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Surfactant Protein D-Mediated Decrease of Allergen-Induced Inflammation Is Dependent upon CTLA4

Ko-Wei Lin,*1 Kai Yu Jen,*1 Carlos Jose Suarez,* Erika C. Crouch,†
David L. Perkins,‡ and Patricia W. Finn*

Pulmonary surfactant protein D (SP-D), a member of the collectin family, is an innate immune molecule critical for defense that can also modulate adaptive immune responses. We previously showed that SP-D-deficient mice exhibit enhanced allergic responses and that SP-D induction requires lymphocytes. Thus, we postulated that SP-D may decrease adaptive allergic responses through interaction with T cells. In this study, we used two forms of SP-D, a dodecamer and a shorter fragment containing the trimeric neck and carbohydrate recognition domains (SP-D NCRD). Both forms decreased immune responses in vitro and in a murine model of pulmonary inflammation. SP-D NCRD increased transcription of CTLA4, a negative regulator of T cell activation, in T cells. SP-D NCRD no longer decreased lymphoproliferation and IL-2 cytokine production when CTLA4 signals were abrogated. Administration of SP-D NCRD in vivo no longer decreased allergen induced responses when CTLA4 was inhibited. Our results indicate that SP-D decreases allergen responses, an effect that may be mediated by increase of CTLA4 in T cells. The Journal of Immunology, 2010, 184: 6343–6349.

Surfactant protein D (SP-D), a member of the collectin family, is primarily produced by alveolar type II cells and nonciliated Clara cells in the lung (1). The structure of SP-D includes four distinct domains, including an N terminus, collagen-like domain, neck domain, and C-terminal carbohydrate recognition domain. SP-D was originally defined as a host defense innate immune molecule (2). Recently, SP-D has been shown to play a role in allergic inflammation by modulating adaptive immune lymphocyte responses. SP-D–deficient mice exhibit enhanced allergic responses, suggesting that SP-D has immunosuppressive properties (3). In addition, allergic and other inflammatory responses are accompanied by increased bronchoalveolar lavage (BAL) and serum SP-D in animal models and human asthmatic patients (4–9). SP-D administration inhibits murine allergic airway responses (10, 11). In lymphocytes isolated from asthmatic children, treatment of SP-D can suppress lymphocyte proliferation in the late phase of bronchial inflammation (12). In lymphocyte-deficient recombinase-activating gene-deficient mice, SP-D is not increased after induction of al- lergen, suggesting that SP-D induction requires lymphocytes (5). Although SP-D modulation of T cells has not been well defined, SP-D has been shown to suppress activated CD3+/CD4+ T cell proliferation in the absence of accessory cells (13).

Activated T cells express negative regulators including CTLA4 (14). Triggering of CTLA4 signaling decreases T cell proliferation and cytokine production by recently activated T cells in inflammatory immune responses (15, 16). Because CTLA4 inhibits T cell activation and plays a central role in maintaining T cell homeostasis, we asked whether the innate molecule SP-D can also impact T cells and influence CTLA4. Our results showed that SP-D decreases allergic inflammation, an effect that may be mediated by an increase of CTLA4 gene expression in T cells, suggesting a potential pathway by which innate immunity interacts with adaptive immunity.

Materials and Methods

Mice
Six- to 8-wk-old BALB/c male mice were purchased from Harlan Laboratories (Indianapolis, IN). The mice were maintained according to the guidelines of the Animal Welfare Program from School of Medicine, University of California at San Diego.

Recombinant human SP-D

The full-length human SP-D dodecamer (SP-D dodec) and a recombinant human N-terminally His-tagged, trimeric neck and carbohydrate recognition domain fusion protein (SP-D NCRD) was generated and purified as previously described (17). SP-D NCRD shows normal folding of the lectin domain and retains the ligand binding properties of the native protein (18). Residual endotoxin levels were quantified using an endpoint chromogenic assay (QCL-1000; Cambrex, East Rutherford, NJ), and only preparations containing <0.2 ng endotoxin per milligram SP-D NCRD protein were used.

Abs
Mouse anti-CTLA4 (UC10-4F10-11) (aCTLA4) and control Ab hamster Ig were purchased from eBioscience (San Diego, CA).

