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Surfactant protein 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 epithelial cells lining the mucosal surfaces. The C-type lectin domain of SP-D performs pattern recognition functions while it binds to putative receptors on immune cells to modify cellular functions. Activation of immune cells and increased serum SP-D is observed in a range of patho-physiological conditions including infections. We speculated if SP-D can modulate systemic immune response via direct interaction with activated PBMCs. In this study, we examined interaction of a recombinant fragment of human SP-D (rhSP-D) on PHA-activated PBMCs. We report a significant downregulation of activation receptors such as TLR2, TLR4, CD11c and CD69 upon rhSP-D treatment. rhSP-D inhibited production of Th1 (TNF-α and IFN-γ) and Th17 (IL-17A) cytokines along with IL-6. Interestingly, levels of IL-2, Th2 (IL-4) and regulatory (IL-10 and TGF-β) cytokines remained unaltered. Analysis of co-stimulatory CD28 and co-inhibitory CTLA4 receptors along with their ligands CD80 and CD86 revealed a selective up-regulation of CTLA4 in the lymphocyte subset. rhSP-D induced apoptosis in the activated but not in non-activated lymphocytes. Blockade of CTLA4 inhibited rhSP-D mediated apoptosis of activated lymphocytes, confirming involvement of CTLA4. We conclude that SP-D restores immune homeostasis. It regulates expression of immunomodulatory receptors and cytokines, which is followed by induction of apoptosis in activated lymphocytes. These findings suggest a critical role of SP-D in immune surveillance against activated immune cells.

1. 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 (Thorburn and Hansbro, 2010; Belkaid and Rouse, 2005). Similarly, galectins exert a range of effects on T cell functions such as signaling, activation, apoptosis, and cytokine secretion in addition to Treg expansion (Rabinovich and Toscano, 2009). Collectins, belonging to C-type lectin family have been shown to be involved in modulating the adaptive immune responses (Borron et al., 1998, 2002; Lin et al., 2010; Mukherjee et al., 2012a,b). Surfactant Protein D (SP-D) is an extensively studied collectin in models of infection, allergy and inflammation. SP-D contains an N-terminal triple-helical collagen region and has homotrimeric carbohydrate recognition domain (CRD) globular heads which interact with a range of viral, bacterial and fungal pathogens and bring about clearance mechanisms that include agglutination, enhanced phagocytosis and superoxidative burst (Qaseem et al., 2013; Kishore et al., 2005). SP-D can also bind allergens, inhibit specific IgE binding and basophil degranulation

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(Madan et al., 1997). SP-D knock-out mice show an exaggerated allergic response due to their hypereosinophilic and IL-13 overexpressing phenotypes (Haczku et al., 2006). A trimeric recombinant fragment of human SP-D (rhSP-D) rescued this phenotype (Madan et al., 2001). SP-D binds and influences the adaptive immune response by inhibiting T cell proliferation and activation (Borron et al., 1998, 2002). SP-D deficient mice show a state of persistent T cell activation and inflammation in the lungs in response to exogenous antigens (Fisher et al., 2002). Lin et al. (2010) provided further insight into SP-D mediated downregulation of allergen-induced inflammation and proliferation showing involvement of an inhibitory receptor CTLA4 in mouse T cells. As with T cells, SP-D also binds to several receptors such as TLR2, TLR4 and CD14 on macrophages and regulates inflammatory response (Ohya et al., 2006; Sano et al., 2000). Systemic immune response is the net result of an interaction of its various components with monocytes and lymphocytes being the major players. However, there are no reports on the immuno-modulatory effects of SP-D in the whole PBMC subset.

Increased levels of SP-D at local sites such as synovial fluid of RA patients (Kankakee, 2006), skin lesions of psoriasis (Akman et al., 2008) and prostate tissue infected with *Escherichia coli* (Oberley et al., 2007) have been reported. Circulating levels of SP-D are elevated in a range of infections such as acute lung injury, chronic obstructive pulmonary disease, and allergy (Bratcher and Gaggar, 2014). While increased serum levels of SP-D act as a biomarker of inflammation, we speculated if SP-D can exert its immunomodulatory effects on circulating immune cells. This could be of great importance in understanding the host response in immune-mediated disorders.

