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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: 000–000.

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 allergen, 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 end-point 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

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Abbreviations used in this paper: aCTLA4, anti-CTLA4 mAb; alum, Al(OH)₃; BAL, bronchoalveolar lavage; dodec, dodecamer; i.t., intratracheally; NCRD, neck and carbohydrate recognition domain; SP-D, surfactant protein D.

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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/d via an ultrasonic nebulizer (model 5000; DeVilbiss). All of the groups were sacrificed at 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 (5×10^5 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 (LINCO Research, St. Charles, MO). The assay is based on conventional sandwich assay technology. The Ab specific to each cytokine is covalently coupled to Luminex 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 Luminex 100 instrument (Luminex, 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 (2×10^5 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 during the last 18 h of Con A stimulation. Seventy-two hours after the initial stimulation, plates were centrifuged, and cells were denatured with FixDenat solution, then incubated for 120 min with 1:100 diluted mouse anti-BrdU mAbs conjugated to peroxidase. After the Ab conjugate was removed, substrate solution was added for 20 min, and the reaction was stopped by adding 1 M H₂SO₄ solution. The absorbance was measured within 30 min at 370 nm with a reference wavelength at 690 nm using an ELISA plate reader.

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 (house-keeping 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 relation to 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 2×10^6 EL4 cells resuspended in 100 µl Nucleofector solution (Lonza, Walkersville, MD) 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 when compared with PBS control mice (*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

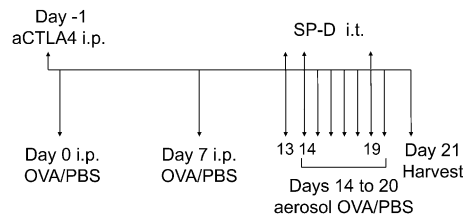


FIGURE 1. Schema of a murine model of allergen-induced responses. Wild-type (BALB/c) mice received allergen OVA or PBS with adjuvant alum by i.p. sensitization on days 0 and 7. Mice were challenged with OVA or PBS by aerosol on days 14–20 and harvested on day 21. In separate experiments, SP-D (either dodec or NCRD) was administered i.t. (3 μ g) on days 13, 14, and 19. In additional experiments, aCTLA4 or control Ig was injected i.p. on day –1 prior to allergen sensitization.

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⁺ murine 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 repression 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 splenocytes ($p < 0.05$; 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 re-

