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CD45RB Ligation Inhibits Allergic Pulmonary Inflammation by Inducing CTLA4 Transcription

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CD45, a type I transmembrane protein tyrosine phosphatase expressed on nucleated hemopoietic cells, is prominently involved in T cell activation. Ligation of CD45RB isoforms has been associated with transplant tolerance. A recent genotyping analysis of asthma indicates a correlation with CD45 splicing. In this study, we administered an anti-CD45RB mAb (aCD45) in a murine model of allergic asthma and found that CD45RB ligation decreases allergic responses. aCD45 decreases allergen-induced pulmonary eosinophilia, bronchoalveolar lavage IL-13, IgE, and airway responses. Also, aCD45 increases the expression of CTLA4, a negative regulator of T cell activation. Furthermore, CD45RB signals no longer decrease allergic inflammation when CTLA4 is inhibited. These data support a role for CTLA4 in CD45RB-mediated inhibition of allergic inflammation. T cells and splenocytes stimulated with aCD45 exhibited increased CTLA4 levels, and analysis of CTLA4 promoter gene constructs identified a CD45RB-inducible regulatory region localized from −335 to −62 bp relative to the transcription start site. Together, these findings suggest that CD45RB signals mediated a novel role in the modulation of allergic inflammation, orchestrated by T cells through induction of CTLA4 transcription. The Journal of Immunology, 2007, 179: 4212–4218.

Immune-mediated disorders, including allergic asthma, are increasing in severity and prevalence (1). Current understanding of the pathogenesis of allergy, autoimmune diseases, and transplantation rejection emphasizes their inflammatory nature. Central to these processes is T cell activation, which is essential in promoting allergic inflammatory disorders, including asthma (2).

CD45 is a type I transmembrane protein tyrosine phosphatase expressed on nucleated hemopoietic cells (3). Alternative mRNA splicing gives rise to multiple CD45 isoforms that differ only in the length and glycosylation of their extracellular domains (4). Although the exact function of each isoform remains unclear, mAbs recognizing CD45RB isoforms differentiate two populations of CD4+ T cells: CD4+CD45RBhigh (5) and CD4+CD45RBlow (6) that secrete different cytokines and have distinct functional properties (7). The CD4+CD45RBhigh population contains naive effector T cells that promote autoimmunity (8) and inflammatory bowel disease (9), while the CD4+CD45RBlow population contains previously activated cells as well as T regulatory (Treg)9 cells, which inhibit T cell effector activity (9, 10).

Previous studies have shown that CD45RB ligation prolongs allograft survival and can induce tolerance in murine models (11–13). Anti-CD45 mAb (aCD45) increases the expression of CTLA4, a negative regulator of T cell activation (7, 14). Interestingly, a recent study of 167 Danish families mapped a functional gene SFRS8 (splicing factor, arginine/serine-rich 8) associated with asthma. This gene regulates the splicing of CD45 (15), suggesting a possible role of CD45RB in allergic asthma. In this study, we asked whether CD45RB signals would impact immune responses in a murine model of allergic inflammation. We also examined the role of CTLA4, known to decrease T cell activation, in CD45RB-mediated inhibition of allergic inflammation. We suggest mechanisms by which aCD45 augments CTLA4 expression and decreases allergic inflammation.

Materials and Methods

Mice
Six- to 8-wk-old BALB/c male mice were purchased from The Jackson Laboratory. The mice were maintained according to the guidelines of the Committee on Animals of the Harvard Medical School and Animal Welfare Program from School of Medicine, University of California, San Diego.

Antibodies
Mouse anti-CD45RB mAb (MB23G2) (aCD45), described previously (16), and mouse anti-CTLA4 (UC10-4F10-11) (aCTLA4) were purchased from Bio Express Culture Services. Rat IgG2a (LO-DNP-16; Serotec) was used as a negative control.

