL-1β is predominately produced by monocytes after superantigen exposure (40). Reduction of IL-1β levels after abatacept treatment both in vitro and in vivo indicates the drug also reduces APC activation. TNF-α levels were significantly reduced by abatacept in murine PBMCs in vitro; however, murine plasma levels of TNF-α remained high at days 3, 6, and 14 after SEB exposure, and these were not affected by abatacept treatment. In human PBMCs, abatacept did not significantly reduce TNF-α production in vitro, whereas ipilimumab, an activator of the immune response and an antagonist of CTLA4, did significantly increase TNF-α production. Thus, modifications of the immune synapse can affect TNF-α secretion levels. Our findings show that weight loss and clinical signs remain unaffected in SEB-treated mice after abatacept therapy. We have demonstrated changes in TNF-α in our studies. However, mice treated with abatacept do not show reduced systemic TNF-α. Importantly, this study indicates that plasma TNF-α levels are not an appropriate biomarker for determining murine responses with abatacept treatment. Furthermore, it implies that TNF-α may not play a critical role in the clinical outcomes in subjects exposed to sublethal levels of superantigens, at least when B7 receptor antagonists are used (38). However, at lethal levels of superantigen exposure, TNF-α antagonist may have utility as a combined therapeutic. It should also be noted that the immunological response to SEB was less obvious in human PBMCs to the murine splenocyte assay and in in vivo studies. Although this difference may be because of species-specific responses, the observed differences were likely because of the cellular composition of the two in vitro models and mouse model used in our study. Although others have found that TNF-α expression is critical to SEB immunopathology (28), our observations suggest that IFN-γ and IL-6 in plasma samples may be better biomarkers for monitoring patient responses with abatacept treatment. This approach should be confirmed in humans.

Abatacept is a commercially licensed drug for the treatment of rheumatoid arthritis and is considered safe (41). The therapeutic doses of abatacept used in our studies are within tolerable dose ranges for rheumatoid arthritis treatment in humans. Abatacept prevents T cell activation by blocking the B7/CD28 receptors, which is preventing the costimulatory signal in the presence of superantigens such as SEB (42). Thus, abatacept may prevent pathology from a broad range of superantigens; for example, abatacept has been previously shown to prevent TSST-dependent mortality in mice (33). In addition to preventing SEB and TSST pathology, it is likely that abatacept will have efficacy at preventing other superantigen-related disease. Indeed, other superantigens produced by *S. aureus*, such as staphylococcal enterotoxins A to G, stimulate T cells and release inflammatory cytokines similar to the cytokine storm observed after SEB exposure (42). This suggests a utility of abatacept to treat SEB effectively, as well as provide a broad range of efficacy to other staphylococcal enterotoxins and TSST-1. Furthermore, recent research has indicated that superantigens selectively anergize T cells to protect the pathogen from the host immune response (43). Thus, abatacept and therapeutic agents targeting the costimulatory pathway may be able to aid treatment of a broad range of bacterial and viral infections.

In conclusion, we have demonstrated that abatacept prevents severe pathology caused by SEB exposure in a murine model, and that a single dose of abatacept given i.v. up to 8 h postexposure is effective. Abatacept prevents lung damage and reduces local and systemic inflammation. The reduction of IL-6 and IFN-γ levels is indicative of the host response to therapy and a potential correlate of protection against SEB intoxication. Further studies on new

