

Inhibition of Th2-Mediated Allergic Airway Inflammatory Disease by CD137 Costimulation¹

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The engagement of CD137 (4-1BB), an inducible T cell costimulatory receptor and member of the TNF receptor superfamily, by agonistic Abs can promote strong tumor and viral immunity mediated by CD8⁺ T cells and stimulate IFN- γ production. However, its role in Th2-mediated immune responses has not been well defined. To address this issue, we studied the function of CD137 engagement using an allergic airway disease model in which the mice were sensitized with inactivated *Schistosoma mansoni* eggs followed by *S. mansoni* egg Ag challenge directly in the airways and Th1/2 cytokine production was monitored. Interestingly, treatment of C57BL/6 mice with agonistic anti-CD137 (2A) during sensitization completely prevents allergic airway inflammation, as shown by a clear inhibition of T cell and eosinophil infiltration into the lung tissue and airways, accompanied by diminished Th2 cytokine production and reduced serum IgE levels, as well as a reduction of airway hyperresponsiveness. At various time points after immunization, restimulated splenocytes from 2A-treated mice displayed reduced proliferation and Th2 cytokine production. In accordance with this, agonistic Ab to CD137 can directly coinhibit Th2 responses in vitro although it costimulates Th1 responses. CD137-mediated suppression of Th2 response is independent of IFN- γ and T regulatory cells. Our study has identified a novel pathway to inhibit Th2 responses in a CD137-dependent fashion. *The Journal of Immunology*, 2006, 177: 814–821.

A member of the TNF receptor superfamily, CD137 (4-1BB) is a T cell costimulatory receptor induced by TCR activation. In addition to its expression on activated T cells (1, 2), CD137 is also expressed on activated NK cells (3), NKT cells (4), CD4⁺CD25⁺ regulatory T cells (Treg)³ (5, 6), and dendritic cells (7, 8). Its natural ligand, CD137L (4-1BBL), a member of the TNF superfamily, has been detected on professional APCs, including B cells, macrophages, and dendritic cells (9–11). This expression pattern indicates that CD137-CD137L interactions may play a role in various innate and adaptive immune responses.

Signaling through CD137 leads to increased TCR-induced T cell activation and functional maturation, as well as prolonged CD8⁺ T cell survival (1, 12, 13). In addition to providing costimulatory signaling for T cells, recent studies showed that CD137 cross-linking also provides costimulation for NK cells (14), dendritic cells (8), and CD4⁺CD25⁺ Treg (15). In addition to the costimulatory roles for CD137, recent data suggest a diametric role of CD137 costimulation in vivo. On one hand, engagement of CD137 with agonistic mAbs against CD137 promotes tumor im-

munity, alloresponses (16–18), and increases primary antiviral CD8⁺ T cell responses (19). In contrast, it has been shown that agonistic anti-CD137 treatment facilitates CD4-mediated acute graft-vs-host disease (GVHD) but inhibits chronic GVHD (18, 20). Similar treatment results in a suppression of CD4⁺ T cell-dependent humoral immunity (21, 22) and the attenuation of multiple CD4⁺ T cell-mediated autoimmune diseases (23–28). The mechanisms involve activation-induced cell death of CD4⁺ cells in some cases and, in others, stimulation of a subset of CD8⁺ T cells and macrophages resulting in the suppression of CD4⁺ T cell-mediated responses in a IFN- γ -dependent manner (23, 24, 27, 28). Taken together, the role of CD137 in Th cell responses is not well defined.

Our goal in the present study was to dissect the role of CD137 engagement in initiating helper T cell differentiation and Th2-mediated responses. Allergic airway inflammatory disease (AAD) is a Th2-dominant disease characterized by increased airway inflammation, increased IgE production, and increased airway hyperactivity (29, 30). AAD can be induced by sensitization with inactivated *Schistosoma mansoni* eggs followed by local antigenic challenge with *S. mansoni*-soluble egg Ag (SEA) (31, 32). Immunization with inactivated *S. mansoni* egg skewed the T cell response toward Th2 and induced a robust IgE response in the absence of infection (31). Following airway challenge with SEA, local cells respond with the production of chemokines and cytokines, which attract Th2 cells to the site of challenge (33). Interestingly, we have observed that administration of agonistic anti-CD137 during sensitization prevents airway inflammation and characteristics of AAD, which is due to the inhibition of Th2 cell priming in vivo independently of IFN- γ and Treg. In accordance with this, agonistic Ab to CD137 can directly inhibit Th2 cytokine production and, at the same time, promote Th1 cytokine production in vitro. Therefore, CD137 can be a coinhibitor for Th2 differentiation and engagement of CD137 leads to inhibition of the Th2 responses. This study identifies a novel pathway to inhibit Th2 differentiation and, therefore, a Th2-mediated disease such as AAD via CD137 engagement on T cells.

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³ Abbreviations used in this paper: Treg, regulatory T cell; GVHD, graft-versus-host disease; AAD, allergic airway inflammatory disease; BAL, bronchoalveolar lavage; BHR, bronchial hyperresponsiveness; LN, lymph node; SEA, *Schistosoma mansoni* egg Ag; EAE, experimental autoimmune encephalomyelitis.

Materials and Methods

Mice

Six- to 10-wk-old C57BL/6 mice were purchased from the Division of Cancer Treatment at the National Cancer Institute. Animals were housed in a specific pathogen-free facility maintained by the University of Chicago Animal Resources Center. All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Chicago. In each experiment, age-matched mice were used.