Cell lines and treatments

Murine splenocytes and EL4 cells (murine thymoma cell line) were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. Murine spleen cells were treated with SP-D NCRD (10 μg/ml) for 24 h at a concentration of 10⁶ cells per milliliter. EL4 cells were treated with SP-D NCRD (10 μg/ml) for 24 h at a concentration of 2 × 10⁶ cells per milliliter. For proliferation and cytokine in vitro assays, murine spleen cells...
were also treated with 5 µg/ml Con A (Sigma-Aldrich, St. Louis, MO) and 5 µg/ml aCTLA4 or remain untreated.

**OVA sensitization and challenge**

Mice were sensitized and challenged with the allergen OVA as previously described (16). OVA mice were sensitized via i.p. injection with 10 µg chicken OVA (Sigma-Aldrich) and 1 mg Al(OH)₃ (alum; Sigma-Aldrich) in 0.2 ml PBS (Sigma-Aldrich), followed by an injection on day 7 with identical reagents. PBS mice received 1 mg alum in 0.2 ml PBS without OVA. On days 14–20, mice received challenges with 6% OVA or PBS, respectively, for 20 min via an ultrasonic nebulizer (model 5000; DeVilbiss). All of the groups were sacrificed on day 21 and analyzed for the allergic parameters described below.

**Treatment protocols**

SP-D dodec (3 µg) or SP-D NCRD (3 µg) was administered to mice intratracheally (i.t.) on days 13, 14, and 19. aCTLA4 (100 µg) was administered i.p. 1 d before sensitization (day −1).

**BAL analysis**

Each mouse underwent BAL as previously described (16). BAL cells were pelleted, and the supernatant was stored at −80°C. Cells were resuspended in RPMI 1640 (5 × 10⁵ cells per milliliter). Slides for differential cell counts were prepared with Cytospin (Thermo Scientific, Waltham, MA) and fixed and stained with Diff-Quik (IMEB, San Marcos, CA). For each sample, an investigator blinded to the treatment groups performed two counts of 100 cells.

**ELISA**

IL-13 was measured by ELISA according to the manufacturer’s specifications (R&D Systems, Minneapolis, MN). Briefly, samples of BAL fluid were aliquoted in duplicate into 96-well plates (50 µl per well) precoated with Ab to specific cytokines and assayed according to the manufacturer’s instructions. OD was measured at 450 nm. Cytokine concentrations were determined by comparison with known standards.

**Cytokine assays**

IL-5 and IL-2 from supernatant was assayed with LINCOplex mouse cytokine assays following manufacturer’s instructions (LINCOResearch, St. Charles, MO). The assay is based on conventional sandwich assay technology. The Ab specific to each cytokine is covalently coupled to Luminesin microspheres, with each Ab coupled to a different microsphere uniquely labeled with a fluorescent dye. The microspheres are incubated with standards, controls, and samples (25 µl) in a 96-well microtiter filter plate for 1 h at room temperature. After incubation, the plate is washed to remove excess reagents. Detection Ab then is added in the form of a mixture containing each of the eight Abs. After 30 min of incubation at room temperature, streptavidin–PE is added for an additional 30 min. After a final wash step, the beads are resuspended in buffer and read on the Luminesin 100 instrument (Luminesin, Austin, TX) to determine the concentration of the cytokines of interest. All of the specimens received were tested in replicate wells. Results were reported as the mean of the replicates.

**Serum IgE**

Total serum IgE levels were determined by ELISA as previously described (16). Total serum IgE concentrations were calculated by using a standard curve generated with a commercial IgE standard (BD Pharmingen, San Diego, CA).

**Lymphocyte proliferation**

The proliferation of murine spleen cells (2 × 10⁵ cells per well) was determined using a colorimetric immunoassay for the quantification of cell proliferation, based on the measurement of BrdU incorporation during DNA synthesis (Roche Diagnostics, Indianapolis, IN). The BrdU ELISA was performed according to the manufacturer’s instructions. Briefly, cells were pulsed with 10 µl per well of 100 µM BrdU solution for 1 h at 37°C. The cells were then stained with anti-CD3 (PECy7), anti-CD4 (allophycocyanin), or anti-His (FITC) Abs or their isotype control Abs (eBioscience). The binding of His-tagged SP-D NCRD to spleen cells is assessed by flow cytometry in the CD3⁺ CD4⁺ T cells and is analyzed by FlowJo software, version 8 (Tree Star, Ashland, OR).

