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Surfactant Protein A Integrates Activation Signal Strength To Differentially Modulate T Cell Proliferation

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Pulmonary surfactant lipoproteins lower the surface tension at the alveolar–airway interface of the lung and participate in host defense. Previous studies reported that surfactant protein A (SP-A) inhibits lymphocyte proliferation. We hypothesized that SP-A–mediated modulation of T cell activation depends upon the strength, duration, and type of lymphocyte activating signals. Modulation of T cell signal strength imparted by different activating agents ex vivo and in vivo in different mouse models and in vitro with human T cells shows a strong correlation between strength of signal (SoS) and functional effects of SP-A interactions. T cell proliferation is enhanced in the presence of SP-A at low SoS imparted by exogenous mitogens, specific Abs, APCs, or in homeostatic proliferation. Proliferation is inhibited at higher SoS imparted by different doses of the same T cell mitogens or indirect stimuli such as LPS. Importantly, reconstitution with exogenous SP-A into the lungs of SP-A−/− mice stimulated with a strong signal also resulted in suppression of T cell proliferation while elevating baseline proliferation in unstimulated T cells. These signal strength and SP-A–dependent effects are mediated by changes in intracellular Ca2+ levels over time, involving extrinsic Ca2+-activated channels late during activation. These effects are intrinsic to the global T cell population and are manifested in vivo in naive as well as memory phenotype T cells. Thus, SP-A appears to integrate signal thresholds to control T cell proliferation. The Journal of Immunology, 2012, 188: 957–967.
gococcal disease and respiratory syncytial virus (22–26). Naive and memory T cells continually migrate through the lung and are present in large numbers in the alveolar region and the lamina propria of the bronchi (27, 28). The SP-A-mediated suppression of T cell proliferation seems surprising in light of the importance of these cells in modulating both adaptive and innate immune responses. Hence, we hypothesized that SP-A may play a differential role in modulating T cell activation depending upon the strength of the activating signal. In the current study, we used a variety of different stimuli, including APC–MHC–peptide interactions, mitogens, and pharmacological agents to demonstrate dose-dependent opposing responses of SP-A interactions in both mouse and human T cells ex vivo and to establish a possible mechanism for the observed effects. Additionally, we demonstrate similar phenotypes in vivo using baseline maintenance proliferation (usually driven by weakly agonist self-peptides) and exogenously added stimuli to mimic weak and strong signals, respectively.

Materials and Methods

Mice and human samples

SP-A−/− mice were generated as previously described (29) and backcrossed to C57BL/6N background for 12 generations. Wild-type (WT) mice were obtained from littersmates in heterogeneous breedings or from Charles River Laboratories (Wilmington, MA). Mice aged 8–12 wk were used for all experiments, which were performed independently with both male and female mice. All mice were housed in a barrier facility, and all procedures were performed according to local and National Institutes of Health guidelines and were approved by the Duke University Institutional Animal Care and Use Committee. Human blood samples from healthy volunteers were collected in BD Vacutainer tubes per institutional review board guidelines and used for isolation of T cells as described later.

SP-A preparation and analyses

SP-A was purified from the lung lavage fluid of patients with alveolar proteinosis as described previously (30). Briefly, the lavage fluid was initially treated with butanol to extract the SP-A. The resulting pellet was then sequentially solubilized in the detergent octylglucoside and 5 mM 2-mercaptoethanol, penicillin–streptomycin (100 U/ml), and 2 mM t-glutamine (all from Life Technologies Invitrogen, Grand Island, NY) was used as the primary culture medium (complete RPMI). All Abs used for activation or flow cytometry were obtained from BioLegend, eBioscience, or BD Pharmingen. Low-endotoxin azide-free anti-CD3 (clone UCHT1 human, 145-2C11 mouse) and anti-CD28 (clone CD28.2 human, 145-2C11 mouse) and anti-CD25, CD44, CD62L, and FR4 to distinguish specific CD8+ cells purified using the Lympholyte Pure system (Cedarlane Labs). T cell purities averaged 96–98% for pan-T cells and 99% for highly purified subpopulations. Bone marrow-derived dendritic cells (BMDCs) were generated using marrow from the tibia and femur bones of mice, which was harvested, washed, and cultured in complete RPMI-supplemented 5% GM-CSF conditioned medium for 6 d (31). Loosely attached cells were harvested and negatively selected with biotinylated Gr-1 Abs (BD Pharmingen) and streptavidin paramagnetic microbeads (Miltenyi Biotec).

Ex vivo activation assays and ELISA

Purified, freshly isolated T cells (~100,000) were incubated under different experimental conditions in 96-well round-bottom plates in complete RPMI. Each well received 0.8 μCi [3H]thymidine (6.7 Ci/mmoll; MP Bio), and cells were incubated for another 15 h. Incorporated radioactivity (as an indicator of proliferation) was measured by liquid scintillation using a Cerenkov counter. Viable cell number was determined by trypan blue exclusion. Also, proliferation was assessed in ELISA by the measurement of TNF-α, IFN-γ, IL-2, or IL-10 using the standard ELISA kit. All ELISAs were performed using eBioscience Ready-Set-Go kits according to the manufacturer’s instructions. The amount of IL-2 in culture supernatants was determined using a four-parameter fit equation derived from values obtained from known amounts of standard cytokine after background subtraction (BMG Labtech/Optima Software).