Antibodies

The generation of mAb 2A, an IgG2a agonistic anti-4-1BB mAb, has been described previously (34) and ascites was produced in SCID mice and purified by passage over a protein G-coupled Sepharose column. Rat IgG was purchased from Sigma-Aldrich and served as a control Ab. Purified anti-CD3 (clone 145-2C11) and anti-CD28 were purchased from BD Biosciences and Stem Cell Technology, respectively.

SEA sensitization and challenge

Inactivated *S. mansoni* eggs and SEA were prepared as previously described (35, 36). Mice were sensitized i.p. on day 0 with 2.5×10^3 inactivated *S. mansoni* eggs and treated i.p. with either 150 μ g 2A or rat IgG. The mice were then challenged on day 7 via intratracheal delivery of 5 μ g SEA. Five days after challenge, mice were sacrificed, bronchoalveolar lavage (BAL) was collected, and lungs were dissected for digestion.

Isolation of lung leukocytes and BAL cells

Mice were sacrificed on day 5 after challenge via i.p. injection of 30 μ g mouse ketamine-HCl. Lung tissues were digested two times by shaking (175 revolutions/min) for 30 min at 37°C in RPMI 1640 medium (Invitrogen Life Technologies) containing 1.5 mg/ml collagenase VIII and 2% heat-inactivated FBS. Lung cells were passed through a Nystex filter, and RBCs were depleted with ammonium chloride-potassium lysing buffer. Lavage was performed by delivering 0.8 ml of cold RPMI 1640 containing 2% FBS into the trachea cannula and gently aspirating the fluid. The first lavage was centrifuged, and supernatant was stored at -20°C for cytokine analysis. Lavage was repeated three times, and cells collected from each wash were pooled for FACS analysis.

Lung homogenate

The lung tissue was homogenized in 500 μ l of PBS containing 1 mM PMSF, 0.01 mg/ml leupeptin, and 0.01 mg/ml aprotinin. The lysate was collected by centrifugation at 12,000 rpm for 15 min.

Histology

Lung tissues for histological examination were fixed in 10% buffered Formalin. Sections of 5 μ m were obtained from the paraffin blocks and stained with H&E.

Measurement of airway reactivity

Assessment of cholinergic airway constrictor responsiveness was done with a computer-controlled small-animal ventilator (Flexivent; SCIREQ). In brief, the mice were anesthetized with 0.1 ml per 10 g body weight of a mixture containing 2 mg/ml xylazine and 40 mg/ml ketamine hydrochloride given i.p. Anesthesia was maintained by supplemental administration of 30% of the initial dose at ~25-min intervals, as required. Heart rate was monitored by electrocardiogram with needle electrodes. After tracheostomy, the trachea was cannulated with a blunted 18-gauge metal needle. The mouse was quasi-sinusoidally ventilated with a nominal tidal volume of 10 ml/kg at a frequency of 150 breaths/min and a positive end expiratory pressure of 2 cm H₂O. To determine the differences in airway response to methacholine between control and 2A-treated mice, each mouse was challenged with seven doses of methacholine aerosol (0, 0.1, 1, 5, 10, 20, and 40 mg/ml in saline) for 12 s. Before each aerosol challenge, the animal was given two deep inspirations to standardize volume history. After each methacholine challenge, respiratory system resistance was recorded during tidal breathing every 10 s for 2 min, and the peak resistance was selected as the bronchoconstrictor response to that methacholine dose. ANOVA was used to analyze the differences in airway response to methacholine between control and 2A-treated mice.

IgE ELISA

The total IgE concentration from the serum was measured by ELISA according to the manufacturer's protocol (BD Pharmingen).

Splenocyte and lung lymphocyte restimulation *ex vivo*

Splenocytes and lung leukocytes were isolated at different time points after priming or challenge as indicated in the text. For blocking IFN- γ and depleting Treg, mice were treated with anti-IFN- γ (XMG1.2) the day of and anti-CD25 (PC61) the day before Ag immunization, respectively. Splenocytes and lung leukocytes were plated at 5×10^5 cells/well in a 96-well U-bottom plate. Cells were cultured with medium alone or 5–10 μ g/ml SEA. The final volume of all wells was 200 μ l. Plates were incubated for 3 days, after which time the supernatants were collected for cytokine analysis. To assess proliferation, additional plates were pulsed with 1 μ Ci/well of [³H]thymidine at day 3 and harvested 12–18 h later.

Real-time quantitative RT-PCR assay

RNA was purified with TRIzol (Invitrogen Life Technologies) and a RNA easy kit (Qiagen) following the instructions provided by the manufacturers. cDNA was synthesized and subject to real-time PCR as described previously (37). Each cDNA sample was amplified for the interested gene and GAPDH with the TaqMan Universal PCR master mixture according to the manufacturer's instructions (Applied Biosystems). The concentration of the interested gene was determined using the comparative threshold cycle number at a cross-point between amplification plot and threshold) method and normalized to the internal GAPDH control. The following primers were used: GATA3 forward primer, 5'-AGAACCGGCCCTTATCAA-3'; GATA3 reverse primer, 5'-AGTTCCGCGCAGGATGTCC-3'; GATA3 probe, 5'-FAM-CCAAGCGAAGGCTGTCCGCGAG-TAMRA-3'; T-bet forward primer, 5'-CAACAACCCCTTTGCCAAAG-3'; T-bet reverse primer, 5'-TCCCCCAAGCAGTTGACAGT-3'; and T-bet probe, 5'-FAM-CCGGGAGAACTTTGAGTCCATGTACGC-TAMRA-3'.