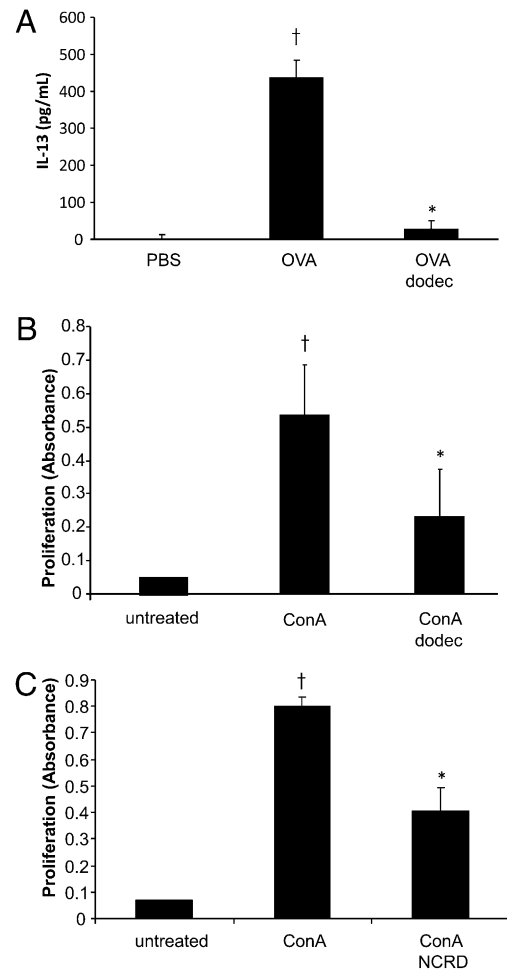


FIGURE 2. SP-D decreases allergen-induced IL-13 in vivo and lymphocyte proliferation in vitro. **A**, Mice (BALB/c) were sensitized and challenged with allergen OVA as described in *Materials and Methods*. Full-length SP-D dodec was administered i.t. (3 μ g) on days 13, 14, and 19. BAL was harvested on day 21 for analysis of IL-13 cytokine by ELISA. Data is shown as geometric mean $\pm$ SEM ($n = 6$ per group for IL-13). [†]OVA versus PBS; *OVA dodec versus OVA ($p < 0.05$). **B** and **C**, Murine splenocytes (BALB/c) were stimulated with mitogen Con A (1 μ g/ml) for 12 h, and treated with SP-D dodec or SP-D NCRD (10 μ g/ml) for 24 h. Proliferation was measured by using BrdU labeling. Values are mean $\pm$ SEM. [†]Con A versus untreated ($p < 0.05$); *Con A dodec or Con A NCRD versus Con A ($p < 0.05$).

sponses (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

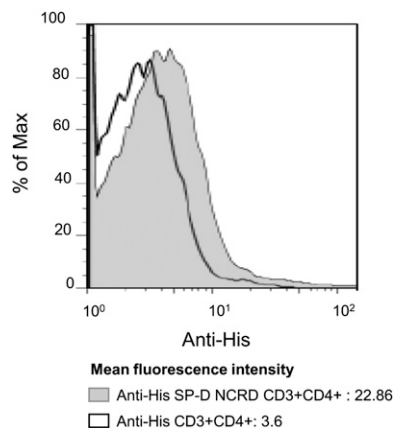


FIGURE 3. SP-D NCRD interacts with T cells. Murine spleen cells (BALB/c) incubated with or without SP-D NCRD-containing a 6X His tag (2 μ g/ml) were stained with anti-CD3, anti-CD4, and anti-His Abs. The binding of His-tagged SP-D NCRD to spleen cells was assessed by flow cytometry in the CD3⁺CD4⁺ T 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.

may be crucial for immunomodulation of adaptive immune responses (3, 5). In this study, we investigated the role of SP-D in adaptive allergic responses. We administered SP-D in vivo and in vitro using two forms of SP-D and focused on a fragment of SP-D containing the functionally important C-terminal lectin domains (SP-D NCRD). SP-D decreased lymphoproliferation and IL-2 cytokine production as well as allergen-induced cytokine levels. Our data suggest that SP-D may modulate T cell function through CTLA4, a negative regulator of T cell activation (19, 21). SP-D regulated CTLA4 transcription in T cells. SP-D no longer decreased lymphoproliferation and IL-2 cytokine production when CTLA4 signals were abrogated. Furthermore, SP-D no longer decreased allergen-induced responses when CTLA4 was inhibited in a murine allergic model. Taken together, these data suggest that SP-D modulation of allergic responses may be mediated by CTLA4.

We have previously found that SP-D-deficient mice exhibit enhanced allergic responses, consistent with an immunosuppressive role (3). Allergic and other inflammatory responses are accompanied by increased SP-D (5, 6). Immunostaining of SP-D is also increased in nonciliated epithelial cells of noncartilaginous airways in a murine allergic model in three murine strains (5). In our study, SP-D administration to OVA sensitized and challenged mice decreased allergic responses. One exception to SP-D effects is that allergen-induced IgE was not decreased by SP-D in our model, whereas other groups have shown that SP-D can reduce allergen-induced IgE (10, 22, 23). Whether use of different forms of SP-D (i.e., recombinant 60 kDa fragment of human SP-D or purified native SP-D), different allergens (i.e., house dust mite (Der 9) or *Aspergillus fumigatus*), or mouse strains (i.e., C57BL/6) 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 recombination-activating gene-deficient mice, suggesting that the allergen-induced increase of SP-D requires T or B lymphocytes (5).

