Surfactant Protein SP-D induces immune quiescence and apoptosis of mitogen-activated peripheral blood mononuclear cells

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Abstract:

Surfactant Protein D (SP-D) is an integral molecule of the innate immunity secreted by the epithelial cells lining the mucosal surfaces. Its C-type lectin domain offers pattern recognition functions while it binds to putative receptors on immune cells to modify cellular functions. Activated PBMCs and increased serum levels of SP-D are observed under a range of pathophysiological conditions including infections. Thus, we speculated if SP-D can modulate systemic immune response via direct interaction with activated PBMCs. Here, we have examined interaction of a recombinant fragment of human SP-D (rhSP-D) on PHA-activated PBMCs. We observed a significant downregulation of TLR2, TLR4, CD11c and CD69 upon rhSP-D treatment. rhSP-D inhibited production of Th1 (TNF-α and IFN-γ) and Th17 (IL-17) cytokines along with IL-6. Interestingly, levels of IL-2, Th2 (IL-4) and regulatory (IL-10 and TGF-β) cytokines were unaltered. Differential expression of co-stimulatory CD28 and co-inhibitory CTLA4 expression along with their ligands CD80 and CD86 revealed selective up-regulation of CTLA4 at both mRNA and protein level. In addition, rhSP-D induced apoptosis only in the activated but not in non-activated PBMCs. Blockade of CTLA4 inhibited rhSP-D mediated apoptosis, confirming an involvement of CTLA4 in induction of apoptosis. We conclude that SP-D restores immune homeostasis: it regulates expression of immunomodulatory receptors and cytokines, which is followed by apoptosis induction of immune-activated cells. These findings appear to suggest a general role for SP-D in immune surveillance against activated immune cells.

Introduction:

The human body is constantly exposed to pathogenic or non-pathogenic foreign antigens triggering a specific antibody or cell-mediated adaptive immune response, which is designed to overwhelm the non-self. However, the immune response needs to be regulated as well as kept quiescent until the next immune challenge. Thus, a fine balance between protective immune response and resolution of prolonged inflammation is required for homeostasis. A number of pathological conditions including inflammatory and autoimmune disorders are characterized by persistent immune activation.

A range of immune mechanisms regulate the systemic or tissue specific-immune activation and inflammation. For instance, regulatory T cells (Tregs) secrete IL-10 and/or TGF-β to regulate chronic immune responses (1, 2). Similarly, galectins exert a range of effects on T cell functions such as signaling, activation, apoptosis, and cytokine secretion in addition to Treg expansion (3). Collectins, belonging to C-type lectin family have been shown to be involved in modulating the adaptive immune responses (4-7). Surfactant Protein D (SP-D) is an extensively studied collectin in the models of infection, allergy and inflammation. SP-D contains an N-terminal triple-helical collagen region and a homotrimeric CRD region which has been shown to interact with a range of viral, bacterial and fungal pathogens and bring about clearance mechanisms that include agglutination, enhanced phagocytosis and superoxidative burst (8, 9). SP-D can also bind allergens, inhibit specific IgE binding and basophil degranulation (10). SP-D knock-out mice show exaggerated allergic response due to their hypereosinophilic and IL-13 overexposing phenotype (11). SP-D binds and influences the adaptive immune response by inhibiting T cell proliferation and activation (4, 5). SP-D deficient mice show a state of persistent T cell activation and inflammation in the lungs in response to exogenous antigens (12). Lin et.

al., provided detailed insights on SP-D mediated downregulation of allergen-induced inflamma-inflammation and proliferation and showed the involvement of an inhibitory receptor CTLA4 in mouse T cells (6). Like with T cells, SP-D also binds to several receptors such as TLR2, TLR4 and CD14 receptor on macrophages and alter their behaviour by regulating inflammatory response (13, 14).

Increased SP-D levels at local sites such as synovial fluid of RA patients (15), skin lesions of psoriasis (16) and *E. coli* infection in prostate tissue (17) have been reported. Circulating levels of SP-D are elevated in a range of infections, acute lung injury, chronic obstructive pulmonary diseases, and allergy (18). While increased serum levels of SP-D acts as a biomarker of inflammation, we speculated if SP-D can exert its immuno-modulatory effects on circulating systemic immune cells. This could be of great importance in understanding host response in immune-mediated disorders.

In the present study, we have assessed the effects of a recombinant fragment of human Surfactant Protein D (rhSP-D) on the expression of different activation markers in PHA-activated PBMCs from healthy donors. rhSP-D bound differentially to activated PBMCs when compared to the non-activated PBMCs. TLR signalling was significantly downregulated upon rhSP-D treatment as evident from TLR2, TLR4 and MyD88 levels. CD69, an activation marker on lymphocytes was also affected. rhSP-D caused a downregulation of pro-inflammatory and Th1 cytokines namely, TNF-α, IFN-γ and IL-6. Th2 cytokines were however unaffected. A marked decrease in levels of IL-17A was also observed in activated PBMCs. Furthermore, rhSP-D significantly and selectively upregulated expression of co-inhibitory signalling receptor CTLA4. Extended exposures of rhSP-D to activated PBMCs induced apoptosis; however, the viability of non-activated PBMCs was not affected. Thus, SP-D appears to modulate immune

response mounted by activated PBMCs by regulating expression of pro-inflammatory cytokines and apoptosis induction. Such suppressive effects could impact upon maintenance homeostasis and be relevant in auto-immunity.