**Binding assay**

Murine spleen cells (BALB/c) were incubated with or without His-tagged SP-D NCRD (2 µg/ml) for 1 h at 37°C. The cells were then stained with anti-CD3 (PECy7), anti-CD4 (allophycocyanin), or anti-His (FITC) Abs or their isotype control Abs (eBioscience). The binding of His-tagged SP-D NCRD to spleen cells is assessed by flow cytometry in the CD3⁺ CD4⁺ T cells and is analyzed by FlowJo software, version 8 (Tree Star, Ashland, OR).

**Real-time quantitative RT-PCR**

Total RNA was isolated with TRI reagent (Sigma-Aldrich). Isolated RNA was reverse-transcribed with SuperScript II RNase reverse transcriptase (Invitrogen, Carlsbad, CA). Specific primer pairs for GAPDH (housekeeping gene) and CTLA4 were designed with Primer Express software (Applied Biosystems, Foster City, CA). Direct detection of the PCR product was monitored by measuring the increase in fluorescence caused by the binding of SYBR Green to dsDNA. With 5 µl cDNA, 5 µl primer, and 10 µl SYBR Green PCR master mix (Applied Biosystems) per well, the gene-specific PCR products were measured continuously by means of a GeneAmp 5700 Sequence Detection System (Applied Biosystems) during 40 cycles. The threshold cycle of each target product was determined and set in both directions for the amplification plot of GAPDH. The difference in threshold cycle values of two genes was used to calculate the fold difference as previously described.

**Cloning of the CTLA4 promoter and construction of deletion constructs**

The mouse CTLA4 promoter containing −1221 bp from the transcription start site was cloned into the pXP2 basic vector, which contains the luciferase reporter gene, as described previously (19). The luciferase construct that contains the 5’ deletion constructs −335 (p335) was sequenced in both directions by dideoxy sequencing (19).

**Transfections and reporter gene assays**

CTLA4 promoter luciferase reporter constructs (described above; 2 µg) and β-galactosidase reporter gene (pGK; 1 µg) were added to 2 × 10⁶ EL4 cells resuspended in 100 µl Nucleofector solution (Lonza, Wiltshire, WI) and electroporated using the C-9 program of the Nucleofector. After 24 h, cells were lysed in reporter lysis buffer (Promega, Madison, WI). Then, 10 µl cell lysate was mixed with 100 µl luciferase assay reagent (Promega), and luciferase activity was measured by a luminometer (Promega, Sunnyvale, CA). Luciferase activity was normalized for transfection efficiency by β-galactosidase activity measured with Galacto-Light systems according to the manufacturer’s instructions (Applied Biosystems). Fold activation was calculated as the ratio of luciferase versus β-galactosidase activity.

**Statistical analysis**

ANOVA was performed by GraphPad Prism software. Bonferroni correction for statistical adjustment of the p value for multiple comparisons was applied as a post hoc analysis. Data are reported as mean ± SEM. Statistical significance was defined by p < 0.05.

**Results**

**SP-D decreases allergen-induced immune responses**

We asked whether administration of SP-D, using full-length SP-D dodec, would impact allergic immune responses in vivo. We examined a murine model of allergen OVA-induced pulmonary inflammation (Fig. 1, schema). As expected, OVA sensitized and challenged mice exhibited a significant increase in BAL IL-13, IL-5, and IFN-γ (p < 0.05; Fig. 2A). Administration of SP-D dodec significantly decreased allergen-induced BAL IL-13 (p < 0.05; Fig. 2A).

**SP-D and T cell interactions**

**SP-D dodec and SP-D NCRD decrease lymphocyte activation.** T cells play a critical role in asthma pathogenesis (20). To investigate whether SP-D would affect T cell function, two forms of SP-D, full-length SP-D dodec and a shorter fragment of SP-D NCRD, were tested in lymphocyte proliferation. Both SP-D dodec
and NCRD significantly decreased lymphocytic proliferation in spleen cells activated with mitogen Con A (p < 0.05; Fig. 2B, 2C).