In the present study, we have assessed effects of the recombinant fragment of human Surfactant Protein D (rhSP-D) on the expression of different activation markers on PHA-activated PBMCs from healthy donors. rhSP-D bound differentially to activated PBMCs (both lymphocyte and monocyte subset) when compared with non-activated PBMCs. TLR signaling 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, TNF-α, IFN-γ and IL-6. Th2 cytokines were however unaffected. A marked decrease in the level of IL-17A was also observed in activated PBMCs. Furthermore, rhSP-D significantly and selectively upregulated the expression of co-inhibitory signaling receptor, CTLA4, but only in the lymphocyte subset. An extended exposure of activated PBMCs to rhSP-D induced apoptosis; however, the viability of non-activated PBMCs was not affected. This apoptotic effect was restricted to the proliferating lymphocytes as activated yet non-dividing monocytes were not affected. Thus, SP-D appears to modulate immune response mounted by activated PBMCs by regulating expression of pro-inflammatory cytokines and induction of apoptosis of activated lymphocytes. Such suppressive effects of SP-D is likely to impact upon the maintenance of homeostasis, and is relevant in the development of auto-immunity.

2. Material and methods

2.1. 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.

2.2. rhSP-D preparation

The recombinant human SP-D (rhSP-D) used in this study comprises of a functional homotrimeric neck and lectin domain. The rhSP-D was expressed in *E. coli*, purified and characterized as described previously (Shrive et al., 2009; Pandit et al., 2014). 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 5.1 pg/μg of rhSP-D.

2.3. Preparation of PBMCs

Blood (5 ml) was collected from 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 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 min at room temperature. The buffy layer containing PBMCs was separated and washed twice with RPMI 1640 medium containing 10% fetal bovine serum (FBS) and gentamycin (50 μg/ml) (Gibe, Life Technologies, USA). PBMCs (5×10^6 cells) were seeded in a T-25 flask and activated using PHA (5 μg/ml) (Gibco, Life Technologies, USA) for 24 h.

2.4. 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 (Mahajan et al., 2008). Briefly, rhSP-D was dialyzed against FITC-labeling buffer (5 mM boric acid and 2 mM sodium chloride, pH 9.2), followed by incubation with FITC for 2 h at room temperature. The labeled proteins were dialyzed against buffer. The labeled protein was quantitated and flurochrome to protein ratio of 5–6:1 was considered optimum for flow cytometric studies. FITC-labeled rhSP-D was incubated with unstimulated and activated PBMCs in the presence of 2 mM $CaCl_2$ and/or 5 mM EDTA at 4 °C for 1 h. Cells were washed thrice by centrifuging at 1200 rpm to remove unbound rhSP-D, fixed with 4% PFA and analysed by flow cytometry.

2.5. Confocal microscopy

To understand whether rhSP-D binding to lymphocytes and monocytes was a surface phenomenon or cytoplasmic localisation, we performed confocal microscopy studies. CD14 positive monocytes were isolated from PBMCs using Dynabeads® Untouched™ Human Monocytes Kit (Life Technologies, USA). Lymphocytes were separated by incubating PBMCs in a tissue culture treated flask for 24 h in RPMI 1640 medium supplemented with 10% FBS. After incubation, the non-adherent cells were collected as lymphocyte population. Lymphocytes and monocytes were smeared on poly-lysine coated glass slides and allowed to dry by cytospin. Smears were then incubated with FITC-labelled rhSP-D in the presence of 2 mM $CaCl_2$ for 1 h at room temperature. Unbound rhSP-D was washed by PBS and the nuclei were counterstained with 6-diamino-2-phenylindole (Sigma, USA) and mounted in vector shield (Vector laboratories, UK). Smears were observed under confocal microscope (Zeiss, Germany).

2.6. rhSP-D treatment of activated PBMCs

Activated PBMCs (stimulated by PHA for 24 h) were washed and resuspended in a fresh complete RPMI 1640 medium. 5×10^5 cells were seeded in a 12-well tissue culture plate (Nalgene Nunc™) and treated with indicated concentrations of rhSP-D for 24 h. After