In vivo studies

Two methods were used to track cell division in vivo. In some experiments, BrdU was instilled in mice to track proliferation. Mice were initially injected i.p. with 200 μl 4 mg/ml BrdU (Sigma). Carrier cages were fitted with deprivation caps, and mice were allowed access to 0.8 mg/ml BrdU in 4 or 5% sucrose-supplemented drinking water ad libitum (32). For some experiments, purified SP-A (unlabeled or AF647 labeled, 15 μg/50 μl dose) and/or PMA plus ionomycin (PMA+I; 250 ng, 250 nM) was administrated intratracheally to determine whether exogenous SP-A could modify the lung T cell phenotype. For CFSE tracking studies, a combination of SP-A, PMA+I, LPS (Escherichia coli 055:B5), Sigma), or CFSE (Molecular Probes) administration was performed by an oropharyngeal aspiration method. Briefly, mice were anesthetized by isoflurane and suspended by their upper incisors with wire on an ~70° inclined frame. The tongue was gently extended, and SP-A/PMA+I/LPS/CFSE solution or USP saline (Sigma) was pipetted into the mouth. Brief occlusion of the nose forced the animal to inhale through the mouth, thereby aspirating the solution into the respiratory tract in one or two breaths. Animals were subsequently removed from the support and observed closely until fully recovered from anesthesia.

Flow cytometry and fluorimetry

Flow cytometry was performed using a BD LSRII (BD Biosciences, San Jose, CA) at the Duke University Human Vaccine Institute Flow Cytometry Core, which is supported by National Institutes of Health Award AI-51445. Multiple panels of multicolor surface staining on T cells were set up using anti-CD3, CD4, CD8, CD25, CD44, CD62L, and FR4 to distinguish specific cell populations. For intracellular staining using Foxp3, IL-4, or anti-BrdU Abs, cells were washed well after surface staining. Fixed in 10% neutral buffered formalin, and permeabilized with 0.3% saponin in HBSS plus 0.5% FBS for BrdU or Foxp3 staining or 0.1% Triton X-100 in HBSS plus 0.5% FBS for cytokine staining. Brefeldin A for intracellular cytokine staining was obtained from eBioscience, and treatment was performed for ~5–6 h prior to staining. Because the anti-BrdU Ab was conjugated to biotin, an additional intracellular staining with either streptavidin–AF647 (Molecular Probes) or streptavidin–AF488 (BioLegend) was performed. All FACS data were analyzed with FlowJo software (Tree Star, Ashland, OR) using default or CFSE-specific plug-ins. Free Ca2+ levels in the cytoplasm were measured by loading the cells with 1 μM fluo-4 aceto-
SP-A enhances T cell proliferation with low-grade stimulation. The fluo-4–direct buffer plus probenicid helps reduce fluorescence caused by dye leakage out of the cells.

Statistics
Data are expressed as mean ± SEM unless otherwise indicated. Statistical significance was tested with an unpaired Student’s t test or non-parametric ANOVA using Prism 4b (GraphPad Software, La Jolla, CA). Statistically significant differences were determined by p < 0.05.

Results
**SP-A**−/− mice display inverse T cell proliferation profiles compared with WT mice in response to low- and high-dose LPS in vivo
To determine the functional role of SP-A in regulating T cell responses to low and high signal strengths, we used the broad-spectrum response invoked by LPS to activate T cells in the lung. The total number of cell divisions in vivo was tracked using CFSE. CFSE is a vital dye that binds covalently to lysine residues of intracellular proteins. Excess, unbound dye is typically cleared in 12–15 h. Because previous studies have determined that it is nontoxic, a high-dose CFSE treatment (125 μM instead of the 0.5–μM dose used for prelabeling cells in vitro) was performed to label cells in vivo. As the cell divides, CFSE fluorescence is reduced by half with every division, giving a range of intermediate fluorescence intensities in actively dividing cells. Thus, the presence of intermediate levels of fluorescence of CFSE (CFSE-int) indicates the occurrence of cell division. WT and SP-A−/− mice were oropharyngeally instilled with different concentrations of LPS, in conjunction with CFSE, in Fig. 1A and 1B. CFSE-int gates were defined based on CFSE-treated lung lymphocytes (undivided, CFSE-hi) and splenocytes (unlabeled, CFSE-lo) isolated from WT saline-treated mice. Less than 0.5% of T cells from spleens of saline-treated mice were present in the CFSE-lo gates, as labeling was performed in the lung. Total CD3+ T cells in the lung remain nearly identical at baseline, with no significant differences. At very low concentrations of LPS (10 ng/mouse), the profile is reversed in SP-A−/− mice and 0.794 ± 0.222% of T cells showed a reduction in homeostatic proliferation in the lung in the SP-A−/− mice (Fig. 2A), whereas no differences were observed in previously activated cells (Fig. 2B). Central and effector memory cells (Fig. 2C, 2D) tended to show reduced proliferation in the absence of SP-A, although statistically significant differences were only seen in effector memory cells. Thus, SP-A enhances T cell proliferation with weak or homeostatic maintenance signals. These results suggest that SP-A affects multiple T cell phenotypes even under normal homeostatic conditions and not just in response to inflammatory or allergic stimuli.