Materials and Methods

Ethics statement

The study was approved by the Institutional Ethics Committee for Clinical Studies, National Institute for Research in Reproductive Health, Indian Council of Medical Research; (Project No.: 148/2008). The study involved collection of blood (5 ml) from healthy donors (27 +/- 7 years). A written informed consent was obtained from each study participant and recommended guidelines were followed during blood collection.

rhSP-D preparation

The recombinant human SP-D (rhSP-D) used in this study comprises of a functional homotrimeric lectin domain followed by the neck region and eight collagen gly-x-y repeats. The rhSP-D was expressed in *E. coli*, purified and characterized as described previously (19, 20). Endotoxin levels in the rhSP-D preparation were determined using the QCL-1000 Limulus amebocyte lysate system (BioWhittaker Inc., USA). The assay was linear over a range of 0.1–1.0 EU/ml (10 EU = 1 ng of endotoxin) and the amount of endotoxin present in the preparations was estimated to be 5.1pg/µg of rhSP-D.

Preparation of PBMCs

Blood (5 ml) was collected from different (N=7) healthy donors in sterile heparinzed vacutainers by vein puncture. PBMCs were isolated using Ficoll (Himedia Laboratories, India). Briefly, blood was diluted in 1:1 ratio with incomplete RPMI 1640 medium (Sigma Aldrich, USA). Diluted blood was overlaid on to the ficoll layer in a 15-ml conical tube and centrifuged at 1800

rpm for 30 mins at room temperature. The buffy layer containing PBMCs was separated and washed twice with RPMI 1640 medium containing 10% FBS and Gentamicin (50µg/ml) (Gibco, Life Technologies, USA). PBMCs ($5\text{x}10^6$ cells) were seeded in a T-75 flask and activated using PHA (5µg/ml) (Gibco, Life Technologies, USA) for 24 h.

Cell binding studies using FITC labeled rhSP-D

To study the binding of rhSP-D to non-activated and activated PBMCs, we tagged FITC dye to the rhSP-D as described earlier (21). Briefly, rhSP-D was dialyzed against FITC-labeling buffer (0.05M boric acid and 0.2M sodium chloride, pH 9.2), followed by incubation with FITC for 2h at room temperature. The labeled proteins were dialyzed against buffer (0.1M Tris–HCl, pH 7.4, 0.1% w/v sodium azide and 0.2M NaCl) and stored at 4°C. The labeled protein was quantitated and flurochrome to protein ratio of 5–6:1 was considered optimum for flow cytometric (FACS) studies. FITC-labeled rhSP-D was incubated with non-activated and activated PBMCs in the presence of 2mM $CalCl_2$ and/or 10mM EDTA at 4°C for 1h. Cells were washed by centrifuging at 1200 rpm to remove unbound rhSP-D, fixed with 4% PFA and analysed by flow cytometry.

rhSP-D treatment of activated PBMCs

Activated PBMCs were washed and resuspended in a fresh complete RPMI 1640 medium. 5 X 10^5 cells were seeded in a 12-well tissue culture plate (Nalgene Nunc$^{\text{TM}}$) and treated with indicated concentrations of rhSP-D. After 24 h incubation, cells were harvested for real time PCR and flow cytometric analysis. Culture supernatants were collected for cytokine analysis.

Real time PCR

Total RNA was isolated using Trizol (Invitrogen, USA). Quality of RNA was assessed by nano-spectrophotometry, and the nucleotide:protein ratio (260:280) was determined. A 3 µg of RNA was reverse transcribed into cDNA using Superscript III first strand synthesis kit (Invitrogen,

USA). The resulting cDNA was diluted and used for real-time polymerase chain reaction (PCR) on the Bio-Rad CFX96 TouchTM real-time PCR detection system using the iQ TM SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). 18s was used as housekeeping control. Each qPCR experiment included cells from a minimum of five donors (biological replicates). Primers were designed using Primer BLAST and thier annealing temperatures and product sizes are mentioned in Table 1. Primers for TLR2 and TLR 4 were used as published elsewhere (22)

Flow cytometry

rhSP-D treated and untreated PBMCs (PHA-activated) were stained using mouse monoclonal antibodies to TLR2, TLR4, (R &D Systems, USA) CD11c (Abcam, United Kingdom) and Mouse anti-CTLA4 (UC10-4F10-11) (eBioscience, San Diego, CA) or matched-isotype control IgG for 1 h at 4^0C. Phycoerythrin-conjugated rat anti-mouse IgG conjugated (Molecular Probes, Eugene, USA) were used as secondary antibodies. Cells were analyzed in a four-color multiparameter flow cytometer, BD FACS Aria III(BD Biosciences, San Jose, California, USA).