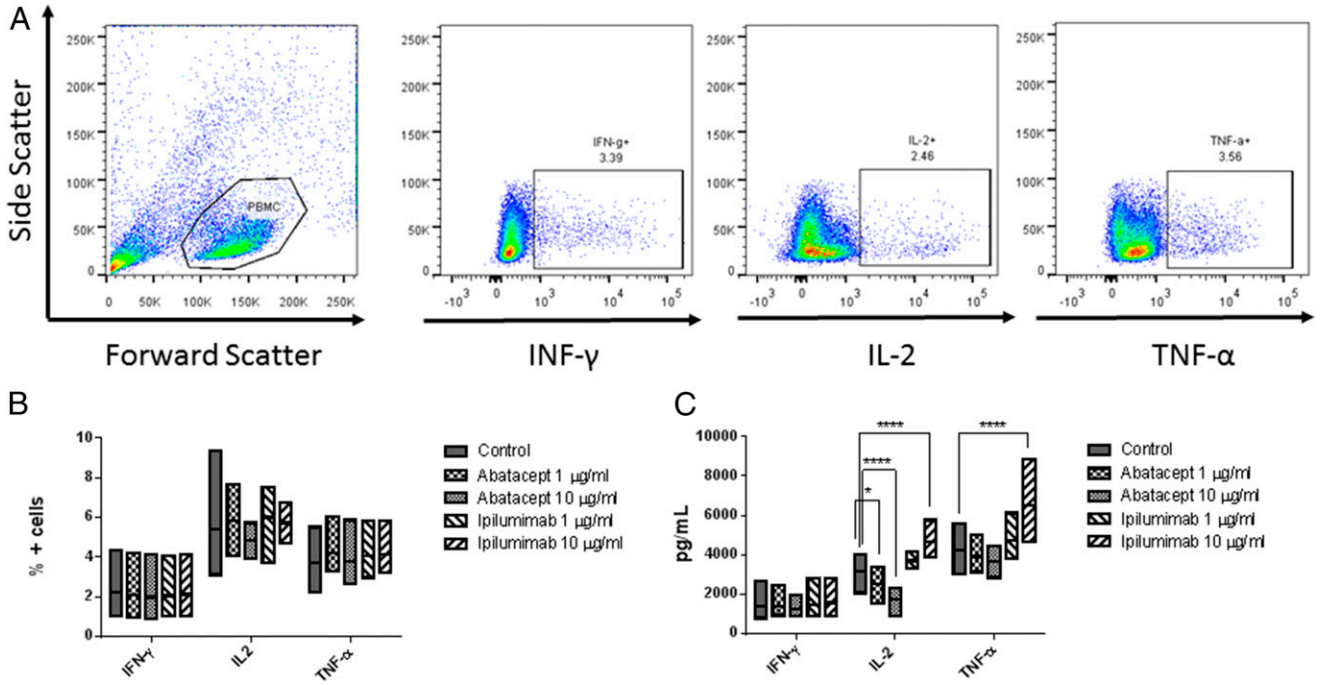

FIGURE 7. Human PBMC stimulation assay for intracellular and secreted cytokine expression. Human PBMCs isolated from volunteers and treated with SEB in combination with PBS (control), abatacept (1 and 10 μg/ml of treatment), or ipilimumab (1 and 10 μg/ml of CTLA4 pathway antagonist). Intracellular cytokine production was determined by flow cytometry for TNF-α, IL-2, and IFN-γ ($n = 3$ per group). Representative gating strategy (**A**) and pooled data are shown (**B**). Supernatants were analyzed by ELISA to determine secretion TNF-α, IL-2, and IFN-γ ($n = 4$ per group) (**C**). Two-way ANOVA with Dunnett multiple comparison test. Significance for intracellular cytokine treatment was $F(2, 6) = 3.25$, $p = 0.6805$ and for ELISA was $F(2, 9) = 14.31$, $p = 0.0016$. Bar graphs represent median 95% CI. Post hoc Dunnett multiple comparison test are summarized as: $^{*}p < 0.05$, $^{****}p < 0.0001$.

compounds that bind to the B7 receptor, such as the CD28 mimetic AB103, would be of interest (44). Further research is needed using nonhuman primate lethal and sublethal aerosolized exposure models to demonstrate the efficacy of abatacept, to prevent pathology from superantigen exposure.

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Disclosures

The authors have no financial conflicts of interest.