**SP-A specifically enhances or suppresses human and mouse T cell activation**
To determine whether human T cells also have a signal strength-dependent response with SP-A, T cells from multiple normal subjects were purified from PBMCs and activated with anti-CD3 in a variety of different doses and presentations to mimic a range of different intensities of TCR stimulation (SoS, Fig. 3A). Plate-bound anti-CD3 immobilized onto tissue culture plates forms a solid support matrix that allows for maximal cross-linking and elicits a stronger SoS compared with soluble anti-CD3, which is flexible and generates a weaker signal (34, 35). The effect of signal strength on cell proliferation was determined by measuring incorporation of [3H]thymidine by actively dividing cells. Increased incorporation corresponds to increased cpm as presentation is switched to a plate-bound format, with comparable this baseline T cell proliferation using BrdU labeling in vivo. BrdU incorporation was tracked over 4 d in both WT and SP-A−/− mice without any exogenous activation, and subpopulations of T cells from the lung were analyzed as described in Materials and Methods. BrdU incorporation by the analyzed cell types in the spleen from WT mice in each experiment was used to normalize the BrdU levels in lung cell populations. Naive phenotype CD3+ T cells showed a reduction in homeostatic proliferation in the lung in the SP-A−/− mice (Fig. 2A), whereas no differences were observed in previously activated cells (Fig. 2B). Central and effector memory cells (Fig. 2C, 2D) tended to show reduced proliferation in the absence of SP-A, although statistically significant differences were only seen in effector memory cells. Thus, SP-A enhances T cell proliferation with weak or homeostatic maintenance signals. These results suggest that SP-A affects multiple T cell phenotypes even under normal homeostatic conditions and not just in response to inflammatory or allergic stimuli.

**SP-A−/− mice have reduced levels of memory T cell proliferation at baseline**
Because SP-A appears to have differential effects depending on the signal strength in vivo, we continued to investigate the significance of SP-A–mediated change from a stimulatory to an inhibitory response. Basal T cell proliferation in vivo is driven by weak agonist self-peptide–MHC interactions. To elaborate further the effects of SP-A in cells with weak signals and to confirm our LPS-mediated low strength of signal (SoS) observations, we examined this baseline T cell proliferation using BrdU labeling in vivo. BrdU incorporation was tracked over 4 d in both WT and SP-A−/− mice without any exogenous activation, and subpopulations of T cells from the lung were analyzed as described in Materials and Methods. BrdU incorporation by the analyzed cell types in the spleen from WT mice in each experiment was used to normalize the BrdU levels in lung cell populations. Naive phenotype CD3+ T cells showed a reduction in homeostatic proliferation in the lung in the SP-A−/− mice (Fig. 2A), whereas no differences were observed in previously activated cells (Fig. 2B). Central and effector memory cells (Fig. 2C, 2D) tended to show reduced proliferation in the absence of SP-A, although statistically significant differences were only seen in effector memory cells. Thus, SP-A enhances T cell proliferation with weak or homeostatic maintenance signals. These results suggest that SP-A affects multiple T cell phenotypes even under normal homeostatic conditions and not just in response to inflammatory or allergic stimuli.

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amounts of anti-CD3 (Fig. 3B). A further increase in thymidine incorporation was observed when exogenous costimulation imparted by anti-CD28 is present, in both soluble and plate-bound anti-CD3–stimulated conditions (Fig. 3C, 3D). As previously reported (17), the addition of purified human SP-A into cultures activated with a relatively high SoS resulted in a suppression of T cell proliferation. However, when SP-A was added to cells activated with the lowest SoS, we observed a consistent 30–50% enhancement in cell proliferation (weak signal, no costimulation, Fig. 3A). These results were further corroborated by cell cycle analyses (Supplemental Table I), where we observed an SP-A–induced increase in proliferation with soluble anti-CD3 and inhibition of proliferation with plate-bound anti-CD3. The stronger signal imparted by anti-CD28–mediated costimulation invariably resulted in inhibition of proliferation in the presence of SP-A.

Neither the structurally similar protein C1q nor a mouse Ig control exhibited SoS-dependent effects on mouse T cell proliferation using low and high doses of the pharmacological mitogens PMA and ionomycin (Fig. 4A), suggesting that the effects observed with SP-A are not shared even by a structurally homologous protein. The other immune surfactant collectin, SP-D, also showed no SoS-dependent effects on proliferation, inhibiting cell division at both low and high SoS, consistent with previous observations (36). These results suggest that SP-A acts as a putative costimulator to specifically enhance cell proliferation in both human and mouse T cells stimulated with weak activation signals.