Multiplex Cytokine Assay

Multiplex cytokine analysis was performed using Evidence Investigator (Randox Laboratories) that uses biochip array technology. Culture supernatants of activated PBMCs treated with or without rhSP-D were centrifuged at high-speed to remove debris and analzyed for a panel of 12 analytes (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, EGF, IFN-γ, MCP-1, TNF-α and VEGF) using cytokine array I kit that comprised of both Th1 and Th2 cytokines.

Viability assay

Non-activated (without PHA) and PHA-activated PBMCs (5×10^4 cells/well) were plated in the 96-well tissue culture plates (Nunc) a day prior to rhSP-D treatment. Cell were treated with rhSP-D (10 or 20µg/ml) and culture medium alone as was untreated control and incubated for

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different time intervals (24, 48 and 72 h). To assess the viability, MTT assay was performed. 10µl MTT (5mg/ml stock) was added to each well and incubated for 4 h. Plate was centrifuged at 1200 rpm and the medium was removed. This was followed by addition of 100µl of acidified iso-propanol to dissolve the formazan crystals and the absorbance was read at 570nm using a spectrophotometer plate reader (Beckman Coulter).

Annexin V Assay

For Annexin-V immunostaining assay, the manufacturer's protocol of Annexin V-FITC apoptosis detection kit (Calbiochem) was followed with some modifications. Briefly, activated PBMCs, treated with indicated concentrations of rhSP-D were harvested at 48 and 72 h. Culture medium was washed with 1x ice cold PBS and cells were incubated with FITC-tagged Annexin V for 20 mins in dark. Later, Annexin V was washed, 1 µl propidium iodide (PI) was added, and cells were immediately analyzed by flow cytometry.

Statistical Analysis

Graphs were plotted and data was analyzed using GraphPad PRISM Software Trial version 6.00 (GraphPad Software Inc., San Diego, CA). Unpaired t-tests was used for comparison among the untreated and rhSP-D treated groups. Data represented as mean +/- SD. Values of $p < 0.05$ were considered statistically significant.

Results:

rhSP-D differentially binds to non-activated and activated PBMCs:

Flow cytometric analysis revealed a dose-dependent binding of rhSP-D to both non-activated and activated PBMCs (Fig 1A). Ca^{2+} was essential for the binding and EDTA (10mM) inhibited the interaction of rhSP-D and PBMCs (Fig 1B) suggesting involvement of the lectin domain. We

observed a significant increase (2 fold) in binding of rhSP-D to activated cells as compared to the non-activated PBMCs, suggesting that rhSP-D may exert differential effects on activation status of PBMCs.

rhSP-D downregulates PHA induced expression and signaling of TLR 2 and TLR4:

Activated PBMCs exhibit clonal propagation of lymphocytes, maturation of myeloid cells and increased expression of activating receptors. PHA activation led to an upregulation of mRNA and protein expression of TLR2 and TLR4 (Fig 2A). We analysed the effect of rhSP-D on expression of TLR2 and TLR4 using real time RT-PCR and flow cytometric analysis. TLR2 transcript was downregulated 4-fold whereas TLR4 downregulation (6-fold) was more pronounced (Fig 2A). Flow cytometric analysis showed a similar downregulation of TLR2 and TLR4 expression by rhSP-D (Fig 2B and C). We also probed the levels of MyD88, a common adapter of TLR2 and TLR4, and a downstream signaling molecule of the TLR pathway. rhSP-D treated, activated PBMCs showed a 4-fold lower levels of MyD88 transcripts (Fig 2A), clearly indicating dampening of the TLR-2/4 inflammatory pathway in the activated PBMCs.

Expression of Integrin CD11c and activation marker CD69 is markedly affected by presence of rhSP-D

RhSP-D effect on expression of CD11c, a marker largely expressed on activated monocytes and lymphocytes (23, 24) was also analysed. A marked reduction of surface CD11c in rhSP-D treated activated PBMCs was observed (Fig 2D). Assessing the classical activation marker on lymphocytes, CD69, revealed that rhSP-D lowered expression of CD69 transcripts by 3-fold,

indicating that the activation and proliferation status of PHA-stimulated PBMCs was significant-significantly affected (Fig 2E).

rhSP-D inhibits Th1- and Th17- cytokines

PHA treated PBMCs showed an exaggerated pro-inflammatory cytokine response including IL-2 (6 fold), IL-6 (9-fold), TNF-α (4-fold), IL-23 (5-fold) and IL-17A (7-fold) along with an increase in anti-inflammatory TGF-β (4-fold) and IL-10 (5-fold) (Fig 4B). To delineate the effect of rhSP-D on PHA induced immune mediators and Th responses, we determined their mRNA and protein levels by real time RT-PCR and multiplex cytokine array, respectively. Transcript levels of IL-6 (4-fold) and TNF-α (2-fold) were down regulated in rhSP-D treated PBMCs (Fig 3A). Cytokine array showed a significant down regulation of secretory IL-6, IFN-γ and TNF-α (Fig 3B). Interestingly, IL-2 was neither affected at m-RNA nor at protein level (Fig 3A, 2B). Among the Th2-cytokines, we found no significant alteration in IL-4 and IL-10, hinting at the potential of rhSP-D in maintaining Th2 balance (Fig 3C, 3D).