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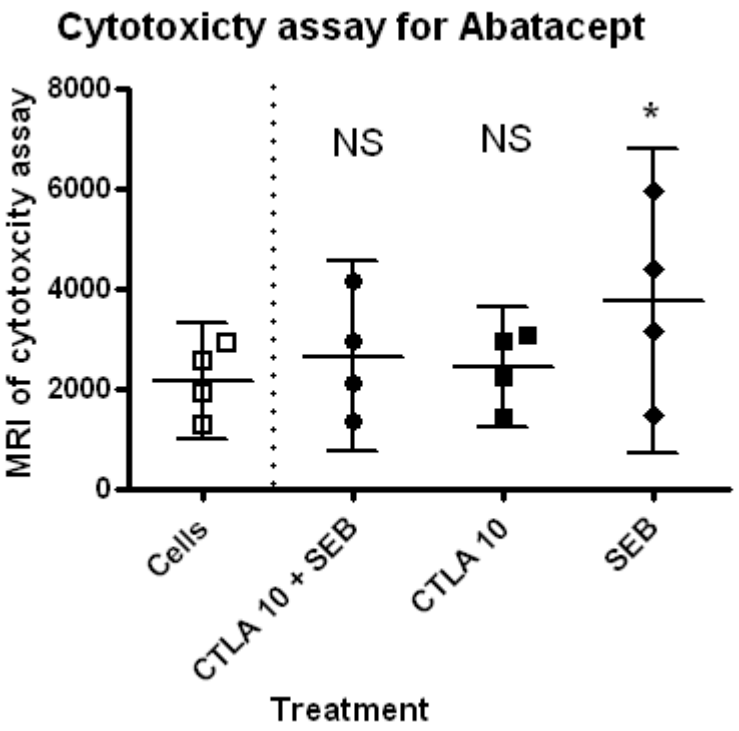
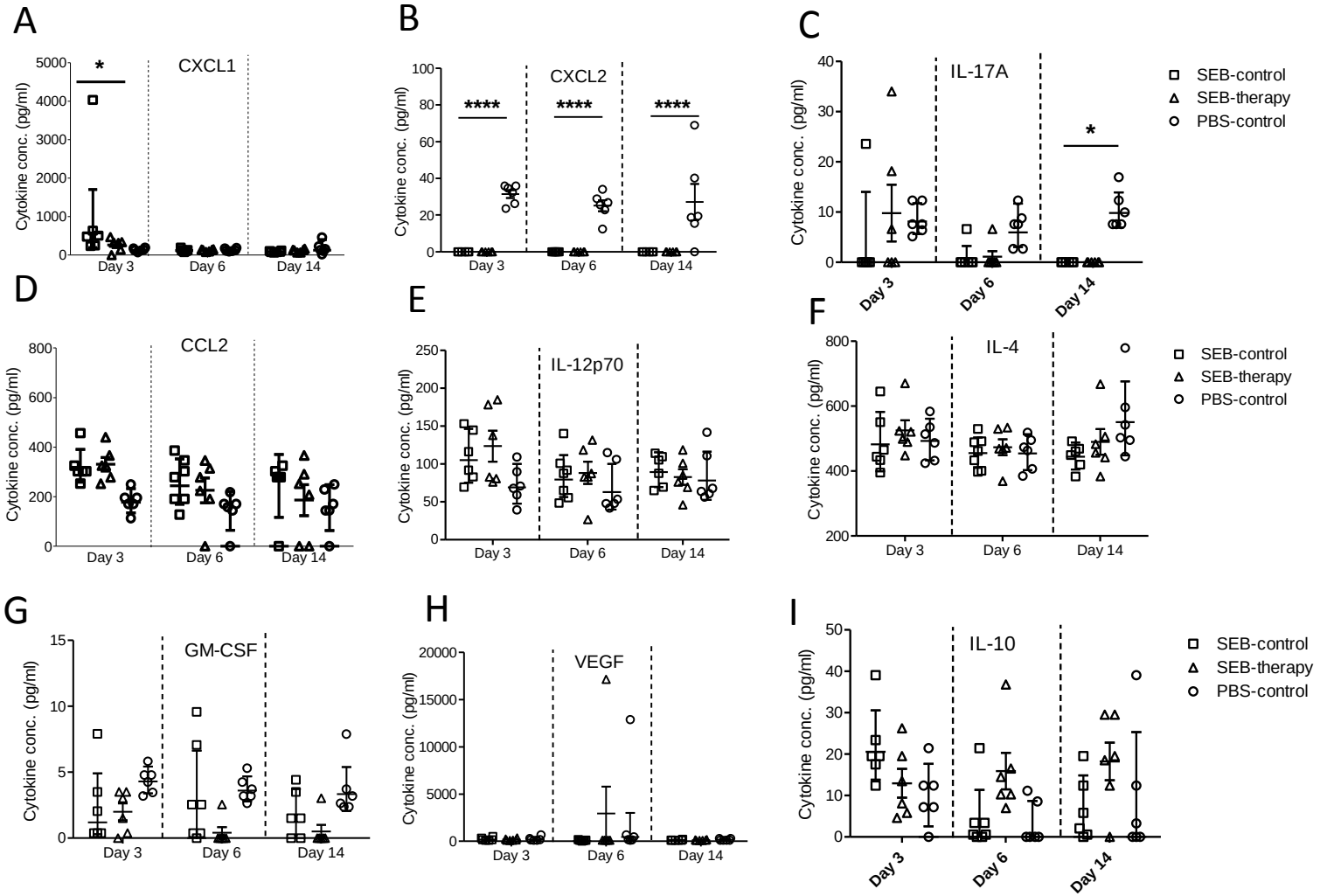
Supplementary Figure 1