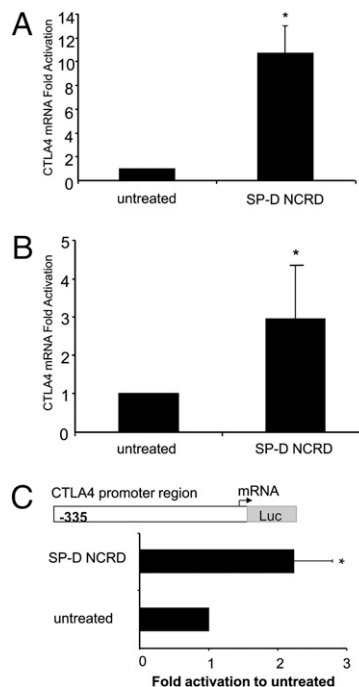


FIGURE 4. SP-D NCRD increases CTLA4 mRNA levels and promoter activity in T cells. *A* and *B*, Murine spleen cells (BALB/c) (*A*) and murine T cells (EL4) (*B*) were treated with SP-D NCRD (500 ng/ml) for 24 h. RNA was purified and analyzed by real-time PCR. Data are expressed as fold difference to untreated murine spleen cells and T cells. Values are mean $\pm$ SEM for spleen cells ($n = 3$) and $\pm$ 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 $\pm$ 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 was not involved. In this study, we used a SP-D NCRD fusion protein containing a His tag useful for tracking binding to cells and analyzed its binding with murine spleen cells. We persistently observed an obvious but small increase in fluorescence intensity. In the absence of SP-D NCRD, no binding of the Ab to CD3⁺CD4⁺ T cells was observed. SP-D NCRD appeared to bind to CD3⁺CD4⁺ T cells in vitro. The specificity of the binding and whether these interactions are direct or indirect require further investigation. The results are consistent with the concept that SP-D may suppress lymphocyte proliferation by interaction with T cells. These data suggest a novel mechanism by which SP-D may bypass the mid-dle men (e.g., APCs) and perhaps modulate T cells directly.

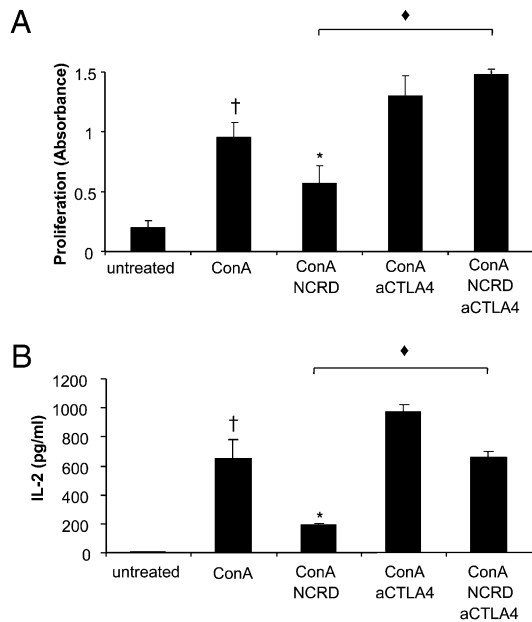


FIGURE 5. SP-D NCRD no longer decreases lymphoproliferation and IL-2 when aCTLA4 is administered. Murine spleen cells (BALB/c) were activated with Con A (1 μ g/ml) for 12 h and treated with SP-D NCRD (10 μ g/ml) for 24 h. aCTLA4 (10 μ g) was administered (i.p.) 12 h before SP-D NCRD administration. Proliferation 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 $\pm$ 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 ($p < 0.05$).