Polarising cytokines such as IL-6 and IL-23 along with TGF-β lead to effector Th17 response and expression of IL-17A (42). In our study, rhSP-D significantly downregulated mRNA expression of IL-23 (3-fold) (Fig 3E). As expected, we observed a marked decrease (3-fold) in IL-17A mRNA expression following rhSP-D treatment (Fig 3E). No alteration in TGF-β expression was observed following rhSP-D treatment of PHA-activated PBMCs.

rhSP-D selectively modulates expression of CTLA4 in activated PBMCs:

SP-D has been shown to enhance expression of CTLA4, a negative regulator of T cell activation and proliferation, in ConA activated murine splenocytes and T cells (6). To assess whether a similar effect is observed in PHA activated human PBMCs, we studied levels of CTLA4, along

with a co-stimulatory competitor CD28 and their ligands CD80 and CD86. A 7-fold increase in mRNA levels of CTLA4 was observed in rhSP-D treated PBMCs whereas CD28 levels were unaltered (Fig 3A). Interestingly, levels of their ligands CD80 and CD86 were also unaltered by rhSP-D, suggesting a selective upregulation of the inhibitory CTLA4 (Fig 4A). We further confirmed the increase in surface expression of CTLA4 by flow cytometric analysis (Fig 4B). Thus, rhSP-D induced alteration in the activation status of PHA-activated PBMCs is due to an upregulated expression of CTLA4. This prompted us to assess the fate of PBMCs with respect to proliferation and apoptosis following rhSP-D treatment.

rhSP-D induces apoptosis of activated PBMCs

It has previously been reported that SP-D inhibits the proliferation of activated T cells (5, 6), however, its effect on the viability of activated cells has not been examined. We performed MTT assay to determine the effect of rhSP-D on viability of activated PBMCs. 24 h after rhSP-D treatment, no significant decrease in viability was observed in either activated or non-activated PBMCs. At 48h and 72h, rhSP-D showed a dose- and time-dependent decrease in viability of activated PBMCs (Fig 5A). The effect on viability was specific for activated PBMCs as no significant decrease in viability of non-activated PBMCs was observed (Fig 5B). Annexin V staining of rhSP-D treated activated PBMCs showed a dose- and time-dependent increase in Annexin V positive cells, suggesting that the reduction in viability is due to induction of apoptosis (Fig 6).

SP-D was shown to inhibit lympho-proliferation through involvement of CTLA4 in murine spleen cells (6). We replicated this observation in human PBMCs. In coherence to their findings, rhSP-D inhibited proliferation of PBMCs and CTLA4 blockade rescued rhSP-D

mediated effects (Fig 7A). To further establish whether the same receptor (CTLA4) is involved in inhibition of apoptosis, we used a pool of activated cells and treated with rhSP-D and/or blocking CTLA4 antibody. rhSP-D led to reduction in viability of activated cells as a result of apoptosis, however, anti-CTLA4 treated PBMCs showed an abrogation of rhSP-D induced apoptotic effects in activated PBMCs (Fig 7B). Thus, it appears that CTLA4 upregulation by SP-D determines the immune response and fate of activated PBMCs.

Discussion

The present study further validates the role of soluble pattern recognition molecule, surfactant protein SP-D, in the regulation of inflammation and maintenance of homeostasis, in addition to pathogen recognition and clearance. SP-D, being present at the mucosal sites and in serum may prevent prolonged survival of activated immune cells, thus, reducing the tissue damage.

Given that activated monocytes and lymphocytes are characterized by an increased expression on activated monocytes and lymphocytes, we assessed their differential regulation in presence of rhSP-D. Most subsets of PBMCs express TLR1-10 transcripts and proteins (25, 26). TLR2 serves as a co-stimulatory receptor on activated T cells and is essential to induce Th1 response (27, 28) whereas TLR4 regulates T cell activation and promotes auto-immune inflammation (29). Both TLR2 and TLR4 are abundantly present on monocytes and result in NF-Kβ translocation to the nucleus and a subsequent pro-inflammatory response (30). SP-D dampens activation of TLR2/4 via its functional C-type lectin domain and markedly inhibits TNF-α production by monocytes and macrophages (13, 31, 32). We, for the first time, report an appreciable decrease in levels of both TLR-2 and TLR-4 in rhSP-D treated PBMCs. Down-regulating TLR-2 and TLR-4 seems a novel mechanism by SP-D in order to curb TLR-2 and

TLR-4 mediated inflammation. During differentiation from monocytes to macrophages, SP-A, a molecule structurally similar to SP-D, leads to down regulation of TLR-2 expression but not of TLR-4 (33). Transcript of MyD88, a common adapter of TLR-2 and TLR-4 (34) was also found to be down-regulated in the rhSP-D treated PBMCs, indicating disruption of TLR signaling cascade. SP-A is also known to affect TLR2 and 4 signaling in macrophages (35, 36 30).