In vitro T cell proliferation

Lymph node (LN) cells were isolated from C57BL/6 mice and cultured with a suboptimal dose of plate-bound anti-CD3 (0.5 μ g/ml) in the presence of anti-CD28 (1 μ g/ml) and/or 2A (30 μ g/ml) for 3 days. Different doses of anti-IFN- γ ascites were added to the culture to neutralize IFN- γ . Alternatively, LN cells were cultured with 0.5 μ g/ml anti-CD3 in the presence of anti-CD28 (1 μ g/ml) or 2A (30 μ g/ml) for 7 days, then live cells were isolated and restimulated with anti-CD3 (1 μ g/ml) for 2 days. Supernatants were collected and IFN- γ and IL-4 levels were analyzed by cytokine beads array (BD Pharmingen).

Cytokine analysis

Cytokine production from the lung lysate, BAL fluid, and culture supernatant was measured by mouse a Th1/Th2 cytokine beads array kit according to the manufacturer's protocol (BD Pharmingen). For intracellular cytokine staining, single-cell suspensions from mediastinal LN and digested lung leukocytes were stimulated with 50 ng/ml PMA plus 500 ng/ml ionomycin for 4 h at 37°C in the presence of Golgi stop (BD Pharmingen). After FcR blockade with 2.4G2 Ab, cells were fixed in 4% formaldehyde and stained intracellularly for IL-4 and IL-5 in the presence of 0.5% saponin for cell permeabilization, followed by staining of the surface marker CD4. All of the Abs used were purchased from BD Pharmingen.

Flow cytometric analysis

All Abs for flow cytometric analysis were purchased from BD Pharmingen. BAL and lung cells were incubated in Fc block, 2.4G2, for 10 min, stained with different fluorescence-labeled Abs in PBS containing 1% FBS (Invitrogen Life Technologies) plus 0.01% NaN₃ for 30 min on ice, and analyzed by flow cytometry on a FACScan (BD Biosciences).

Statistical analysis

All statistics were done using an unpaired Student two-tailed *t* test. Error bars represent SD.

Results

Agonistic anti-CD137 treatment prevents Th2-mediated airway inflammation

We have previously (34) shown that 2A, an agonistic Ab to CD137, showed a strong costimulatory effect on T cell proliferation and CD8⁺ T cell-mediated tumor rejection. Such treatment also inhibited Th1-mediated experimental autoimmune encephalomyelitis (EAE) by increasing Th1 cell apoptosis following initial

activation (23). The role of CD137 in Th cell differentiation however, is not as well described. To study the role of CD137 costimulation in the induction of Th2 cell-mediated airway inflammation, female C57BL/6 mice were sensitized i.p. on day 0 with 2.5×10^3 inactivated *S. mansoni* eggs and treated with 150 μ g of either control rat IgG or agonistic anti-CD137 (2A). One week later, the mice were challenged with 5 μ g of SEA delivered by intratracheal instillation. Five days after challenge, the animals were sacrificed and analyzed for airway inflammation. As shown in Fig. 1A, the lungs of control rat IgG-treated mice manifested massive eosinophil and lymphocyte infiltration predominantly in the peribronchial and perivascular areas. In contrast with mice treated with control Ig, 2A-treated mice showed significantly reduced airway infiltration. We further examined the percentage and number of infiltrating T cells and CCR3-positive eosinophils within the airways. Eosinophilia in both the lung and BAL is characteristic of AAD. We found that the percentage of CCR3⁺ eosinophils was dramatically reduced in 2A-treated mice compared with control-treated mice in both BAL (72.1 ± 2.3 vs 19.5 ± 5.2 , Fig. 1B) and the lung (34.1 ± 3.5 vs 13.9 ± 2.5 , Fig. 1C). The CD4⁺ T cell percentage was not significantly changed by 2A treatment in BAL and lung (Fig. 1, B and C), although the CD8⁺ T cell percentage increased. However, due to a reduction in the total cell number in BAL (2.9 ± 0.26 in control-treated vs 0.1 ± 0.02 million in 2A-treated group) and lung (9.3 ± 1.5 in control-treated vs 2.7 ± 0.4 million in 2A-treated group), the absolute cell number of eosinophils and CD4⁺ T cells was reduced in BAL (Fig. 1D) and lung (Fig. 1E) of 2A-treated mice. In the BAL, eosinophil numbers were reduced >100-fold (2.1 ± 0.26 in control-treated vs 0.02 ± 0.01 million in 2A-treated mice) and the number of CD4⁺ T cells decreased >30-fold (0.41 ± 0.04 in control- vs 0.015 ± 0.008

million in 2A- treated mice). Similarly, in the lung, eosinophil numbers were reduced 8- to 9-fold (3.4 ± 0.8 in control- vs 0.38 ± 0.05 million in 2A-treated mice) and CD4⁺ T cells decreased ~4-fold (1.7 ± 0.44 in control- vs 0.4 ± 0.05 million in 2A-treated mice). These results show that CD137 engagement significantly inhibits Th2-mediated airway inflammation.