**SP-A affects T cell responses with multiple activation models to modulate signal strength in vitro**

To elucidate the role of signal strength and SP-A in T cell activation, we used the pharmacological agents PMA+I. These agonists provide a defined activation signal that stimulates two key signal transduction pathways in T cells (PI3K–NF-κB and Ca2+–NFAT, respectively), and their concentrations can be titrated to show a linear response. These experiments demonstrated a switch from a SP-A–mediated stimulatory response at low PMA+I to
FIGURE 4. SP-A–mediated modulation of T cell proliferation is specific and depends upon the strength of activating signal. A–C, Primary mouse T cells were purified and activated with the indicated proteins (20 μg/ml) and defined amounts of PMA+I or anti-CD3 with or without anti-CD28 for 50 h with a [³H]thymidine pulse during the last 15 h. Data are represented as mean cpm (A) or as fold difference normalized to the lowest dose of PMA+I (B) or soluble anti-CD3 alone (C) and are representative of three to six independent experiments with pooled cells obtained from five to nine mice per experiment. A total of nine different concentrations of PMA and ionomycin (I) covering a range of defined signal strengths are depicted. *p < 0.05, **p < 0.005 (compared with respective no SP-A treated condition). D, Purified T cells from OVA-primed mice were activated with different amounts of OVA and varying ratios of OVA-primed BMDCs to establish different levels of signaling. Ratios depicted include those with 10,000 BMDCs for 100,000 purified T cells (1:10) or 3,350 BMDCs for 100,000 T cells (1:30). Control Ig or exogenous SP-A (20 μg/ml) treatment was performed as indicated in the plots. The activation culture was performed for a total of 65 h, with a [³H]thymidine pulse during the last 15 h. Data are represented as mean fold difference to corresponding ratios of nonprimed BMDCs and T cells without OVA. An increased cpm in the control conditions corresponds to greater signal strength, as indicated by the scale bar on the plots. Data are representative of three to five independent experiments with pooled cells obtained from three to four mice per experiment. *p < 0.05, **p < 0.02 (compared with respective control Ig-treated conditions).
a suppressive response as the concentration of PMA+I was increased (Fig. 4B), consistent with SoS-dependent effects of SP-A observed with direct stimulation of the TCR (Fig. 3). Signal strength in this system showed a greater dependence on ionomycin-induced [Ca\(^{2+}\)], flux compared with PI3K activation by PMA, as evidenced by the gradation of response in the left panel in Fig. 4B, with low PMA and increasing amounts of ionomycin. However, both PMA and ionomycin were required for a T cell response (also shown in Ref. 37).

SP-A–induced enhancement of proliferation was also observed with purified mouse T cells activated with low-dose soluble anti-CD3 (Fig. 4C). As previously observed with human T cells, stronger signals (manifested by increased incorporation of \(^{3}H\)thymidine compared with cells stimulated with soluble anti-CD3 alone) imparted by plate-bound cross-linked anti-CD3 or the presence of CD28 resulted in the suppression of proliferation in the presence of exogenous SP-A. These SP-A–mediated effects saturated at 12.5–15.0 \(\mu g/ml\) SP-A from four low-endotoxin SP-A preparations. Thus, as the strength of activating signal imparted to T cells increases, the SP-A–mediated enhancement of proliferation is replaced by an inhibitory response.

To determine whether SP-A exhibited SoS-dependent effects in the presence of APC-mediated activation, we also stimulated lung T cells from i.p. OVA-treated mice with different ratios of OVA-primed BMDCs and different amounts of OVA. Proliferation was assayed by \(^{3}H\)thymidine incorporation, and different ratios of APCs and OVA amounts were tested. In this system, signal strength is non-linearly defined by both ratio of BMDCs/T cells (within limits, lower numbers of BMDCs counter-intuitively provided better T cell stimulation) and concentration of OVA. Hence, we used a series of different ratios and amounts of OVA, and three representative conditions with steadily increasing mean cpm are shown in Fig. 4D (from left to right panels) and reported as fold difference compared with unstimulated control (T cells plus unprimed BMDCs). In the weakly activating condition, at a 1:10 ratio of BMDCs to T cells with 40 \(\mu g/ml\) OVA, lung-derived T cells showed \(\sim 30\%\) enhanced proliferation in response to exogenously added SP-A. However, when the ratio is changed to 1:30 with the same amount of OVA (40 \(\mu g/ml\)), these T cells showed a 40% suppression of proliferation in the presence of SP-A. Even greater suppression was observed when OVA concentration increased to 200 \(\mu g/ml\), 1:10 BMDC/T cell ratio. Thus, proliferation over baseline proportionately increased as the total signal strength increased from left to right, with the greatest degree of SP-A–mediated suppression at the highest signal strength.

**SP-A enables early division cycles in lung T cells with low and high signal strength but suppresses later divisions with a strong signal**

Next, we determined the kinetics of the SP-A–mediated activation signal integration mechanism. CFSE-labeled purified T cells were activated with different SoS in the absence or presence of SP-A, and cell division analysis was performed. As expected, low SoS resulted in a lower magnitude of proliferative response, with a large proportion of undivided cells, and few cells with two or more complete division cycles (Fig. 5A). At low signal strength in the presence of SP-A, we observed \(\sim 50\%\) decrease in undivided cells with a concomitant 3-fold increase in the number of cells with two or more cell divisions, as well as significantly more cells with a greater number of cell divisions (Fig. 5B). This profile was reversed with high SoS (Fig. 5C, 5D), where fewer cell divisions were observed in the presence of SP-A. Notably, as the initial cell divisions are very comparable in both conditions, it appears that the presence of SP-A under strong activation signals does allow for initial rounds of cell division. As seen in Fig. 5C and 5D, cells within the first three divisions account for 73.3 and 84.34% of total T cells in the absence or presence of SP-A, respectively.
However, in the presence of a strong signal in the absence of SP-A, 26.7% of cells are at four or more divisions compared with 15.66% in the presence of SP-A. Thus, the suppressive effect becomes predominant by the third and fourth rounds of cell division.