CD11c is predominantly expressed by myeloid cells mainly by DCs, however, a small fraction of activated CD8 T cells also express CD11c (23, 24). CD11c mRNA was increased by SP-D deficiency (37). We found a profound decrease in surface expression of CD11c on rhSP-D treated PHA-activated PBMCs. Further, we also determined the levels of CD69, a classical early activation marker on lymphocytes, which was significantly upregulated in lymphocytes of SP-D knock-out mice. As evident by lowered CD69 expression, our observation corroborates with the previous finding that SP-D regulates T cell activation (12).

PHA activation results in an efflux of both Th1 and Th2 cytokines (38). An increase in IL-2, IL-6, IFN-γ and TNF-α indicated an upregulated Th1 and proliferative response under influence of PHA. In addition, mRNA levels of IL-4, IL-10 and TGF-β were increased along with IL-23. At 24 h. rhSP-D suppressed levels of Th1 cytokines IFN-γ, TNF-α and IL-6; however levels of IL-2 remained unaltered. It is well-established that SP-D inhibits T cell proliferation with or without involving IL-2 (4, 5). Intrestingly, Th2 cytokines (IL-4 and IL-10) and TGF-β were not affected by rhSP-D suggesting that SP-D appears to maintain an anti-inflammatory milieu, a key to maintain immune homeostasis. Moreover, exogenous administration of rhSP-D restores Th1: Th2 balance in SP-D knock out mice model of allergy (39, 40). It is worth noticing the retention of IL-10 and TGF-β levels that are largely expressed by Tregs, another important mechanism in immune homeostasis (41). SP-A has been

shown to induce Tregs via TGF-β (7). TGF-β along with IL-6 and IL-23 directs differentiation of Th cells to Th17, the Th subset involved in autoimmunity (42 - 44). However, in the presence of rhSP-D, the levels of IL-6 and IL-23 were downregulated, and thus, not significant enough to induce Th17 differentiation and IL-17A, a cytokine produced by Th17 cells. Reduced levels of IL-17A in the rhSP-D treated PBMCs suggest that SP-D inhibited Th17 differentiation. This is in coherence with decreased IL-17A expression by SP-D treated, ConA activated murine splenocytes and their increased inclination towards Tregs (45). Taken together, this modulation in the levels of cytokines is suggestive of a regulatory role of SP-D in fine-tuning the prolonged systemic inflammation.

Several studies have shown interplay between activation markers, cytokines and Th responses. Interestingly, the expression of TLR2 is essential to induce a Th1 response in PBMCs by stanols and plant sterols (28). IL-4, a Th2 cytokine, down regulates TLR4 expression (46), whereas Th1 cytokines, IL-6 and TNF-α, positively regulate TLR2 expression (47, 48). Activated TLR2 and TLR4 are known to drive pro-inflammatory cytokine response and pathogenic Th17 response (49, 29). Similarly, activation marker CD69 also governs Th17 response (50). In peripheral tissues, TGF-β inhibits T cell activation and proliferation, effector T-cell differentiation and maintains Treg cells. SP-D driven downregulation of TLR2, TLR4, CD69, Th1 and Th17 cytokines appear to emphasize its contribution immune quiescence.

It has been well documented that SP-A and SP-D bind to lymphocytes and suppress T cell proliferation (4, 5). In mice splenocytes, SP-D inhibits allergen induced inflammation and proliferation through up regulating CTLA-4, a negative regulator of T cell activation (6). In our study, we confirm that rhSP-D increase expression of CTLA4, and thus contributing to T cell homeostasis. However, co-stimulatory receptor CD28 was unaltered by rhSP-D treatment along

with their ligands CD80 and CD86 that are present on the antigen presenting cells. By this ob-observation, SP-D selectively regulates the expression of homeostatic molecule by direct interaction with T cells through receptor molecule(s) yet to be defined. We observed a higher binding of rhSP-D to activated cells than the non-activated cells, indicating that the effects observed on the activated cells could plausibly due to more receptor availability and increased binding. The interaction could be glycan-mediated as rhSP-D used in the study contains only a functional CRD domain.

To determine the fate of activated PBMCs following a significant modulation of cytokine profile, activation status and CTLA4 expression by rhSP-D, we assessed their viability over time. Activated PBMCs showed a significant reduction in viability which was due to induction of apoptosis. This effect was only observed at a later time point, only in activated PBMCs. The fact that CTLA4 can induce anergy (51) and TLR2 and 4 is associated with T cell survival, (52, 29) rhSP-D mediated differential regulation of these markers could have led to apoptosis at a later time-point. Moreover, our observation that CTLA4 blockade abrogates rhSP-D mediated reduction in viability could be as a result of regaining the proliferative activity in the absence of CTLA4. It had been shown that CTLA4 can trigger apoptosis and anti-CTLA4 leads to reduced apoptosis in tumor cells (53). Also, we have earlier reported that SP-D could act as a potential immune-survelliance molecule as it selectively induced apoptosis in activated eosinophils and in several cancer cell lines (21, 54, Dodagatta-Marri et al, unpublished data). rhSP-D mediated apoptosis in AML14.3D10 eosionophilic cancer cell line was p53 mediated and involved caspase 9 (54). However, key receptors in SP-D binding and apoptotic pathway involved in activated PBMCs remains to be elucidated. In summary, SP-D sequentially regulates inflammation,

modulates expression of activating molecules and later induces apoptosis specifically of the acti-activated systematic immune cells to maintain homeostasis.