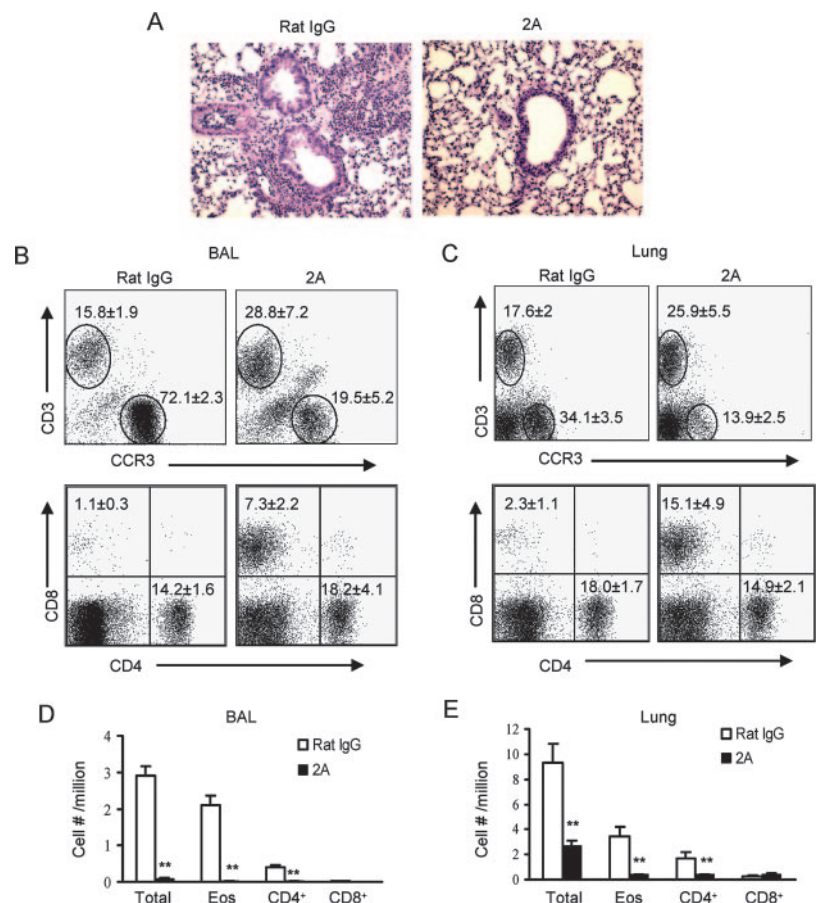
CD137 costimulation inhibits Th2 cytokine production in airway and lung

It has been reported (38–40) that the observed eosinophilia in the airway is related to the local production of Th2 cytokines, such as IL-4 and IL-5. Therefore, we tested IL-5 and IL-4 production in BAL and lung. The results showed that IL-5 and IL-4 levels were significantly reduced in the BAL (Fig. 2A) and lung (Fig. 2B) of 2A-treated mice compared with the control group. In accordance with this, intracellular cytokine staining demonstrated that there was a reduced percentage of CD4⁺ T cells producing either IL-5 or IL-4 in the lung of the 2A-treated group compared with the control group (Fig. 2C). When leukocytes isolated from digested lung were restimulated ex vivo with SEA Ag, the control group produced large amounts of the Th2 cytokines IL-4 and especially IL-5. However, the 2A-treated group did not respond to SEA restimulation to produce IL-4 and IL-5 (Fig. 2D). These studies suggest that strong CD137 engagement can inhibit Ag-specific Th2 responses in both airway and lung.

CD137 costimulation inhibits IgE production and bronchial hyperresponsiveness (BHR)

S. mansoni egg administration induces a strong IgE response which is the prototypical characteristic of parasitic infection and indicative of a Th2 response. It has also been shown (41) that an

FIGURE 1. Agonistic anti-CD137 treatment prevents airway inflammation. Mice were sensitized i.p. on day 0 with inactivated *S. mansoni* eggs and treated with either control rat IgG or 2A, followed by SEA challenge on day 7 via intratracheal delivery. Five days after challenge (A), lung tissues were harvested, fixed, and embedded in paraffin. Sections of 5 μ m were stained with H&E. Shown is a representative field from the lung of control and 2A-treated mice. Original magnification, $\times 200$. BAL cells (B) and lung infiltrating cells (C) were isolated, stained with FITC-conjugated anti-CCR3, and PE-conjugated anti-CD3, or FITC-conjugated anti-CD4 and PE-conjugated anti-CD8, and analyzed by flow cytometry. Cell number in BAL (D) and lung (E). The total number of nucleated cells in control (□) and 2A treated (■) was counted and the cell number of each subset was determined by total cell number multiplied with respective percentage from the FACS profiles ($n = 3$). **, Values of $p < 0.001$. The results are representative of three independent experiments.