**SP-D NCRD interacts with murine T cells.** We next asked whether SP-D NCRD might interact with T cells. SP-D NCRD fusion protein (as described in Materials and Methods) contains a His tag, a fusion protein useful for tracking binding to cells. When His-tagged SP-D NCRD was incubated with spleen cells, a small increase of FITC signal was detected by flow cytometry in CD3+CD4+ mouse T cells, suggesting that SP-D NCRD may interact with T cells (Fig. 3). The mean fluorescence intensities were 22.86 and 3.6, respectively. In the absence of SP-D NCRD, no binding of the Ab to CD3+CD4+ T cells was observed, supporting the concept that SP-D NCRD may bind to CD3+CD4+ T cells.

**SP-D NCRD induces CTLA4 promoter activation and transcription in T cells.** On the basis of SP-D suppression of T cell activation, we evaluated SP-D NCRD modulation of T cell markers. We focused on CTLA4, a negative regulator of T cell activation, found primarily in T cells. Spleen cells and T cells (EL4) were activated with mitogen Con A and treated with SP-D NCRD. In primary spleen cells, SP-D NCRD augmented the levels of CTLA4 mRNA (11-fold relative to untreated, p < 0.05) (Fig. 4A). Also, SP-D NCRD increased the levels of CTLA4 mRNA in T cells (3-fold relative to untreated, p < 0.05) (Fig. 4B). We have previously shown that adaptive stimuli increase CTLA4 gene expression and are regulated at the transcriptional level (21). We asked whether SP-D NCRD would influence CTLA4 transcriptional activation. We examined a CTLA4 promoter construct consisting of 335 bp (p335) relative to the transcription start site. SP-D NCRD induced CTLA4 promoter region activation, as determined by a significant increase in the luciferase activity relative to untreated (p < 0.05; Fig. 4C).

**Administration of aCTLA4: influence on SP-D NCRD-mediated immune responses**

We next asked whether SP-D repressing of allergic responses might be mediated by CTLA4. We examined the effect of aCTLA4 on SP-D NCRD-mediated lymphoproliferation and IL-2 secretion (Fig. 5). SP-D NCRD significantly decreases lymphoproliferation and IL-2 production from activated splencytes (p < 0.05; Fig. 5). In contrast, SP-D NCRD no longer decreases lymphoproliferation and IL-2 production in the presence of aCTLA4. The abrogation of the ability of SP-D NCRD to inhibit lymphoproliferation and IL-2 production suggests that SP-D may decrease T cell activation through modulation of CTLA4 pathways.

To test the effect of CTLA4 blockade on SP-D modulation of allergic responses in vivo, we administered aCTLA4 to allergen sensitized and challenged mice (Fig. 6). Administration of aCTLA4 without SP-D NCRD resulted in maintained allergic responses (Fig. 6), which was not modified by control Ig (data not shown). aCTLA4 administration at different time points (i.e., day −1/7 and day −1/13) had similar effects (data not shown). Administration of aCTLA4 with SP-D NCRD results in maintained or enhanced allergen-induced eosinophilia or IgE (Fig. 6A, 6B, respectively). SP-D NCRD administration significantly decreased allergen-induced IL-13 and IL-5 (Fig. 6C, 6D). Administration of aCTLA4 with SP-D NCRD did not result in decreased allergen-induced IL-13 and IL-5 (Fig. 6C, 6D), in contrast to what was observed with administration of SP-D NCRD alone.

**Discussion**

An increase in allergic inflammatory diseases, including asthma, has necessitated increased investigation of allergic immune mechanisms. In addition to analysis of adaptive Ag-dependent immunity in asthma, recent scrutiny has turned to innate non-Ag-dependent immunity. Specifically, SP-D is critical for innate defense against exogenous molecules derived from pathogens and...
FIGURE 3. SP-D NCRD interacts with T cells. Murine spleen cells (BALB/c) incubated with or without SP-D NCRD-containing a 6X His tag (2 μg/ml) were stained with anti-CD3, anti-CD4, and anti-His Abs. The binding of His-tagged SP-D NCRD to spleen cells was assessed by flow cytometry in the CD3+CD4+ T cells. When His-tagged SP-D NCRD was incubated with spleen cells, higher levels of FITC signal were detected by flow cytometry (gray) compared with untreated cells (black line). The figure is representative of three independent experiments.