Cell lines and treatment
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 of penicillin/ml, and 50 μg of streptomycin/ml. EL4 cells were treated with aCD45 (3 μg) for 24 and 48 h at 2 × 10^6 cells/ml. As a positive control, EL4 cells or spleen cells were also treated with 5 μg/ml Con A (Sigma-Aldrich) and 100 ng/ml PMA (Sigma-Aldrich). As a negative control, both EL4 cells and murine splenocytes remained untreated.
**OVA sensitization and challenge**

Mice were sensitized and challenged with the allergen OVA as previously described (17–20). OVA mice were sensitized via i.p. injection with 10 µg of chicken OVA (Sigma-Aldrich) and 1 mg of Al(OH)₃ (alum; Sigma-Aldrich) in 0.2 ml of PBS (Sigma-Aldrich), followed by an injection on day 7 with identical reagents. PBS mice received 1 mg of alum in 0.2 ml of PBS without OVA. On days 14–20, mice received aerosolized challenges with 6% OVA or PBS, respectively, for 20 min/day via an ultrasonic nebulizer (model 5000; DeVilbiss). All groups were sacrificed at day 21 and analyzed for the allergic parameters described below.

**Bronchoalveolar lavage (BAL) analysis**

Each mouse underwent BAL as previously described (21, 22). BAL cells were pelleted and the supernatant was stored at –80°C. Cells were resuspended in RPMI 1640 (5 × 10⁵ cells/ml). Slides for differential cell counts were prepared with Cytospin (Shandon) and fixed and stained with Diff-Quik (Dade Behringm). For each sample, an investigator blinded to the treatment groups performed two counts of 100 cells.

**ELISA**

BAL IL-13 was measured by ELISA according to the manufacturer’s specifications (R&D Systems). Briefly, samples of BAL fluid were aliquoted in duplicate into 96-well plates (50 µl/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.

**Serum IgE levels**

Total serum IgE levels were determined by ELISA as previously described (17, 22). Total serum IgE concentrations were calculated by using standard curve generated with commercial IgE standard (BD PharMingen).

**Determination of airway measurements**

Airway measurements were assessed using whole-body plethysmography 4 h after the final aerosol OVA challenge. Mice were placed in individual chambers and increasing doses of methacholine were nebulized into the chambers via an inlet at concentrations of 20, 40, 80, and 100 mg/ml for 2 min as previously described (17, 20). Readings were averaged over 5 min after the nebulization. The whole-body plethysmography system measures changes in box pressure during expiration and inspiration; peak expiratory and peak inspiratory pressures, respectively; inspiratory time; expiratory time; and relaxation time (Tr = time of the pressure decay to 36% of total box pressure during expiration) and generates a value called enhanced pause (enhanced pause = peak expiratory pressure/peak inhibitory pressure [expiratory time – Tr]/Tr) that relates to airway measurements in BALB/c mice (23).

**Treatment protocols**

BALB/c mice received 100 µg of aCD45 via i.p. injection 1 day before sensitization (day –1) and/or 100 µg of aCTLA4 i.p. 2 days before sensitization (day –2). Control BALB/c mice received IgE 2 days before sensitization (day –2).

**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 Life Technologies). Specific primer pairs for GADPH (housekeeping gene), CTLA4, IFN-γ, IL-2, IL-4, IL-5, IL-6, and IL-13 were designed with Primer Express software (Applied Biosystems). Direct detection of the PCR product was monitored by measuring the increase in fluorescence caused by the binding of SYBR Green to dsDNA. Using 5 µl of cDNA, 5 µl of primer, and 10 µl of SYBR Green 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 (24).

**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 (25). The luciferase construct that contains the –1221-bp fragment (p1221) was used as a template for further 5’ deletion constructs –335 (p335), –238 (p238), and –62 (p63). All constructs were sequenced in both directions by dideoxy sequencing (25).

**Transfections and reporter gene assays**

CTLA4 reporter 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 of Nucleofector solution (Amaxa Biosystems) and electroporated using the C-9 program of the Nucleofector. After 24 h, cells were lysed in reporter lysis buffer (Promega). Then, 10 µl of the cell lysate was mixed with 100 µl of luciferase assay reagent (Promega) and luciferase activity was measured by a luminometer (Turner Biosystems). 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 vs β-galactosidase activity.
Bioinformatics

Transcription factor binding sites from CTLA4 promoter region were identified using TESS (www.cbil.upenn.edu/cgi-bin/tess/tess33).