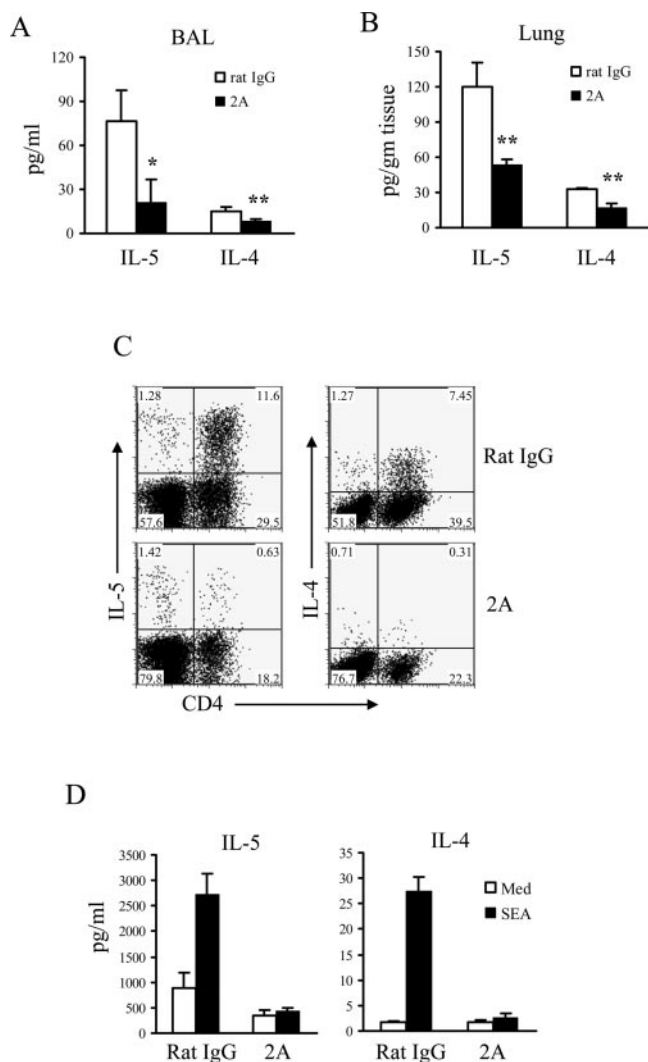


FIGURE 2. CD137 costimulation inhibits Th2 cytokine production in airway and lung. Mice were primed and challenged as in Fig. 1. IL-4 and IL-5 levels in BAL (A) and lung homogenate (B) were measured by cytokine bead array from rat IgG-treated mice (□) and from 2A-treated mice (■) ($n = 3$). *, $p < 0.005$; **, $p < 0.001$. C, Intracellular cytokine staining of lung-infiltrating cells. D, Lung-infiltrating cells were isolated and cultured with SEA ex vivo for 3 days. Supernatants were collected and IL-5 and IL-4 levels were measured by cytokine bead array. The results are representative of three independent experiments.

IgE-dependent mechanism is associated with a Th2 immune response and the subsequent infiltration of eosinophils into the airways. Similarly to the reduced Th2 cytokine production and diminished eosinophilia in airway, 2A treatment significantly diminished the levels of serum IgE to that of naive mice (~200 ng/ml) compared with control animals (Fig. 3A). Allergen-linked IgE signaling participates in the initiation of immediate hypersensitivity reaction and BHR by triggering mast cell degranulation (41, 42). To evaluate the severity of BHR in 2A-treated mice, we used intratracheal delivery of methacholine to assess airway constrictor responsiveness in anesthetized, tracheostomized, mechanically ventilated mice, using the Flexivent computerized mouse ventilator to calculate the respiratory resistance (43). The 2A-treated mice showed a significantly diminished response to methacholine when compared with control rat IgG-treated mice. Interestingly, the response of 2A-treated mice is close to that of naive mice. The peak resistance in the bronchoconstrictor response to

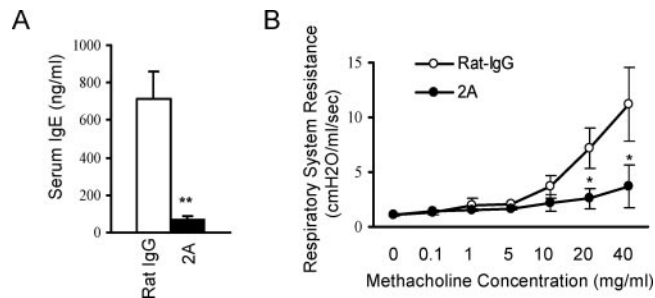


FIGURE 3. Agonistic anti-CD137 treatment suppresses IgE production and BHR to nonspecific challenge. Mice were primed and challenged as in Fig. 1. A, Five days after challenge, sera were collected and IgE levels in control rat IgG (□)- and 2A (■)-treated mice were detected by ELISA. B, Methacholine dose-response curves for respiratory system resistance in a control rat IgG- vs 2A-treated mouse. Invasive measurement was used to determine respiratory system resistance in anesthetized, tracheostomized, and mechanically ventilated mice. The peak resistance from each dose from pooled data from three experiments was analyzed to compare both groups ($n = 3$). *, $p < 0.005$; **, $p < 0.001$.

increasing doses of methacholine from control and 2A-treated mice is shown in Fig. 3B. These data confirmed that CD137 engagement inhibits classic features of Th2-mediated responses in the airway, including local eosinophilia, IgE responses, and bronchial constriction.