Reconstitution with exogenous SP-A results in elevated levels of proliferation at baseline and decreased proliferation with high-dose PMA

To confirm our in vitro observations, we studied the proliferative response to defined signals in both WT and SP-A−/− mice in vivo. Mice were instilled with saline or PMA+I in the absence or presence of exogenous AF647-labeled SP-A or left completely untreated. Single-cell suspensions from the lungs showed ∼5–12% labeling with CFSE (CFSE-hi), giving us a sampling efficiency of 1 of every 8–20 cells isolated. As previously observed with BrdU labeling with CFSE (CFSE-hi), giving us a sampling efficiency of

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The Ca2+ flux, trig-

in baseline proliferation was observed in saline-treated SP-A

incorporation in Figs. 2

A

of every 8–20 cells isolated. As previously observed with BrdU

labeling with CFSE (CFSE-hi), giving us a sampling efficiency of

1 and C, a small but significant decrease in baseline proliferation was observed in saline-treated SP-A−/− mice compared with WT mice (Fig. 5E, p < 0.05). This was completely reversed when exogenous SP-A was instilled into the lungs of SP-A−/− mice prior to stimulation with PMA+I (p < 0.05), suggesting that SP-A was playing a role in maintaining basal proliferation levels. Local instillation of PMA+I in vivo leads to increased levels of CFSE-int cells that had undergone one or more cell divisions in both WT and SP-A−/− mice (*p < 0.01). However, SP-A−/− mice showed a significant increase in the proportion of divided cells over their WT counterparts (p < 0.05). The presence of exogenously added SP-A–AF647 resulted in a sharp decrease in percentage of divided T cells (p < 0.05) in both WT and SP-A mice activated with PMA+I in vivo. No significant differences in the activation profiles were observed in parallel experiments performed to compare any functional differences between unlabeled and AF647-labeled SP-A (data not shown). Thus, these results indicate that SP-A enhances cell division with low-dose activation and inhibits later-stage division with strong activation.

SoS and SP-A dependent levels of [Ca2+]i over several hours provides a mechanism for differential T cell activation

Based on our experiments with PMA+I where the dose of ionomycin appeared to play a dominant role in determining the signal thresholds, we also analyzed levels of intracellular Ca2+ in the presence and absence of SP-A several minutes to hours after activation with various SoS. Initial cell divisions appeared to proceed normally even when SP-A eventually suppresses proliferation (Fig. 5), and lack of significant changes in the initial [Ca2+]i spike (data not shown) suggests that changes in initial proximal signaling are not playing a critical role in the observed effects of SP-A. Therefore, we also assayed for [Ca2+]i levels at varying intervals from 30 min to 80 h postactivation with low or high doses of PMA+I in the absence or presence of SP-A. The concentrations of PMA+I used were derived from experiments in Fig. 4B to cover weak and strong signals (10 ng/ml + 100 nM and 50 ng/ml + 250 nM PMA+I, respectively). The Ca2+ flux, triggered by the depletion of intracellular stores has been previously reported to play a critical role in maintenance of B and T cell activation (38, 39). At intermediate periods of activation between 15 and 30 h (the 20-h time point is shown in Fig. 6A), [Ca2+]i levels mirrored the final T cell proliferative response. At low SoS, the presence of SP-A enhanced [Ca2+]i by ∼140%, whereas at high SoS, [Ca2+]i levels were reduced ∼60% compared with control. However, this difference in Ca2+ capacitance disappeared over time (e.g., at 42 h postactivation), even as total [Ca2+]i levels gradually dropped to near baseline levels.

To attribute these effects to ionomycin-mediated increase in [Ca2+]i, we used another compound, thapsigargin (Thg), in combination with PMA (10 ng/ml). Thg specifically increases [Ca2+]i by blocking [Ca2+]i transport to the sarcoplasmic and endoplasmic reticulum and opens inositol triphosphate-gated channels in the endoplasmic reticulum. Although we still observed a small but significant increase with SP-A–treated cells in the presence of a weak signal, the magnitude of this effect was much reduced. In addition, EGTA, which acts as an extracellular Ca2+ chelator, completely eliminated the SP-A–mediated effect while simultaneously reducing total fluorescence (Fig. 6B). Because Thg preferentially acts on intracellular reserves of Ca2+, these results suggest that SP-A helps modulate the influx of extracellular Ca2+ into the cell. Because previous experiments used the pharmacological agents PMA, ionomycin, and Thg, which activate T cells bypassing TCR–CD3 signaling, we used anti-CD3, which triggers activation via the surface TCR–CD3 complex to corroborate further these observations (Fig. 6C). T cells were activated with low-dose (0.2 µg/ml) soluble anti-CD3 alone, which delivers a weak signal, or high-dose anti-CD3 plus anti-CD28. Weak anti-CD3–mediated activation led to a marginal (<10%) increase in [Ca2+]i compared with unactivated cells. However, [Ca2+]i was enhanced nearly 60% over baseline in the presence of exogenous SP-A. The opposite effect was observed with high-dose anti-CD3 plus anti-CD28, where SP-A reduced [Ca2+]i by ~34%. The addition of either BAPTA-AM, an intracellular calcium chelator, or extracellular EGTA dramatically reduced [Ca2+]i, and T cell proliferation. The dependence of T cell activation on extracellular Ca2+ fluxes has been very well established. EGTA has profound effects on T cell activation irrespective of the presence or absence of SP-A and acts as an experimental control to block activation to determine the relative importance of internal stores (with Thg-mediated activation) versus total calcium (with anti-CD3–mediated activation) with weak and strong activation signals. Thus, the Ca2+ channel-induced Ca2+ flux might play a key role in precipitating the final outcome of SP-A–mediated modulation of T cell activation.