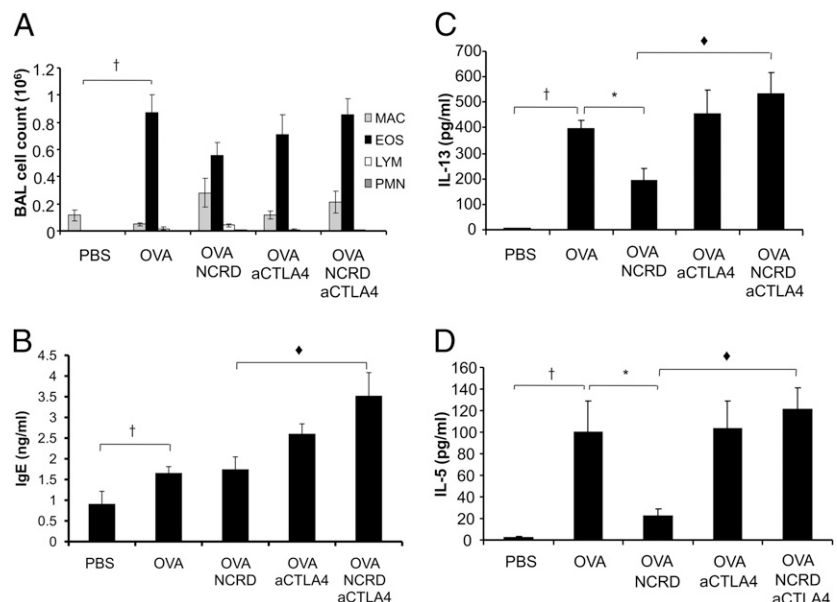
To investigate the potential pathways by which SP-D NCRD modulates T cell activation, we focused on CTLA4, a negative regulator of T cells. Previous studies indicate that polymorphisms in the CTLA4 gene are associated with asthma, bronchial hyper-responsiveness, and an increase of serum IgE (28–30). A polymorphism found in the CTLA4 promoter (–318C/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 CTLA4 promoter region that is important for inducible CTLA4 expression in activated T cells (21). Analysis of this CTLA4 upstream promoter region (–335 to –62) reveals potential DNA binding sites for NFAT, NF- κ B, 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- κ B in alveolar macrophages (37). SP-D also suppresses Der

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 μ g) or control Ab hamster Ig (100 μ g) 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 $\pm$ 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-induced activation of macrophages through suppression of the CD14/TLR signaling pathway (38). NFAT binding induces CTLA4 expression (19, 39, 40). We have previously shown that a TLR2 agonist induces CTLA4 (41). TLR4 signaling may increase CTLA4 in T cells because CTLA4 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- κ B or NFAT) to the CTLA4 promoter. The specific pathways by which SP-D induces CTLA4 remain to be determined.

SP-D inhibits allergen-induced pulmonary responses and increases CTLA4 mRNA levels and promoter activation in T cells. Blockade of CTLA4 decreases the ability of SP-D to inhibit allergen-induced immune responses. Taken together, these results indicate that SP-D regulates CTLA4 and modulates allergen-induced pulmonary inflammation. 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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References