CD137 engagement inhibits Th2 cell function in vivo

The diminished Th2 cytokine production and inflammation in airway and lung in 2A-treated mice could be attributable to a defect of Th2 cell migration to airway or to defective effector function from Th2 cells in these mice. To dissect the mechanism involved, we tested Ag-specific T cell function on day 5, 7, and 12 after priming with *S. mansoni* eggs by testing ex vivo proliferation and cytokine production in the presence of SEA. We found that splenocytes from control mice showed robust proliferation in the presence of SEA at all of the time points tested. However, splenocytes from 2A-treated mice exhibited much less proliferation than the control 5 days after priming (Fig. 4A, left panel), and almost no proliferative response to SEA stimulation on days 7 and 12 (Fig. 4A, center and right panels). Splenocytes from control mice produced increasing amounts of the Th2 cytokine IL-5 in the presence of SEA ex vivo. On the contrary, splenocytes from 2A-treated mice did not produce significant IL-5 at any time point under the same conditions (Fig. 4B). To determine whether the inhibition of Th2 responses by CD137 engagement is due to the costimulatory effect of CD137 on Th1 cells through promoting IFN- γ production, we also monitored Ag specific IFN- γ production. The results showed splenocytes from control mice produced a small amount of Th1 cytokine IFN- γ early (days 5 and 7), but then subsides after day 12. The level of IFN- γ from the 2A-treated group is slightly lower than that of the control group (days 5 and 7) and maintains this level even after day 12 (Fig. 4C). These data suggest that it is unlikely that the complete lack of IL-5 production is caused by a 2A-mediated increase of the Th1 response; instead, they imply that CD137 engagement may directly inhibit Th2 cell skewing and its function in vivo.

Inhibition of the Th2 response by CD137 costimulation is IFN- γ and CD4⁺CD25⁺ Treg independent

Previous studies (24, 27) have shown that IFN- γ plays a critical role in agonistic anti-CD137-mediated suppression of Th1-mediated autoimmune disease. To test whether IFN- γ was also involved

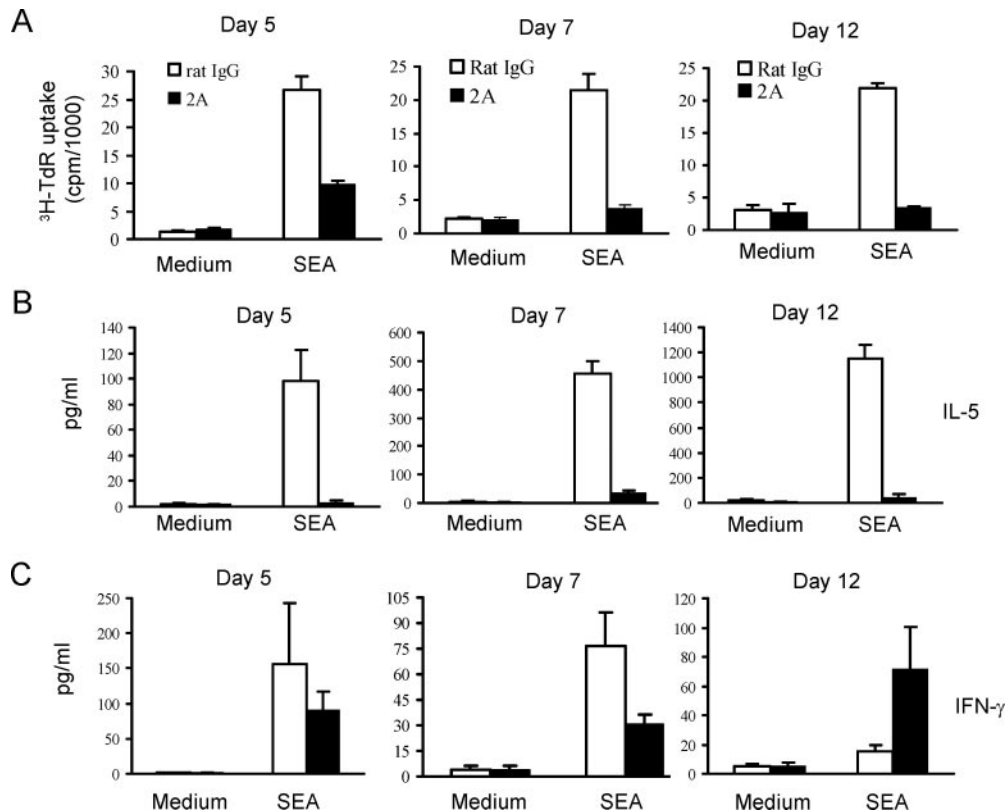


FIGURE 4. CD137 engagement inhibits Th2 cell function in vivo. Mice were sensitized i.p. on day 0 with inactivated *S. mansoni* eggs and treated with either control rat IgG or 2A. Splenocytes from rat IgG ($\square$)-treated and 2A ($\blacksquare$)-treated mice were isolated on days 5, 7, and 12 and restimulated with 5–10 $\mu\text{g}/\text{ml}$ SEA ex vivo. A, T cell proliferation was detected by [^3H]thymidine incorporation. Th2 cytokine IL-5 (B) and Th1 cytokine IFN- γ (C) levels in 3-day culture supernatant were detected by cytokine bead array ($n = 3$). The results are representative of two independent experiments.

in the inhibition of Th2 responses by CD137 engagement in vivo, we treated mice with anti-IFN- γ Ab on the same day of immunization with *S. mansoni* egg and 2A treatment. The results showed that the IFN- γ blockade did not reverse the inhibition of Ag-specific IL-5 (Fig. 5A) and IFN- γ (Fig. 5B) production mediated by agonistic anti-CD137 treatment. IL-4 production showed a similar pattern as IL-5; however, the overall IL-4 cytokine level was much lower (data not shown). This study suggests the inhibition of Th2 responses by CD137 costimulation uses an IFN- γ -independent mechanism.