Another class of β-galactoside specific, innate immune molecules called galacteins have come across as a major regulators of immune responses besides their classical pathogen recognising ability. Galectin 1, 2 and 9 have been shown to inhibit secretion of Th1 and Th17 cytokines and promote Th2 cytokines, in addition to inducing T cell apoptosis *in vitro,* thus, establishing a link between cytokine deviation and selective induction of apoptosis of T cells (3). Although the mechanism by which galectins and SP-D may regulate inflammation and immune response could be different, there exists a common endpoint in terms of linking and regulating innate and adaptive immune axis. Mechanisms underlying apoptosis of activated PBMCs and skewing of the Th responses warrant further studies in animal models.

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Footnotes:

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Running Title: SP-D regulates immune activation of PBMCs

Abbreviations used in this paper:

Figure Legends:

Fig1. rhSP-D binds more effectively to activated PBMCs.

A. Binding of rhSP-D to non-activated PBMCs. Black line histogram represents unstained, grey filled histogram shows rhSP-D binding to non-activated PBMCs as compared to unstained, pattern filled histogram shows inhibition of binding by EDTA. Each experiment was repeated four times.

B. Binding of rhSP-D to activated PBMCs. Black line histogram represents unstained, grey filled histogram shows rhSP-D binding to activated PBMCs as compared to unstained, pattern filled histogram shows inhibition of binding by EDTA. Each experiment was repeated four times.

C. Quantitation of Mean Fluorescence Intensity (MFI) of rhSP-D binding to non-activated and activated PBMCs. Data represents mean ± S.D of four independent experiments. *indicates statistical significance and *$p<0.05$ relative to binding rhSP-D binding on non-actiavted PBMCs.

Fig 2. rhSP-D down-regulates expression of TLR2, TLR4, MyD88, CD11c and CD69 on activated PBMCs

A.Real time RT-PCR analysis showed an upregulation of transcripts of TLR 2, TLR4, MyD88 in PHA-activated PBMCs as compared to non-activated PBMCs (taken as 1 on Y-axis). rhSP-D (10µg/ml) treatment for 24 h down regulated transcripts of TLR2, TLR 4 and

MyD88 when compared to untreated. Each bar represents the mean ± S.D of three inde-independent experiments. $*,^{\#}$ indicates statistical significance and $^{\#}$p<0.001 and **p<0.01 relative to rhSP-D untreated controls.

B. FACS analysis of TLR2 and TLR4 expression.(i)The black line histogram indicates TLR2 expression by PHA-activated PBMCs (83.7%); the grey line indicates expression by rhSP-D treated PBMCs (64.8%); (ii) The black line histogram indicates TLR4 expression by PHA-activated PBMCs (76.3%) and the grey line indicates expression by rhSP-D treated PBMCs (29.4%). Each experiment was repeated three times.

C. FACS analysis of CD11c expression on activated PBMCs as analysed by flow cytometry. The black line histogram indicates CD11c expression by PHA-activated PBMCs (88.4%); the grey line indicates expression by rhSP-D treated PBMCs (60.03%). Each experiment was repeated three times.

D. Real time RT-PCR analysis of CD69 mRNA levels of CD69 transcripts were upregulated in PHA-activated PBMCs as compared to non-activated PBMCs (taken as 1 on Y-axis). rhSP-D (10μg/ml) treatment for 24 h downregulated CD69 mRNA levels when compared to the untreated cells. Each bar represents the mean ± S.D of three independent experiments. * indicates statistical significance **p<0.01 relative to rhSP-D untreated controls.

Fig 3. Effect of rhSP-D on cytokine production in activated PBMCs:

A. Differential expression levels of IL-2, IL-6 and TNF-α mRNA (n = 5). Real-time RT-PCR analysis shows an upregulation of transcripts of IL-2, IL-6 and TNF-α in PHA-activated PBMCs as compared to non-activated PBMCs (taken as 1 on Y-axis). rhSP-D treated PBMCs expressed lowered levels of IL-6 and TNF-α transcripts and no alteration in IL-2

levels was found. * indicates statistical significance and *p<0.05 and **p<0.01 relative to rhSP-D untreated controls.

B. Levels of IL-2, IL-6, TNF-α and IFN-γ in the culture supernatant of rhSP-D treated PBMCs were significantly lower in comparison to culture supernatants of untreated PBMCs. Each bar represents the mean ± S.D in pg/ml and of five independent experiments. Statistical significance of *P <0.001 relative to rhSP-D untreated controls.

C. Differential expression levels of transcripts of IL-10 and TGF-β by real-time RT–PCR analysis (n = 5). Real time RT-PCR analysis showed an upregulation of transcripts of IL-10 and TGF-β in PHA-activated PBMCs as compared to non-activated PBMCs (taken as 1 on Y-axis). In presence of rhSP-D, no significant alteration in transcripts of IL-10 and TGF-β were observed in comparison to untreated PBMCs.