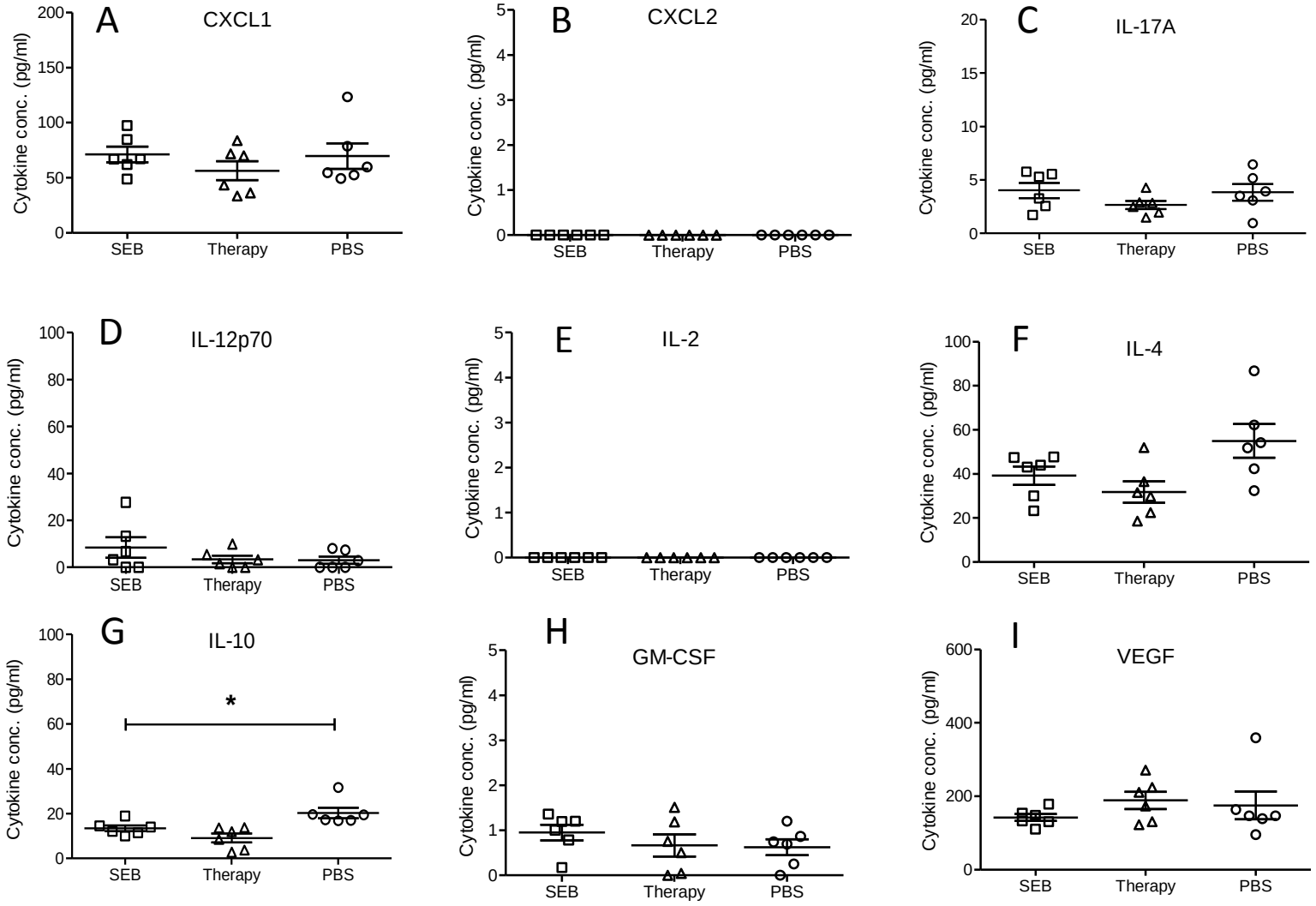
Supplementary Figure 1. Murine splenoctye cytotoxicity assay. Isolated splenocytes were treated with PBS (negative control), SEB (positive control) or with SEB and abatacept. After 24hr cell cytotoxicity assays were performed. The ability of abatacept to inhibit the cytotoxic effect of SEB was investigated using a Promega MultiTox-Flour Multiplex Cytotoxicity Assay according to the manufacturer's instructions (Promega, USA). Cell toxicity assays were performed on 4 separate occasions using splenocytes only; splenocytes treated with abatacept; splenocytes exposed to SEB and splenocytes exposed to SEB and treated with abatacept. Dead cell numbers were determined using fluorescence at 485Ex / 535Em 3hr after addition of the 5x MultiFlour 5 reagent prepared according to the manufacturers guidance. Repeated measures ANOVA showed that the effect of treatment was significant for proliferation $F(3, 9)=4.989$, $p=0.0262$. Means of 5 replicates and SEM are represented Post hoc analysis using Dunnett's multiple comparison are indicated ns = $p>0.05$; * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$; **** = $p<0.0001$.