FIGURE 4. SP-D NCRD increases CTLA4 mRNA levels and promoter activity in T cells. A and B, Murine spleen cells (BALB/c) (A) and murine T cells (EL4) (B) were treated with SP-D NCRD (500 ng/ml) for 24 h. RNA was purified and analyzed by real-time PCR. Data are expressed as fold difference to untreated murine spleen cells and T cells. Values are mean ± SEM for spleen cells (n = 3) and ±SEM for T cells (n = 6). *Untreated versus SP-D NCRD (p < 0.05). C, T cells (EL4) were transfected by nucleofection with a CTLA4 promoter luciferase-based reporter construct (−335 relative to the transcription starting site) and β-galactosidase reporter gene (pGK) construct. After 24 h, cells were treated with SP-D NCRD (500 ng/ml) for 24 h followed by analysis of reporter gene expression using a luminometer. Fold activation of relative light units is calculated as the ratio of luciferase activity versus β-galactosidase activity. Fold induction is the ratio SP-D NCRD-treated to untreated T cells. Data are mean ± SEM (n = 4). *Untreated versus SP-D NCRD (p < 0.05).

Activation of lymphocytes is important during allergic immune responses. Several studies have shown that SP-D inhibits lymphocyte activation. SP-D–deficient mice exhibit a persistent T cell activation (25). Full-length recombinant rat SP-D dodec inhibits IL-2 production and proliferation of human PBMCs stimulated by lectin and anti-CD3 (26). Native SP-D suppresses lymphocyte proliferation of PBMCs from children with asthma in the late phase of bronchial inflammation (12). SP-D can directly suppress CD3+/CD4+ T cell proliferation without accessory cells (13). SP-D and T cell interactions have been previously analyzed using SP-D purified from human BAL incubated with a T cell line (Jurkat) (27). SP-D appeared to interact with late apoptotic cells in a Ca2+- and maltose-independent fashion, suggesting that the lectin site of SP-D may differentially impact IgE remains to be determined. There are also prior examples of the divergent effects on allergic responses by different reagents, which may be due to timing or systemic effects (24). Related to SP-D impact on allergic responses, Haczku et al. (6) showed that allergen-induced SP-D is IL-4/IL-13–dependent, preventing further activation of sensitized T cells. SP-D administration in our study significantly decreased allergen-induced IL-13. SP-D also decreases IL-4, albeit not significantly (data not shown). SP-D is not induced in lymphocyte-deficient recombinase-activating gene-deficient mice, suggesting that the allergen-induced increase of SP-D requires T or B lymphocytes (5).
To investigate the potential pathways by which SP-D NCRD modulates T cell activation, we focused on CTLA4, a negative regulator of T cells. Previous studies indicate that polymorphisms in the CTLA4 gene are associated with asthma, bronchial hyperresponsiveness, and an increase of serum IgE (28–30). A polymorphism found in the CTLA4 promoter (2318C/T) is associated with an increase of total serum IgE in patients with asthma (30). Polymorphisms identified in the CTLA4 gene are also involved in the regulation of IgE levels and development of allergic asthma (29, 30). Because CTLA4 is involved in maintaining the balance of the immune system, the disruption of CTLA4 signaling transduction by either gene polymorphisms or blockade may foster allergic inflammation. We and others have shown that blockade of CTLA4 signals in vivo enhances or maintains airway inflammation (31, 32). Overall, these findings suggest that modulation of CTLA4 signaling may impact T cells responses, thus modulating pulmonary allergic inflammation.

T cell activation is an adaptive immune response that plays an essential role in allergic inflammation. We previously showed that modulation of adaptive immunity can regulate CTLA4 function and decrease allergic inflammatory responses in vivo (16). We tested whether SP-D, an innate molecule, regulates CTLA4 signaling in T cells. Inhibition of CTLA4 signaling affected the ability of SP-D NCRD to abrogate T cell activation. CTLA4 activation during T cell differentiation inhibits polarization of naive CD4+ T cells to a Th2 cell subset as well as Th2 cytokine production (33–35). These data are consistent with our findings that SP-D no longer decreases allergen-induced immune responses when CTLA4 signaling is blocked. One interpretation is that SP-D may interact with activated T cells and enhance CTLA4 activation, leading to suppression of T cell cytokine production (33). Overall, SP-D–mediated modulation of CTLA4 may be a novel immune regulatory pathway that decreases allergic inflammatory responses.