Statistics

ANOVA was performed for dose-response curves (see Fig. 5c). Statistical analysis of normally distributed data was performed by t test, and non-parametric data were analyzed by a Mann-Whitney U test (Sigma Stat 3.0 software). Bonferroni correction for statistical adjustment of the p value for multiple comparisons was applied as a post hoc analysis. Data are reported as means ± SEM. Statistical significance was defined by p < 0.05.

Results

Pulmonary allergic inflammation is maintained following aCTLA4 administration

We analyzed allergic parameters using a model of allergen OVA-induced pulmonary inflammation (17, 18, 26, 27). OVA-sensitized and -challenged mice showed a significant increase in BAL eosinophilia, IL-13, and total serum IgE when compared with PBS control mice (Figs. 1, a and b, and 2). CTLA4 is a negative regulator of T cell activation and administration of a blocking mAb to CTLA4 (aCTLA4) to OVA-sensitized and -challenged mice increased allergen-induced eosinophils (Fig. 1a), total serum IgE (Fig. 1b), and BAL IL-13 (Fig. 2). These data indicate that CTLA4-mediated influences are manifested in allergic pulmonary inflammation.

aCD45 increases CTLA4 levels in murine spleen cells

Previous studies in transplantation models indicate that aCD45 prolongs allograft survival through induction of CTLA4 expression in T cells (7, 14). In concert, incubation of murine splenocytes for 24 h with aCD45 resulted in an ~5-fold increase in CTLA4 mRNA levels (Fig. 3).

Allergen-induced pulmonary inflammation is decreased by administration of aCD45

The role of CD45RB signals has not previously been examined in allergic immune responses. Although aCD45 induces CTLA4, its effectiveness in allograft models may also relate to deviation toward a CD45RBlow phenotype and induction of Th2 cytokine expression (28–29). Although in allergic responses, Th2 (as well as Th1) cytokines have been shown to promote allergic inflammation, it is not clear whether aCD45 would be effective in this setting (30, 31). We therefore examined the effect of aCD45, which was administered 2 days before OVA sensitization and challenge. Administration of aCD45 in an established OVA murine model of allergic inflammation markedly decreases allergen-induced BAL eosinophilia, total serum IgE, and BAL IL-13, such that they resemble those observed in control mice “immunized” with PBS (Fig. 4). These changes were not evident when mice were treated with control Ig rather than aCD45 (data not shown).

aCD45 no longer decreases allergen-induced pulmonary inflammation with blockade of CTLA4 signals

We next examined the effect of CTLA4 blockade on protection from allergic responses mediated by administration of aCD45 to OVA-sensitized plus -challenged mice. In mice that also received aCTLA4, aCD45 no longer decreased OVA-induced pulmonary eosinophilia and BAL IL-13 secretion or airway measurements (Fig. 5). The abrogation of the ability of aCD45 to inhibit allergic responses suggests that aCD45 decreases allergic inflammation through CTLA4 pathways.

aCD45 induces CTLA4 levels during activation of T cells

aCD45 increased CTLA4 in vivo and this pathway appears critical for CD45RB-mediated suppression of allergic inflammation. To
determine potential mechanisms by which aCD45 increases CTLA4 expression in T cells, murine T cells (EL4) were treated with aCD45 in vitro. Untreated EL4 cells did not express detectable levels of CTLA4 mRNA expression. Increased CTLA4 levels were observed 24 and 48 h after treatment with aCD45 (Fig. 6). Treatment with Con A plus PMA (similar to TCR signals) was used as a positive control and showed an increase of 6-fold at 24 h (data not shown). Thus, aCD45 may directly augment CTLA4 transcription.

Localization of aCD45-inducible region for T cell activation in the CTLA4 promoter region

To define the regulatory regions involved in aCD45-inducible CTLA4 gene expression in T cells, we analyzed a panel of CTLA4 luciferase reporter gene constructs. These luciferase constructs were defined at positions: −335 (p335), −238 (p238), and −63 (p63) bp relative to the start site for CTLA4 transcription. aCD45 induces p335 and p238 promoter region activation, as determined by an increase in luciferase activity (Fig. 7a). A larger construct located within 1.2 kb (p1221) of the transcription start site also increased luciferase activity (5-fold relative to medium; data not shown). Deletion from positions −335 (p335) to −62 (p63) in the CTLA4 promoter abrogates luciferase activity. The p335 and p238 constructs showed a 5- and 4-fold increase, respectively, in luciferase activation, indicating the presence of positive regulatory elements for CTLA4-inducible expression by aCD45. In addition, we