CD137 signaling has also been shown to costimulate CD4 $^+$ CD25 $^+$ regulatory cells (15). We found that 2A treatment increased CD4 $^+$ CD25 $^+$ Foxp3 $^+$ Treg cell numbers in the spleen (data not shown), but CD25 depletion did not reverse 2A-mediated Th2 cytokine production (Fig. 5C), suggesting that CD137-mediated inhibition of Th2 responses is independent of these cells. Interestingly, the reduced IFN- γ production by 2A treatment was reversed with Treg depletion (Fig. 5D). This study implies Treg are dispensable in the mechanism of CD137-mediated suppression of Th2 responses.

Anti-CD137 treatment reduces GATA3 expression

GATA3 and T-bet are critical transcription factors for Th2 and Th1 cell development, respectively (44); therefore, we tested the effect of anti-CD137 treatment on their expression. We found 4 days after immunization that 2A-treated mice showed a reduced GATA3 mRNA level in the spleen compared with that of control mice; however, the mRNA level of T-bet remains unchanged (Fig. 5E). These data suggest that CD137 costimulation induces downregulation of Th2-specific transcription factor GATA3.

CD137 engagement inhibits the Th2 cell response in vitro

To test whether CD137 costimulation directly inhibited Th2 cell function, we used an in vitro culture system. LN cells were cultured with a suboptimal dose of plate-bound anti-CD3 in the presence of anti-CD28 and/or anti-CD137 (2A) for 3 days. As shown in Fig. 6A, both CD28 and CD137 costimulation induced similar levels of IFN- γ production. IL-4 production, however, was greatly reduced in the presence of anti-CD137 compared with anti-CD28. Furthermore, although anti-CD137 synergizes with anti-CD28 in inducing IFN- γ production, it inhibited IL-4 production induced by CD28 costimulation. Even in the presence of increasing amounts of anti-IFN- γ , which almost completely blocked IFN- γ in the culture, IL-4 production was not recovered. This suggests that the inhibition of the Th2 response by CD137 costimulation is due to its specific effect on Th2 cell differentiation rather than the promotion of IFN- γ -producing cells.

To further test the effect of CD137 costimulation on naive T cell differentiation to Th1 and Th2, we cultured LN cells with suboptimal doses of plate-bound anti-CD3 in the presence of anti-CD28 or anti-CD137 as described above for 7 days for priming and differentiation; live cells were then isolated and restimulated for 2 days with anti-CD3. The results showed that although CD28 costimulation induced both Th1 cytokine IFN- γ and Th2 cytokine IL-4 and IL-5 production, CD137 costimulation induced only Th1 but not Th2 responses. Furthermore, CD137 engagement together with anti-CD28 inhibits Th2 cytokine production (Fig. 6B). These data suggest that CD137 engagement provides costimulatory signaling to Th1 cells while inhibitory signaling to Th2 cell activation.