Supplemental Figure 2



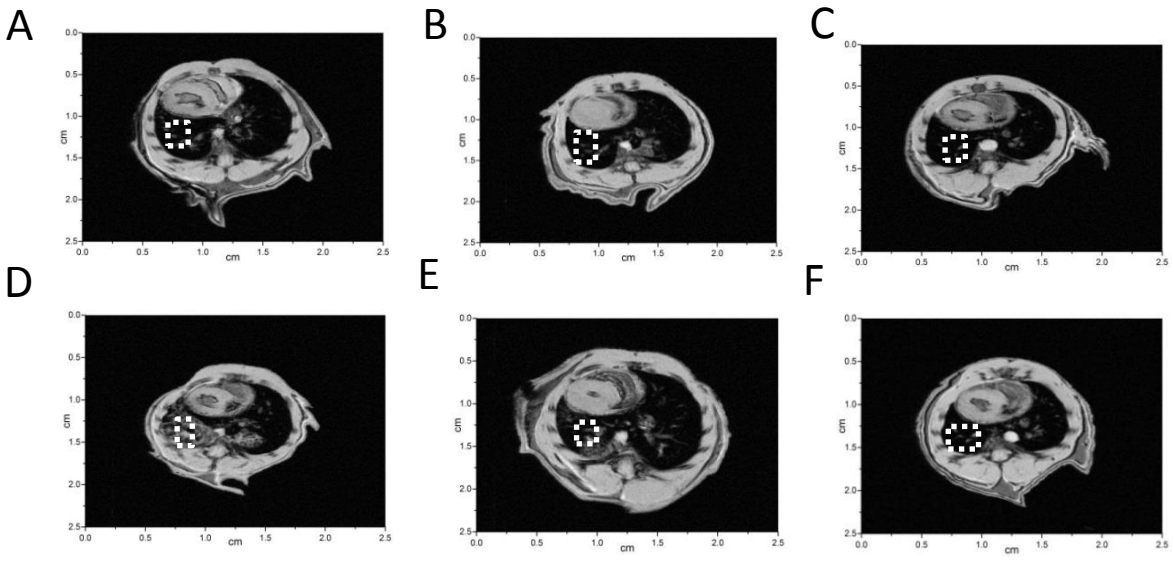
Supplemental Figure 2. Plasma concentrations of CXCL1, CXCL2, IL-17A, CCL2, IL-12p70, IL-4, GM-CSF, VEGF and IL-10. Plasma concentrations CXCL1, CXCL2, IL-17A, CCL2, IL-12p70, IL-4, GM-CSF, VEGF and IL-10 (**A to I respectively**) were measured for 6 animals in the SEB negative control group, PBS positive control and SEB treated groups at day 3 days post SEB exposure. Two-way ANOVA analysis was performed for cytokines with post hoc Bonferroni multiple comparison test. Significance for treatment was CXCL1 $F(2, 46)=1.60$, $p=0.2134$; CXCL2 $F(2, 46)=65.15$, $p<0.0001$; IL-17A $F(2, 46)=6.47$, $p=0.0033$; CCL2 $F(2, 46)=6.85$, $p=0.0025$; IL-12p70 $F(2, 46)=2.62$, $p=0.0839$ IL-4 $F(2, 46)=1.48$, $p=0.2383$; GM-CSF $F(2, 46)=9.05$, $p=0.0005$; VEGF $F(2, 46)=0.5$, $p=0.5645$ and IL-10 $F(2, 46)=3.32$, $p=0.0451$. Significance for time was CXCL1 $F(2, 46)=2.98$, $p=0.0608$; CXCL2 $F(2, 46)=0.29$, $p=0.7460$; IL-17A $F(2, 46)=2.97$, $p=0.0613$; CCL2 $F(2, 46)=3.75$, $p=0.0309$; IL-17p70 $F(2, 46)=2.07$, $p=0.1375$; IL-4 $F(2, 46)=1.62$, $p=0.209$; GM-CSF $F(2, 46)=0.88$, $p=0.4204$; VEGF $F(2, 46)=2.05$, $p=0.1403$ and IL-10 $F(2, 46)=2.59$, $p=0.0858$. Graphs depict mean cytokine values with SEM. Post hoc Bonferroni multiple comparison test are summarised as ns = $p>0.05$; * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$; **** = $p<0.0001$