- Crouch, E., D. Parghi, S. F. Kuan, and A. Persson. 1992. Surfactant protein D: subcellular localization in nonciliated bronchiolar epithelial cells. *Am. J. Physiol.* 263: L60–L66.
- Crouch, E., and J. R. Wright. 2001. Surfactant proteins a and d and pulmonary host defense. *Annu. Rev. Physiol.* 63: 521–554.
- Schaub, B., R. M. Westlake, H. He, R. Arestides, K. J. Haley, M. Campo, G. Velasco, A. Bellou, S. Hawgood, F. R. Poulain, et al. 2004. Surfactant protein D deficiency influences allergic immune responses. *Clin. Exp. Allergy* 34: 1819–1826.
- Wang, J. Y., C. C. Shieh, C. K. Yu, and H. Y. Lei. 2001. Allergen-induced bronchial inflammation is associated with decreased levels of surfactant proteins A and D in a murine model of asthma. *Clin. Exp. Allergy* 31: 652–662.
- Haley, K. J., A. Ciota, J. P. Contreras, M. R. Boothby, D. L. Perkins, and P. W. Finn. 2002. Alterations in lung collectins in an adaptive allergic immune response. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 282: L573–L584.
- Haczku, A., Y. Cao, G. Vass, S. Kierstein, P. Nath, E. N. Atochina-Vasserman, S. T. Scanlon, L. Li, D. E. Griswold, K. F. Chung, et al. 2006. IL-4 and IL-13 form a negative feedback circuit with surfactant protein-D in the allergic airway response. *J. Immunol.* 176: 3557–3565.
- Kasper, M., G. Sims, R. Koslowski, H. Kuss, M. Thuemmler, H. Fehrenbach, and R. L. Auten. 2002. Increased surfactant protein D in rat airway goblet and Clara cells during ovalbumin-induced allergic airway inflammation. *Clin. Exp. Allergy* 32: 1251–1258.
- Cheng, G., T. Ueda, T. Numao, Y. Kuroki, H. Nakajima, Y. Fukushima, S. Motojima, and T. Fukuda. 2000. Increased levels of surfactant protein A and D in bronchoalveolar lavage fluids in patients with bronchial asthma. *Eur. Respir. J.* 16: 831–835.
- Koopmans, J. G., J. S. van der Zee, E. J. Krop, C. E. Lopuhaä, H. M. Jansen, and J. J. Batenburg. 2004. Serum surfactant protein D is elevated in allergic patients. *Clin. Exp. Allergy* 34: 1827–1833.
- Madan, T., U. Kishore, M. Singh, P. Strong, H. Clark, E. M. Hussain, K. B. Reid, and P. U. Sarma. 2001. Surfactant proteins A and D protect mice against pulmonary hypersensitivity induced by *Aspergillus fumigatus* antigens and allergens. *J. Clin. Invest.* 107: 467–475.
- Kishor, U., T. Madan, P. U. Sarma, M. Singh, B. C. Urban, and K. B. Reid. 2002. Protective roles of pulmonary surfactant proteins, SP-A and SP-D, against lung allergy and infection caused by *Aspergillus fumigatus*. *Immunobiology* 205: 610–618.
- Wang, J. Y., C. C. Shieh, P. F. You, H. Y. Lei, and K. B. Reid. 1998. Inhibitory effect of pulmonary surfactant proteins A and D on allergen-induced lymphocyte proliferation and histamine release in children with asthma. *Am. J. Respir. Crit. Care Med.* 158: 510–518.
- Borron, P. J., E. A. Mostaghel, C. Doyle, E. S. Walsh, M. G. McHeyzer-Williams, and J. R. Wright. 2002. Pulmonary surfactant proteins A and D directly suppress CD3⁺/CD4⁺ cell function: evidence for two shared mechanisms. *J. Immunol.* 169: 5844–5850.
- Linsley, P. S., W. Brady, M. Urnes, L. S. Grosmaire, N. K. Damle, and J. A. Ledbetter. 1991. CTLA-4 is a second receptor for the B cell activation antigen B7. *J. Exp. Med.* 174: 561–569.