FIGURE 5. Allergic immune responses are increased after administration of aCTLA4 in mice treated with aCD45. Mice were sensitized and challenged with OVA. Mice received aCTLA4 (100 μg) i.p. at day −2 and aCD45 (100 μg) at day −1 before allergen sensitization (see Materials and Methods). Cell counts were determined by differential staining of cells isolated from BAL fluid. BAL cytokine was measured by ELISA (R&D Systems). a, BAL eosinophilia (EOS); b, BAL IL-13; c, airway measurements (enhanced pause (Penh)) by whole-body plethysmography. Statistical analysis as described in Materials and Methods. Data are shown as geometric mean and geometric SEM (n = 4–6/group). *, OVA aCTLA4 vs OVA (p < 0.05); #, OVA aCD45 vs OVA aCTLA4 vs OVA aCD45.

aCD45 increases CTLA4 mRNA levels in T cells. Murine T cells (EL4) were incubated with or without aCD45 (3 μg/ml) or Con A (5 μg/ml) plus PMA (100 ng/ml) (data not shown) for 24 h. RNA was purified and analyzed by real-time PCR (see Materials and Methods). Data are from one experiment, representative of n = 3.

FIGURE 6. aCD45 increases CTLA4 mRNA levels in T cells. Murine T cells (EL4) were incubated with or without aCD45 (3 μg/ml) or Con A (5 μg/ml) plus PMA (100 ng/ml) (data not shown) for 24 h. RNA was purified and analyzed by real-time PCR (see Materials and Methods). Data are from one experiment, representative of n = 3.

FIGURE 7. Localization of regulatory regions in CTLA4 promoter inducible by aCD45. a, Murine T cells (EL4) were transfected by nucleofection (see Materials and Methods) with three CTLA4 promoter luciferase (Luc)-based reporter constructs (−335, −238, −62) as well as a promoterless vector (pXP2) and β-galactosidase reporter gene (PGK) constructs. After 24 h, cells were stimulated with or without aCD45 for 24 h followed by analysis of reporter gene expression using a luminometer. Fold induction is the ratio of activated to resting sample of luciferase plasmids and pXP2. Fold activation was calculated as the ratio of luciferase activity vs β-galactosidase activity. Data are mean ± SD values of three independent experiments. b, Localization of binding sites for transcription factors in the CTLA4 promoter region from positions −335 to −1 relative to the transcription starting site. Open square, AP-1 sites; dash-dotted square, IL6REBP sites; square with dots, NF-IL2A site; open square with bold line, NFAT-1 sites; −1, CTLA4 transcription start site.
analyzed this CTLA4 promoter region (−335 to −1) by TESS (see Materials and Methods). Binding sites for AP-1, NFAT-1, IL-6/RE-BP, and NF-IL2A were identified, in addition to others (Fig. 7b). Notably, these data indicate that aCD45 may increase CTLA4 by directly augmenting its transcription.

**aCD45 increases IL-2, IL-6, IL-4, IL-5, and IL-13 mRNA levels in T cells**

Positive regulatory elements for induction of CTLA4 by aCD45 may include putative sites for a number of transcription factors involved in cytokine production. We next examined cytokine transcript levels in EL4 cells that were or were not treated with aCD45 (Fig. 8). IL-2, IL-4, IL-5, IL-6, and IL-13 mRNA levels were increased after 24 h of stimulation with aCD45 as analyzed by real-time PCR. aCD45 does not increase the mRNA levels of IFN-γ.

**aCD45 increases IL-2, IL-4, IL-5, and IL-13 mRNA levels in murine spleen cells**

Murine spleen cells treated with aCD45 and untreated cells were examined for cytokine transcript levels at two different time points (24 and 48 h; Fig. 9). IL-2 and IL-13 mRNA levels were increased at 24, but not at 48 h (data not shown), while IL-4 and IL-5 mRNA levels were increased at 48 h, but not at 24 h (data not shown). IFN-γ levels increased 3-fold relative to untreated at both time points.