Supplemental Figure 3



Supplemental Figure 3. Lung tissue extract concentrations of CXCL1, CCXL2, IL-17A, Il-12p70, IL-2, IL-4, IL-10, GMCSF and VEGF. Lung tissue extract concentrations CXCL1, CXCL2, IL-17A, IL-12p70, IL-2, IL4, IL-10, GM-CSF and VEGF (**A to I respectively**) were measured for 6 animals in the SEB negative control group, PBS positive control and SEB treated groups at day 3 days post SEB exposure. One-way ANOVA analysis was performed for cytokines with post hoc Dunnett's multiple comparison test. Significance for treatment was CXCL1 $F(2, 15)=0.7569$, $p=0.4862$; IL-17A $F(2, 15)=1.33$, $p=0.2932$; IL-12p70 $F(2, 15)=1.180$, $p=0.3342$; IL-4 $F(2, 15)=4.259$, $p=0.0343$; IL-10 $F(2, 15)=8.632$, $p=0.0032$; GM-CSF $F(2, 15)=0.7689$, $p=0.4809$ and VEGF $F(2, 15)=0.8173$, $p=0.4603$. Graphs depict mean cytokine values with SEM. Post hoc Dunnett's multiple comparison test are summarised as ns = $p>0.05$; * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$; **** = $p<0.0001$

Supplemental Figure 4



Supplementary Figure Supplemental Figure 4. Magnetic Resonance Imaging of lungs following SEB intoxication in mice. Groups of mice (3 per group) were dosed with SEB i.n. and PBS intravenously (**A and D**), PBS i.n. and PBS intravenously (**Figure B and E**) or SEB i.n. and abatacept (10mg/Kg mouse) (**C and F**). Intravenous PBS or abatacept was administered 8hr post SEB i.n. challenge. Mice were sacrificed on days 3 whole body imagery was performed. Representative MRI scans for each group in the study are shown (**A, B & C**), where white boxes indicate the lung cavity and grey shading indicates fluid accumulation in the lungs.