Taken together, as summarized in the model in Fig. 7, these data suggest that SP-A seems to affect T cell proliferation by functioning like a signal strength-dependent rheostat, enhancing [Ca2+]i levels and influx of extracellular Ca2+ throughout the course of activation with low signals while reducing [Ca2+]i levels with strong activation signals.

Discussion

This study demonstrates that SP-A differentially modulates responses of T cells from both humans and mice depending on the strength of activating signal. At high levels of signal strength, interaction with SP-A suppresses T cell activation, irrespective of the type of applied signal: pharmacological agents, anti-CD3 (soluble or plate bound), or MHC class II–peptide presentation by APC. This suppression by SP-A is consistent with previous reports (15, 16, 40). Although we did not observe enhanced neutrophil or TNF-α levels in SP-A−/− mice at 3 d after LPS exposure, it is possible that some of the enhanced proliferation observed with high-dose LPS in the SP-A−/− may be due to greater levels of initial inflammation at earlier time points. Reconstitution with exogenous SP-A into SP-A−/− mice stimulated with a strong signal also resulted in suppression of T cell proliferation (Fig. 5E). However, at low integrated SoS, SP-A interactions with T cells serve to enhance T cell activation and proliferation in the bulk T cell population, independent of the presence of accessory cells (Figs. 3, 4). Indeed, this effect was observed in both naive and memory phenotype CD4+ T cells in the
lung. SP-A–mediated enhanced proliferation at low SoS was observed on T cells irrespective of the source (human PBMCs, mouse spleen, lung, as well as mediastinal, mesenteric, andinguinal lymph nodes). Finally, using CD4+CD25+ (regulatory T cell or preactivated) and CD4+CD44+ (memory phenotype) T cell-depleted populations in proliferation assays, we determined that the observed SoS-dependent responses to SP-A were a property of the bulk T cell population and were global T cell effects that were not significantly altered by native regulatory, preactivated, or memory T cells (Supplemental Fig. 1). Additionally, no significant differences in overall numbers of T cells sourced from spleen, lung, or inguinal, mediastinal or mesenteric lymph nodes were observed between WT and SP-A−/− mice.

SP-A enabled extended numbers of cell divisions in T cells activated with low SoS, as evidenced by the greater number of cell division events and greater proportions of cells in the higher-generational spans of CFSE analysis (Fig. 5). Although fold differences are not large, the effects on proliferation are additive—in both weak and strong activating signals. Notably, IL-2 ELISAs from supernatants revealed that there is some disconnect between cytokine production and inhibition of proliferation. T cell proliferation in vitro usually requires the presence of IL-2, which is both produced and used in an autocrine and paracrine manner during the course of activation. We observed enhanced levels of IL-2 with increasing signal strengths, even when proliferation was suppressed in the presence of SP-A (Supplemental Fig. 2), suggesting IL-2–independent effects on proliferation. A similar effect was also observed with soluble and plate-bound anti-CD3–mediated activation (Supplemental Fig. 2; both human and mouse T cells) or using various concentrations of OVA peptide in DO11.10 TCR transgenic T cells (data not shown). We also assayed for expression of the high-affinity IL-2 receptor, CD25, to determine if a lack of expression of the receptor was responsible for the accumulation of IL-2. However, as seen in Supplemental Fig. 2, no significant differences in CD25 expression were observed. At the same time, as previously observed with human cells, no enhanced apoptosis was observed by cell cycle analysis (Supplemental Table I).

The maintenance of cell division in the presence of SP-A with otherwise weak stimuli might be a direct result of increased levels and Ca2+ signaling over the first ∼30 h of activation. In contrast, when T cells are stimulated with high SoS, the profile is reversed in the presence of SP-A, with lower Ca2+ levels in the first ∼30 h of activation. Overall levels of [Ca2+]i drop steadily over the
FIGURE 7. A schematic model summarizing the differential role of SP-A in modulating T cell activation and proliferation depending upon the strength of activating signal. SP-A allows for low-grade, basal levels of proliferation in part by maintaining [Ca\(^{2+}\)] levels and thus may help maintain the immune repertoire in T cells. Initial expansion in response to strong signals does occur in the presence of SP-A, but it acts as a brake to prevent runaway inflammation at later stages of strong T cell responses. Because extensive proliferation and differentiation to terminal effector cells that ultimately undergo activation-induced cell death is avoided, the generation of multifunctional T cells that eventually form memory cells might be enhanced in the presence of SP-A.

course of activation, and during later stages of activation, no significant differences were observed. In addition, SP-A did not affect the intensity maxima of the calcium spike. These results differ from those previously observed by Borron et al. (16), where a reduction in the intensity of the initial Ca\(^{2+}\) spike was observed using purified normal bovine SP-A rather than the human SP-A used in the current studies. Because sustained [Ca\(^{2+}\)]\_capacitance rather than the initial spike is thought to be responsible for maintaining NFAT signaling responsible for optimal T cell proliferation (41–43), this kinetic effect may explain the delay in later cell divisions and reduced total numbers of 4-generation cells in the presence of SP-A and a strong activating signal. NFAT and NF-\(\kappa\)B are two key signaling pathways common to activation induced by PMA+I, anti-CD3, or APC–MHC–peptide. Although these pathways in tandem have an integral role in productive activation, Ca\(^{2+}\)/calcineurin signaling that solely goes through the NFAT pathway induces a limited set of anergy-associated genes (41). This phenomenon is worth noting because we have not observed enhanced cell death associated with SP-A–mediated suppression (Supplemental Table I). Together, these results demonstrate that T cells, in conjunction with SP-A, respond uniquely to different activation conditions and strength of activating signal.