- Walunas, T. L., D. J. Lenschow, C. Y. Bakker, P. S. Linsley, G. J. Freeman, J. M. Green, C. B. Thompson, and J. A. Bluestone. 1994. CTLA-4 can function as a negative regulator of T cell activation. *Immunity* 1: 405–413.
- Jen, K. Y., M. Campo, H. He, S. S. Makani, G. Velasco, D. M. Rothstein, D. L. Perkins, and P. W. Finn. 2007. CD45RB ligation inhibits allergic pulmonary inflammation by inducing CTLA4 transcription. *J. Immunol.* 179: 4212–4218.
- Crouch, E., Y. Tu, D. Briner, B. McDonald, K. Smith, U. Holmskov, and K. Hartshorn. 2005. Ligand specificity of human surfactant protein D: expression of a mutant trimeric collectin that shows enhanced interactions with influenza A virus. *J. Biol. Chem.* 280: 17046–17056.
- Wang, H., J. Head, P. Kosma, H. Brade, S. Muller-Loennies, S. Sheikh, B. McDonald, K. Smith, T. Cafarella, B. Seaton, and E. Crouch. 2008. Recognition of heptoses and the inner core of bacterial lipopolysaccharides by surfactant protein D. *Biochemistry* 47: 710–720.
- Finn, P. W., H. He, Y. Wang, Z. Wang, G. Guan, J. Listman, and D. L. Perkins. 1997. Synergistic induction of CTLA-4 expression by costimulation with TCR plus CD28 signals mediated by increased transcription and messenger ribonucleic acid stability. *J. Immunol.* 158: 4074–4081.
- Mamessier, E., A. Nieves, A. M. Lorec, P. Dupuy, D. Pinot, C. Pinet, D. Vervloet, and A. Magnan. 2008. T-cell activation during exacerbations: a longitudinal study in refractory asthma. *Allergy* 63: 1202–1210.
- Perkins, D., Z. Wang, C. Donovan, H. He, D. Mark, G. Guan, Y. Wang, T. Walunas, J. Bluestone, J. Listman, and P. W. Finn. 1996. Regulation of CTLA-4 expression during T cell activation. *J. Immunol.* 156: 4154–4159.
- Strong, P., P. Townsend, R. Mackay, K. B. Reid, and H. W. Clark. 2003. A recombinant fragment of human SP-D reduces allergic responses in mice sensitized to house dust mite allergens. *Clin. Exp. Immunol.* 134: 181–187.
- Liu, C. F., Y. L. Chen, C. C. Shieh, C. K. Yu, K. B. Reid, and J. Y. Wang. 2005. Therapeutic effect of surfactant protein D in allergic inflammation of mite-sensitized mice. *Clin. Exp. Allergy* 35: 515–521.
- Fleming, C. M., H. He, A. Ciota, D. Perkins, and P. W. Finn. 2001. Administration of pentoxifylline during allergen sensitization dissociates pulmonary allergic inflammation from airway hyperresponsiveness. *J. Immunol.* 167: 1703–1711.
- Fisher, J. H., J. Larson, C. Cool, and S. W. Dow. 2002. Lymphocyte activation in the lungs of SP-D null mice. *Am. J. Respir. Cell Mol. Biol.* 27: 24–33.
- Borron, P. J., E. C. Crouch, J. F. Lewis, J. R. Wright, F. Possmayer, and L. J. Fraher. 1998. Recombinant rat surfactant-associated protein D inhibits human T lymphocyte proliferation and IL-2 production. *J. Immunol.* 161: 4599–4603.
- Jäkel, A., H. Clark, K. B. Reid, and R. B. Sim. 2009. The human lung surfactant proteins A (SP-A) and D (SP-D) interact with apoptotic target cells by different binding mechanisms. *Immunobiology*.
- Hizawa, N., E. Yamaguchi, E. Jinushi, S. Konno, Y. Kawakami, and M. Nishimura. 2001. Increased total serum IgE levels in patients with asthma and promoter polymorphisms at CTLA4 and FCER1B. *J. Allergy Clin. Immunol.* 108: 74–79.
- Howard, T. D., D. S. Postma, G. A. Hawkins, G. H. Koppelman, S. L. Zheng, A. K. Wysong, J. Xu, D. A. Meyers, and E. R. Bleeker. 2002. Fine mapping of an IgE-controlling gene on chromosome 2q: analysis of CTLA4 and CD28. *J. Allergy Clin. Immunol.* 110: 743–751.