We have previously determined regulatory elements in the CTLA4 promoter for an adaptive induced increase of CTLA4 transcription (19, 21). We identified a 335-bp upstream region of the CTLA4 promoter (2318 to 262) that is important for inducible CTLA4 expression in activated T cells (21). Analysis of this CTLA4 upstream promoter region reveals potential DNA binding sites for NFAT, NF-κB, AP-1, as well as NF-IL2A (Oct-1) and IL6-REBP (16, 21). Interestingly, SP-D also increases the same 335-bp regulatory region of the CTLA4 promoter in T cells as is increased by adaptive stimuli (Fig. 4C). SP-D may interact with T cells, and CTLA4 expression appears to be induced after this interaction. SP-D NCRD has been shown to bind to the extracellular domains of soluble forms of recombinant TLR2 and TLR4, with a different mechanism than its binding to phosphatidylinositol and LPS (36).

Prior analysis of transcription factors suggests potential pathways for SP-D–induced CTLA4 expression. SP-D negatively regulates NF-κB in alveolar macrophages (37). SP-D also suppresses Der FIGURE 5. SP-D NCRD no longer decreases lymphoproliferation and IL-2 when aCTLA4 is administered. Murine spleen cells (BALB/c) were activated with Con A (1 μg/ml) for 12 h and treated with SP-D NCRD (10 μg/ml) for 24 h. aCTLA4 (10 μg) was administered (i.p.) 12 h before SP-D NCRD administration. Proliferation was measured by using BrdU labeling (A), and supernatant was assayed for IL-2 by a bead-based multiplex sandwich immunoassay (B). Values are mean ± SEM. †Con A versus untreated (p < 0.05); *Con A NCRD versus Con A (p < 0.05); #Con A NCRD aCTLA4 versus Con A NCRD (p < 0.05).

FIGURE 6. SP-D NCRD no longer decreases allergen-induced responses when aCTLA4 is administered. Mice (BALB/c) were sensitized and challenged with allergen OVA. aCTLA4 (100 μg) or control Ab hamster Ig (100 μg) was administered i.p. at day −1, and SP-D NCRD was administered i.t. at days 13, 14, and 19. Cell counts were determined by differential staining of cells isolated from BAL fluid. Total serum IgE and BAL IL-13 cytokine were measured by ELISA. BAL IL-5 was assayed by a bead-based multiplex sandwich immunoassay: BAL eosinophilia (A); serum IgE (B); BAL IL-13 (C); BAL IL-5 (D). Data are shown as mean ± SEM (n = 6–15 per group). †OVA versus PBS (p < 0.05); *OVA NCRD versus OVA (p < 0.05); #OVA NCRD aCTLA4 versus OVA NCRD (p < 0.05).
p-activated induction of macrophages through suppression of the CD14/TLR signaling pathway (38). NFAT binding induces CD1A4 expression (19, 39, 40). We have previously shown that a TLR2 agonist induces CD4A4 (41). TLR4 signaling may increase CD4A4 in T cells because CD4A4 is decreased in TLR4-defective CD4+ T cells (42). We speculate that SP-D may bind to known (TLR2/TLR4) or unknown receptors, perhaps through its carbohydrate recognition domain, which leads to modification of transcription factor binding (e.g., NF-kB or NFAT) to the CD4A4 promoter. The specific pathways by which SP-D induces CD4A4 remain to be determined.

SP-D inhibits allergen-induced pulmonary responses and increases CD4A4 mRNA levels and promoter activation in T cells. Blockade of CD4A4 decreases the ability of SP-D to inhibit allergen-induced immune responses. Taken together, these results indicate that SP-D regulates CD4A4 and modulates allergen-induced pulmonary immune responses. Future investigations regarding SP-D modulation of T cells may be applicable to distinct therapeutic approaches for allergic diseases.

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Disclosures

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