Previous studies in animals and humans, using in vitro and in vivo models, have demonstrated that SP-A exhibits potent regulatory effects on immunity and inflammatory reactions in the lung, although the mechanism(s) by which SP-A’s effects are mediated remains undefined. Investigations to identify specific receptors for SP-A have been confounded by the fact that SP-A is a “sticky” protein and promiscuously interacts with high affinity with several ligands including myosin, Igs, SP-D, TLR4, CD93, and CD91/calreticulin, as well as lipids and Abs (19, 20, 40, 44–46). Most “classical” receptors that have been reported to bind SP-A (e.g., TLR4, CD93) are not present on naive T cells, even though there are multiple studies that highlight a functional role of SP-A on T cells. Another candidate for a receptor is SP-R210 (18), now identified as unconventional myosin 18A (47), which is largely expressed on monocytes and on a fraction of T cells. A recent report showed that expression of SP-R210 was markedly increased in T lymphocytes after stimulation by Mycobacterium tuberculosis (48). Thus, it is possible that activated T cells that express SP-R210 may become more susceptible to the effects of SP-A, which could then inhibit cell cycle progression of T cells and attenuate intracellular Ca\(^{2+}\) levels. Another possible binding partner is CTLA4 (CD152), which has long been implicated in differential suppression of T cell responses and recently shown to interact with SP-D (49). Considered together, our work and other data show that SP-A mediates not only T cell proliferation induced by direct T cell agonists such as anti-CD3, anti-CD28, PMA, and ionomycin, but also that induced via other cells. This is evidenced by the differential SP-A–mediated T cell responses to low- and high-dose LPS in Fig. 1 in vivo (where neutrophils and macrophages followed by T cells are primary responders).

This study has many immunological implications. The SoS has been reported to have multiple effects on functional responses by T cells (50–52, 34). For example, in T cells, low-affinity peptides generate reduced Ca\(^{2+}\) levels that result in the transcription of IL-4, whereas a stronger signal that activates both the Ca\(^{2+}\)–NFAT and MAPK pathways is required to induce IFN-\(\gamma\) for Th1 responses. In the presence of a strong signal in the lung, such as oropharyngeally administered high-dose PMA+I, or an invading pathogen, SP-A may attenuate the later phases of the response, thereby preventing an overzealous runaway inflammatory response that can be very damaging to the delicate lung tissue. After activation of T cells with a strong signal, the SP-A–mediated suppression might also help prevent the generation of T cells that express exclusively high-affinity TCRs. SP-A–mediated interactions with T cells allow the initial immune response to occur irrespective of TCR avidity (which corresponds to signal strength). However, later preferential inhibition of T cells with a high-affinity TCR would decrease their competitive advantage and conceivably prevent these clones from dominating an immune response in perpetuity. The lungs and their associated secondary lymphoid tissues such as BALT and mediastinal lymph nodes have among the largest environmental exposure of any organ system in the body. This makes the lungs an ideal locale to ensure the generation of an adequate memory response capability by maintaining the diversity of Ag-specific cells. When stimulation results in lower activation of T cells, SP-A–mediated interactions enhance T cell proliferation. This would be exemplified by low TCR signal due to low-affinity peptides or cross-reactive peptides (all of which have lower Ca\(^{2+}\) responses in T cells). Thus, the presence of SP-A might enable the prosurvival, basal (homeostatic) low-grade “maintenance” proliferation from TCR–MHC–self-peptide interactions (with no or minimal exogenous stimulation) to occur normally in the lung. The reduced BrdU incorporation observed in naive and central memory T cells from SP-A\(^{-/-}\) mice (Fig. 2) gives credence to this mechanism. However, even though Corse et al. (53) make convincing arguments in terms of mimicking these weak maintenance signals with p-MHC or soluble Abs, it is very difficult to draw direct comparisons between these effects in vivo versus in vitro activation. Duration and extent of TCR occupancy (exemplified by the immunological synapse and “serial triggering” of the TCR) function as nonexclusive mechanisms to enhance the natural low affinity of the TCR–MHC complex. It is likely that, by enhancing the generation of low-affinity TCR clones, SP-A interactions might broaden T cell responses to cross-reactive Ags that may play important roles as pathogens mutate to overcome the host immune response and delay the onset of T cell immunosenescence (54, 55) (summarized in Fig. 7). Future studies will address additional paracrine effects of SP-A interactions on T cell tolerance, homeostasis, and trafficking, as well as explore the role of accessory cells such as the lung alveolar type II cells that produce SP-A.
In summary, SP-A modulates the activation threshold and functional outcome of both mouse and human T cell activation, depending upon the strength of activating signal, at least in part by a “store operated” [Ca2+] entry-mediated mechanism. Multiple T cell surface receptors are known to contribute to T cell activation. Thus, cumulative signals from these receptors might be “integrated” to derive the lower threshold for enhanced proliferation and the upper threshold to halt excessive proliferation (Fig. 7). SP-A interactions, possibly by cross-linking these surface receptors, enable SP-A to act as a molecular rheostat that integrates activation signals of varying strengths to modulate T cell activation.