- Munthe-Kaas, M. C., K. H. Carlsen, P. J. Helms, J. Gerritsen, M. Whyte, M. Feijen, B. Skiningsrud, M. Main, G. N. Kwong, B. A. Lie, et al. 2004. CTLA-4 polymorphisms in allergy and asthma and the T_H1/T_H2 paradigm. *J. Allergy Clin. Immunol.* 114: 280–287.
- Hellings, P. W., P. Vandenbergh, A. Kasran, L. Coorevits, L. Overbergh, C. Mathieu, and J. L. Ceuppens. 2002. Blockade of CTLA-4 enhances allergic sensitization and eosinophilic airway inflammation in genetically predisposed mice. *Eur. J. Immunol.* 32: 585–594.
- Alenmyr, L., V. Matheu, L. Uller, L. Greiff, M. Malm-Erjefält, H. G. Ljunggren, C. G. Persson, and M. Korsgren. 2005. Blockade of CTLA-4 promotes airway inflammation in naive mice exposed to aerosolized allergen but fails to prevent inhalation tolerance. *Scand. J. Immunol.* 62: 437–444.
- Oosterwegel, M. A., D. A. Mandelbrot, S. D. Boyd, R. B. Lorschach, D. Y. Jarrett, A. K. Abbas, and A. H. Sharpe. 1999. The role of CTLA-4 in regulating Th2 differentiation. *J. Immunol.* 163: 2634–2639.
- Ubaldo, V., L. Gatta, L. Pace, G. Doria, and C. Pioli. 2003. CTLA-4 engagement inhibits Th2 but not Th1 cell polarisation. *Clin. Dev. Immunol.* 10: 13–17.
- Alegre, M. L., H. Shiels, C. B. Thompson, and T. F. Gajewski. 1998. Expression and function of CTLA-4 in Th1 and Th2 cells. *J. Immunol.* 161: 3347–3356.
- Ohya, M., C. Nishitani, H. Sano, C. Yamada, H. Mitsuizawa, T. Shimizu, T. Saito, K. Smith, E. Crouch, and Y. Kuroki. 2006. Human pulmonary surfactant protein D binds the extracellular domains of Toll-like receptors 2 and 4 through the carbohydrate recognition domain by a mechanism different from its binding to phosphatidylinositol and lipopolysaccharide. *Biochemistry* 45: 8657–8664.
- Yoshida, M., T. R. Korfhagen, and J. A. Whitsett. 2001. Surfactant protein D regulates NF- κ B and matrix metalloproteinase production in alveolar macrophages via oxidant-sensitive pathways. *J. Immunol.* 166: 7514–7519.
- Liu, C. F., M. Riviere, H. J. Huang, G. Puzo, and J. Y. Wang. 2010. Surfactant protein D inhibits mite-induced alveolar macrophage and dendritic cell activations through TLR signalling and DC-SIGN expression. *Clin. Exp. Allergy* 40: 111–122.
- Miller, R. E., J. D. Fayen, S. F. Mohammad, K. Stein, S. Kaderit, K. D. Woods, R. M. Sramkoski, J. W. Jacobberger, D. Templeton, S. B. Shurin, and M. J. Laughlin. 2002. Reduced CTLA-4 protein and messenger RNA expression in umbilical cord blood T lymphocytes. *Exp. Hematol.* 30: 738–744.

40. Gibson, H. M., C. J. Hedgcock, B. M. Aufiero, A. J. Wilson, M. S. Hafner, G. C. Tsokos, and H. K. Wong. 2007. Induction of the CTLA-4 gene in human lymphocytes is dependent on NFAT binding the proximal promoter. *J. Immunol.* 179: 3831–3840.
41. Schaub, B., M. Campo, H. He, D. Perkins, M. W. Gillman, D. R. Gold, S. Weiss, E. Lieberman, and P. W. Finn. 2006. Neonatal immune responses to TLR2 stimulation: influence of maternal atopy on Foxp3 and IL-10 expression. *Respir. Res.* 7: 40.
42. Frisancho-Kiss, S., S. E. Davis, J. F. Nyland, J. A. Frisancho, D. Cihakova, M. A. Barrett, N. R. Rose, and D. Fairweather. 2007. Cutting edge: cross-regulation by TLR4 and T cell Ig mucin-3 determines sex differences in inflammatory heart disease. *J. Immunol.* 178: 6710–6714.