**Discussion**

A recent study of asthmatic Danish families mapped a functional gene associated with asthma responsible for the regulation of the splicing of the CD45 molecule (15). Although the functional consequences are unknown, aCD45 alters CD45 splicing (28), suggesting that aCD45 might impact allergic responses. Indeed, we found that CD45RB signals suppress allergic responses in a murine model of allergic inflammation. Our results showed that CD45RB decreases allergen-induced pulmonary eosinophilia, BAL IL-13, total serum IgE, and airway responses. Furthermore, CD45RB signals can no longer suppress allergic inflammation when CTLA4 is inhibited. These data support a role for CTLA4 in CD45RB-mediated decreases of allergic inflammation. In this regard, we found that aCD45 may directly augment CTLA4 transcription. To determine the regulatory elements involved, we performed a reporter gene analysis with CTLA4 promoter constructs. We identified an aCD45-inducible regulatory region localized from −335 to −62 bp relative to the CTLA4 transcription start site. Together, these findings suggest that CD45RB signals may play a novel role in the modulation of allergic inflammation.

Previous studies have shown that aCD45 is a potent tolerogenic agent that prolongs allograft survival in murine models (7, 11, 14, 28, 29, 32). The immunomodulatory function of aCD45 may occur through increased CTLA4 on T cells, as evidenced in transplantation models (7, 14). To address the mechanisms by which CD45RB decreases allergic immune responses, we also examined the role of CTLA4 in allergic inflammation. Consistent with previous models, blockade of CTLA4 maintained or increased allergic responses (33, 34). Also, in the presence of aCD45, aCD45 no longer suppressed IL-13 production. aCD45 also enhances cytokines in vitro. One interpretation is that in the presence of allergen stimulation in vivo, aCD45 is capable of enhancing Th2 cytokines, including IL-13, but it appears that CTLA4-mediated inhibition predominates. Overall, CD45RB-mediated modulation of CTLA4 is a crucial immune regulatory pathway.

To elucidate the molecular mechanisms by which CD45RB signals modulate CTLA4 expression, T cells were stimulated with aCD45 and CTLA4 mRNA levels were analyzed. Our data indicate that aCD45 increases transcription of the CTLA4 promoter region in T cells, a finding supporting direct induction of CTLA4 by aCD45. It is already known that T cell activation leads to CTLA4 induction. However, this generally requires full T cell activation and entry into the cell cycle (25). Remarkably, aCD45 appears able to induce CTLA4 even though this mAb is not mitogenic and does not induce activation markers such as CD69 or CD44 (Refs. 7 and 35 and D. Rothstein, unpublished data). Our reporter gene analysis indicates that the regulatory region for CD45RB-inducible CTLA4 expression is located within −335 to −62 bp relative to the transcription start site. Interestingly,
CD45RB signals induced the CTLA4 promoter region that we previously ascertained to be TCR signal inducible (25, 36). Thus, although aCD45 does not induce overt T cell activation, it clearly results in signals that partially overlap with those mediated by the TCR. The importance of this finding is that T cells may be induced to express CTLA4 without undergoing normal activation, proliferation, and development of their full-fledged effector repertoire.

Computer analysis of the CTLA4 upstream region (−335 to −62) also revealed potential DNA binding sites for NF-IL2A (Oct-1), IL6REBP as well as AP-1 and NFAT. We focus on NF-IL2A and IL6REBP because they have been shown to regulate the gene expression of IL-2 and IL-6 (37, 38). Interestingly, in T cells stimulated with aCD45, we found an increase of IL-2 and IL-6 levels. Whether CD45RB signals may regulate these cytokines by acting on specific CTLA4 promoter regions remain to be determined. In this scenario, CD45RB signals may influence NF-IL2A and IL6RE-BP DNA binding sites localized within this CTLA4 promoter upstream region. We hypothesize that induction of CTLA4 before TCR stimulation inhibits subsequent activation of allergic immune responsiveness. This CTLA4 induction may specifically dampen the CD44low subset as would be expected if CD45RBhigh cells are readily visualized (7, 28). Moreover, there is no depletion of leukocyte populations and signaling pathways that may be targets for the effects of CD45RB signals will be crucial in the understanding of how to manipulate this critical pathway to decrease allergic responses.

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Disclosures

The authors have no financial conflict of interest.

References

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