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Disclosures
The authors have no financial conflicts of interest.

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15. Scanlon, S. T., T. Milovanova, S. Kierstein, Y. Cao, E. N. Atochina, Y. Tomer, and J. R. Wright. 2006. Surfactant protein A differentially modulates T cell proliferation depending upon the strength of activating signal, at least in part by a “store operated” Ca2+ entry-mediated mechanism. Multiple T cell surface receptors are known to contribute to T cell activation. Thus, cumulative signals from these receptors might be “integrated” to derive the lower threshold for enhanced proliferation and the upper threshold to halt excessive proliferation (Fig. 7). SP-A interactions, possibly by cross-linking these surface receptors, enable SP-A to act as a molecular rheostat that integrates activation signals of varying strengths to modulate T cell activation.


SP-A mediated modulation of T cell proliferation is an intrinsic property of the bulk T cell population.

Equal numbers of purified T cells, or purified cells that were subject to C′-mediated depletion and magnetic selection to eliminate CD25+ or CD44+ T cells. Cells were then activated with different concentrations of PMA+I as previously described. For comparison of the different populations, the mean cpm obtained from replicate culture wells was normalized to undepleted, purified CD4+ cells activated with the lowest concentration of PMA+I. *p<0.02 compared to respective controls from 3 independent experiments.

Cells activated with relatively low signal strengths show an increase in proliferation in the presence of SP-A, whereas cells activated with stronger signals are inhibited. This activation profile was unaffected by depletion of either CD4+CD25+ (~regulatory) or CD4+CD44+ (~memory) T cells, suggesting that the SP-A/signal strength mediated 'switch' mechanism is an intrinsic property of the global T cell population.
Supplementary Figure S2

SP-A exerts IL-2 secretion- and CD25 expression-independent effects on T cell proliferation

Human (A) or mouse (B and C) T cells were activated with weak or strong signals as indicated. Supernatants were collected at 24-25 h post-activation and an IL-2 ELISA was performed. Figures S2A, B and C are companion experiments to Figures 3, 4C and 4B from the manuscript, with measurements from 9 human subjects, or 3-6 independent mouse experiments with pooled cells obtained from 5-9 mice per experiment.

Levels of IL-2 increased with progressively stronger activation signals, even when T cell proliferation was suppressed by SP-A.

(D) Activated mouse T cells with or without exogenous SP-A were washed well and subjected to flow cytometric analysis to determine CD25 expression 30 h post-activation. The % of CD3+CD4+ T cells expressing CD25 in replicate wells from one of three experiments is shown. CD25 expression is enhanced with higher doses of PMA+I, in keeping with its known role as the high affinity IL-2 receptor and activation marker. However, no significant differences were observed with SP-A treatment, suggesting that the observed effects on cell division and proliferation are independent of intermediate stage activation events such as IL-2 production and CD25 expression.
Supplementary table 1:
SP-A enhances proportion of actively cycling S/G2M phase cells on activation with a weak signal, but suppresses cell division in the presence of strong activating signals

<table>
<thead>
<tr>
<th>Activation condition</th>
<th>Mean fold difference</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S/G2M phase cells</td>
<td>Hypodiploid cells</td>
<td></td>
</tr>
<tr>
<td>Anti-CD3 (sol)</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Anti-CD3 (sol) + SP-A</td>
<td>1.60 ± 0.22</td>
<td>1.51 ± 0.42</td>
<td></td>
</tr>
<tr>
<td>Anti-CD3 (sol) + anti-CD28</td>
<td>4.97 ± 0.39</td>
<td>0.63 ± 0.19</td>
<td></td>
</tr>
<tr>
<td>Anti-CD3 (sol) + anti-CD28 + SP-A</td>
<td>3.02 ± 0.34</td>
<td>1.15 ± 0.26</td>
<td></td>
</tr>
<tr>
<td>Anti-CD3 (pb)</td>
<td>3.19 ± 0.47</td>
<td>1.11 ± 0.19</td>
<td></td>
</tr>
<tr>
<td>Anti-CD3 (pb) + SP-A</td>
<td>2.21 ± 0.33</td>
<td>1.47 ± 0.28</td>
<td></td>
</tr>
<tr>
<td>Anti-CD3 (pb) + anti-CD28</td>
<td>5.61 ± 0.72</td>
<td>1.11 ± 0.14</td>
<td></td>
</tr>
<tr>
<td>Anti-CD3 (pb) + anti-CD28 + SP-A</td>
<td>3.34 ± 0.50</td>
<td>1.30 ± 0.27</td>
<td></td>
</tr>
</tbody>
</table>

T cells were purified from human PBMCs as described in Materials and Methods, and activated with soluble anti-CD3 (0.2 μg/ml; weak signal) or plate-bound anti-CD3 (crosslinked, strong signal). SP-A (20 μg/ml) and anti-CD28 (0.5 μg/ml) were added as indicated. After 50-55 h of activation, cells were surface stained with anti-CD3 and –CD4, permeabilized in a citrate buffer containing 0.1% Triton X-100 and RNAse. Cell suspensions were stained overnight with propidium iodide, and acquired on a BD LSRII flow cytometer. Data is normalized to the S/G2M and hypodiploid populations present in cells activated with sol anti-CD3 (mean ± SEM). n=5 experiments (subjects)