D. Levels of IL-4 and IL-10 in culture supernatant of rhSP-D treated PBMCs were not significantly altered in comparison to culture supernatants of untreated PBMCs. Each bar represents the mean ± S.D in pg/ml of five independent experiments.

E. Differential expression levels of transcripts of IL-17 and IL-23 by (n = 5). Real time RT-PCR analysis showed an upregulation of transcripts of IL-10 and TGF-β in PHA-activated PBMCs as compared to non-activated PBMCs (taken as 1 on Y-axis). In presence of rhSP-D, no significant alteration in transcripts of IL-10 and TGF-b were observed in comparison to untreated PBMCs.

Fig. 4 rhSP-D selectively upregulates expression of CTLA4 in activated PBMCs:

A. Real time RT-PCR analysis showed an upregulation of transcripts of CD28, CTLA4, CD8 and CD86 in PHA-activated PBMCs as compared to non-activated PBMCs (taken as 1 on

Y-axis). rhSP-D (10ug/ml) treatment for 24 h downregulated transcript of CTLA4 alone when compared to untreated. Each bar represents the mean ± S.D of three independent experiments. * indicates statistical significance and **p<0.01 relative to rhSP-D untreated controls.

B. Expression of CTLA4 as analysed by flow cytometry. The black line histogram indicates CTLA4 expression on PHA-activated PBMCs (56.5%) and the grey line indicates expression by rhSP-D treated PBMCs (89.8%) The experiment is a representative of five independent experiments.

Fig. 5 rhSP-D treatment results in reduction in viability of PBMCs.

A. Effect of rhSP-D (10 and 20μg/ml concentration) on activated PBMCs following 24, 48 and 72 h treatment via MTT assay. Each bar represents % viability ± S.D of four independent experiments. * indicates statistical significance and *p<0.05 relative to rhSP-D untreated controls.

B. Effect of rhSP-D on non-activated PBMCs at concentrations 10 and 20μg/ml for 24, 48 and 72h as assessed by MTT assay. Each bar represents % viability ± S.D of four independent experiments.

Fig. 6 Induction of apoptosis in activated PBMCs following rhSP-D treatment

Dot blots showing Annexin V-FITC and PI staining of activated PBMCs at 10μg/ml at 48 and 72 h to evaluate apoptosis of activated PBMCs. Q1 represents necrosis ; Q2 show FITC and PI positivity indicating late apoptosis, Q3 represents FITC positive cells indicating early apoptotic cells; and Q4 is unstained. (i) FSC and SSC scatter (ii) 48h untreated control (iii) 48h rhSP-D treated PBMCs (iv) 72h untreated control (iii) 72h rhSP-D treated PBMCs. The experiment is a representative of three independent experiments.

Fig 7. Effect of anti-CTLA4 antibody on proliferation and apoptosis upon rhSP-D treatment

A. Effect of rhSP-D on proliferating (in the presence of PHA) PBMCs at the 10 and 20μg/ml concentration after 48h in the presence of anti-CTLA4 blocking antibody, assessed by MTT assay. Each bar represents % viability ± S.D of three independent experiments.

B. Effect of rhSP-D on PMA-activated PBMCs at 10 and 20μg/ml concentration after 48h in the presence of anti-CTLA4, assessed by MTT assay. Each bar represents % viability ± S.D of three independent experiments.

Table I. Primers Sequences

Transcripts	Forward Primer (5'-3')	Reverse Primer (5'-3')	Tm (°C)	Product Size (in bp)
TLR 2	GGCCAGCAAATTACCTGTGTG	AGGCGGACATCCTGAACCT	61.8	67
TLR 4	CAGAGTTTCCTGCAATGGATCA	GCTTATCTGAAGGTGTTGCACAT	56	88
MyD 88	CCGCGACGACGTGCTGCT	ACTGGATGTCGCTGGGGCAAT	70	230
CD69	GGTCCTTCCAAGTTCCTGTCC	ATGGCTGTCTGATGGCATTGA	66	140
IL2	CCAAACTCACCAGGATGCTC	CACTTCCTCCAGAGGTTTGAGT	64	107
IL6	AATGAGGAGACTTGCCTGGTG	TGGCATTTGTGGTTGGGTCA	66	202
TNF-α	GATCGGCCCCCAGAGGGAAGAG	GGCATTGGCCCGGCGGTTC	64	169
IL10	CAAGGCCGTGGAGCAGGTGAA	GGTTTCTCAAGGGGCTGGGTCA	68	231
TGF-β	AGGGCTTTCGCCTTAGCGCC	GCCGCACGCAGCAGTTCTTC	66	246
IL23	CTCGGTGAACAACTGAGGGA	GGTGGAATCTCTGCCCACTT	64	90

IL17	CTGAGGACAAGAACTTCCCCC	ACTTTGCCTCCCAGATCACAG	64	174
CTLA4	CTGGCCCTGCACTCTCCTGT	CAGCCTGCCGAAGCACTGTC	68	176
CD28	AAATGACCGCCATCTCCAGC	GGCCATGGCACTGTAAGACT	64	159
CD80	GAAGGGAAAGTGTACGCCCT	TACGTAAAGGGCAAGGTGGG	60	143
CD86	AGAACCAAGAATTCAACTATCGAGT	CGCGTCTTGTCAGTTTCCAG	66	158
18S	GGAGAGGGAGCCTGAGAAAC	CCTCCAATGGATCCTCGTTA	64	174

Fig.   1

(A)
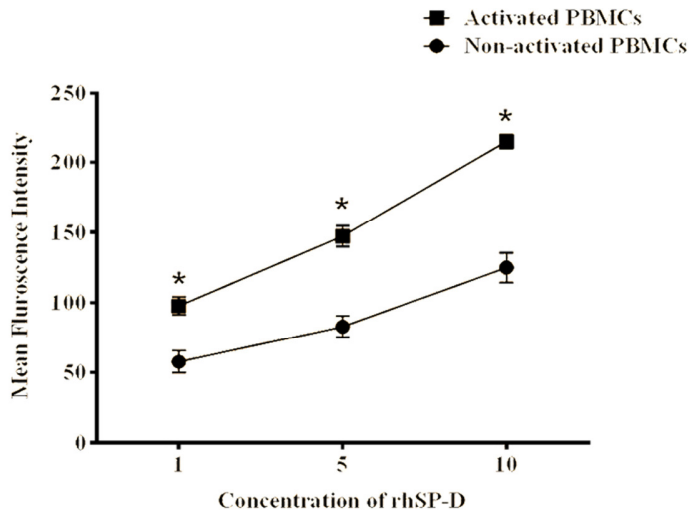
(B)
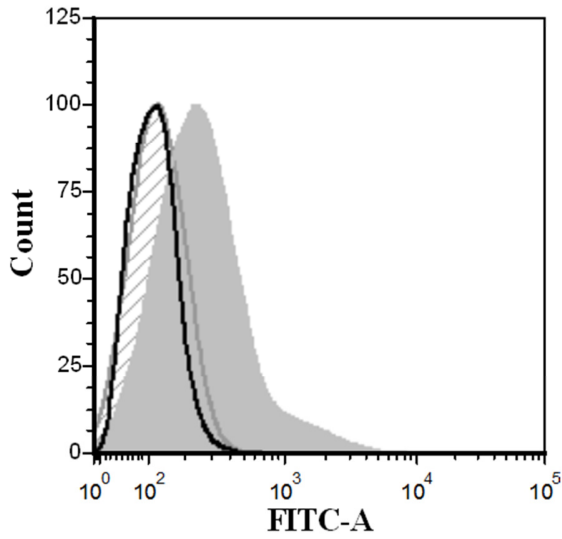
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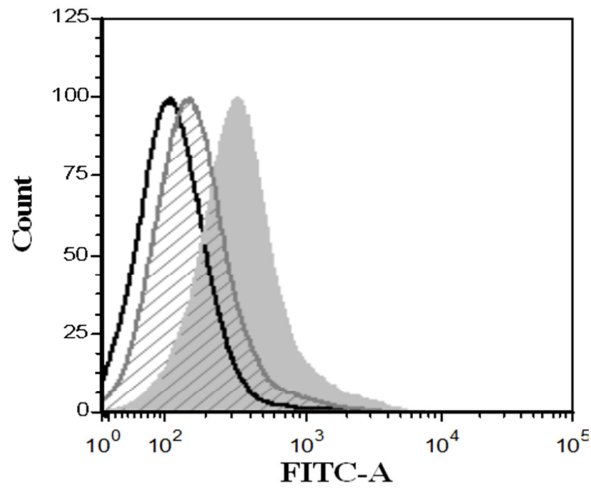
Fig. 2

(A)
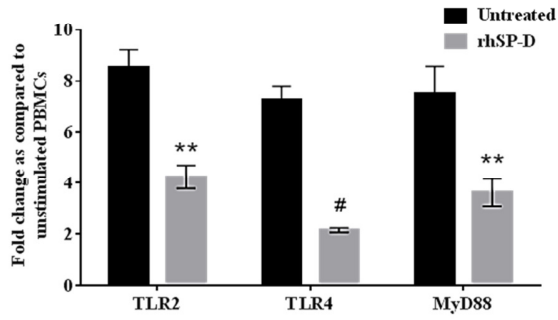
(D) CD11c
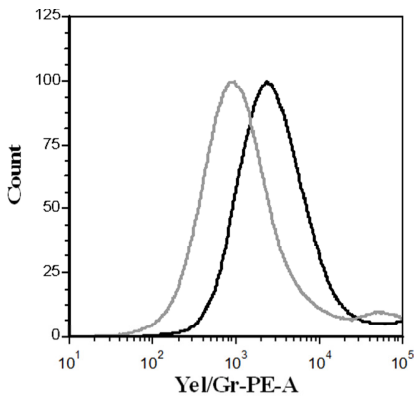
(B) TLR2

(C) TLR4

(E)

Fig. 3.

(A)

(B)

Fig. 4.

(A)

(B)

(C)

(D)

(E)

Fig. 5.

(A)

(B)

Fig. 6.

Fig. 7.