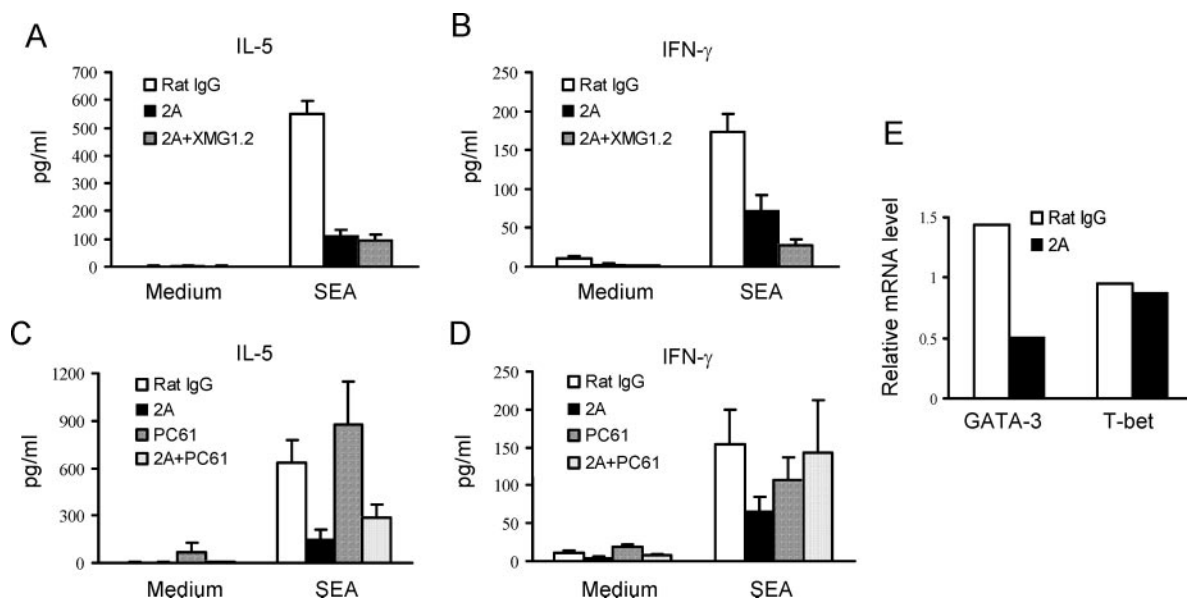


FIGURE 5. Inhibition of a Th2 response by CD137 costimulation is IFN- γ and Treg independent. Mice that were injected with XMG1.2 (A and B) the day of or PC61 (C and D) the day before mice were immunized i.p. with inactivated *S. mansoni* eggs and treated with either control rat IgG or 2A. A and B, Splenocytes from rat IgG ($\square$), 2A ($\blacksquare$), and 2A plus XMG1.2 (striped line) were isolated 7 days after immunization and restimulated with 5 μ g/ml SEA ex vivo. C and D, Splenocytes from rat IgG ($\square$), 2A ($\blacksquare$), PC61 (striped line), and 2A plus PC61 (dotted line) were isolated 7 days after immunization and restimulated with 5 μ g/ml SEA ex vivo. Th2 cytokine IL-5 (A and C) and Th1 cytokine IFN- γ (B and D) levels in 3-day culture supernatants were detected by cytokine bead array ($n = 3$). E, Real-time PCR analysis was conducted on cDNA prepared from DNase-treated RNA extracted from the spleen of mice immunized with inactivated *S. mansoni* eggs and treated with either control rat IgG or 2A for 4 days. Relative abundance of mRNA levels of GATA3 and T-bet is shown. The results are representative of two independent experiments.

Discussion

Allergic airway inflammatory disease, a prototypic Th2-dominant response, is a major health hazard in developed countries, resulting in an increase in mortality and morbidity. Selective inhibition of Th2-dominant responses in the airway could be an ideal treatment for allergic airway inflammation. CD137 engagement provides a potent costimulatory signal of T cell responses, especially CD8-mediated responses. Interestingly, the results presented in this study have demonstrated that strong CD137 engagement prevents Th2-mediated allergic airway disease. The use of a single dose of agonistic Ab to CD137 can potently diminish eosinophilia, CD4⁺ T cell infiltration, inhibit Th2 cytokine production in airway and lung, and dramatically reduce IgE production and BHR to non-specific Ag challenge.

The role of anti-CD137 in helper T cell-mediated responses is rather complicated. Lee et al. (45) recently found that CD137-deficient CD4⁺ T cells showed hyperresponsive responses to protein Ag in vivo, suggesting a negative role for CD137. Accordingly, agonistic anti-CD137 treatment suppresses multiple Th1-mediated autoimmune diseases, including EAE, collagen-induced arthritis, and experimental autoimmune uveitis models (23, 26–28). Our studies have shown it also inhibited Th2-mediated AAD. Although a CD137 agonist inhibits both Th1- and Th2-mediated immune responses in vivo, the mechanisms involved appear to be different. CD137 plays a diametric role in Th1 responses. Our previous study (23) showed in Th1-mediated EAE, CD137 costimulation did not inhibit T cell priming, instead, it initially promoted proliferation of CD4⁺ T cells, and Th1 cytokines such as IFN- γ and GM-CSF production shortly after immunization. It then potentiated the activation-induced cell death of activated T cells resulting in the disappearance of activated Th1 cells and the diminished Th1 response (23). The outcome of dual phases of CD137 costimulation on CD4⁺ T cells was also observed in another Th1-mediated experimental autoimmune uveitis model (28), as well as

a GVHD model, in which agonistic anti-CD137 treatment facilitates CD4-mediated acute GVHD but inhibits chronic GVHD (18, 20). In support of the positive role of CD137 on Th1 responses, in a tumor model, Li et al. (46) reported that CD137 engagement, in addition to anti-CD3/anti-CD28 activation, preferentially up-regulated Th1 cytokines IFN- γ and GM-CSF secretion by murine tumor-draining LN cells, whereas concomitantly reducing IL-10, which led to effective tumor regression. Similarly, human CD137 cross-linking regulated anti-CD28 costimulation to promote Th1 cytokine IFN- γ (47). Therefore, CD137 engagement may have a dual effect on Th1 development depending on the status of activation. In the AAD model we used in this study, strong CD137 engagement completely inhibited the initiation of Th2 response, which is different from its effect on Th1 responses. Ag-specific Th2 cytokine production could not be detected at any time point (early and late) after immunization, suggesting a single-phase inhibitory role of CD137 on Th2 responses. In vivo, however, it is difficult to demonstrate a direct costimulatory or coinhibitory role. Therefore, we performed in vitro experiments to test whether CD137 costimulation can inhibit Th2 cytokine production in response to TCR cross-linking. CD137 costimulation in vitro promoted Th1, but not Th2 cytokine production, in response to a suboptimal dose of anti-CD3, and inhibited Th2 cytokines but promoted Th1 cytokine production in the presence of both anti-CD3 and anti-CD28. In support of the negative role of CD137 on Th2 responses, a recent study by Maerten et al. (48) has shown that CD137-deficient CD4⁺ T cells exhibited increased Th2 responses in an adoptive transfer model of colitis. Together, these studies support that CD137 engagement can be a coinhibitor for Th2-mediated responses.

A previous study (27) has shown that the inhibition of Th1 responses by a CD137 agonist is dependent on IFN- γ produced primarily by a CD8⁺CD11c⁺ cell population. Activation of a Th1 response or an enhancement of IFN- γ by either CD4⁺ or CD8⁺

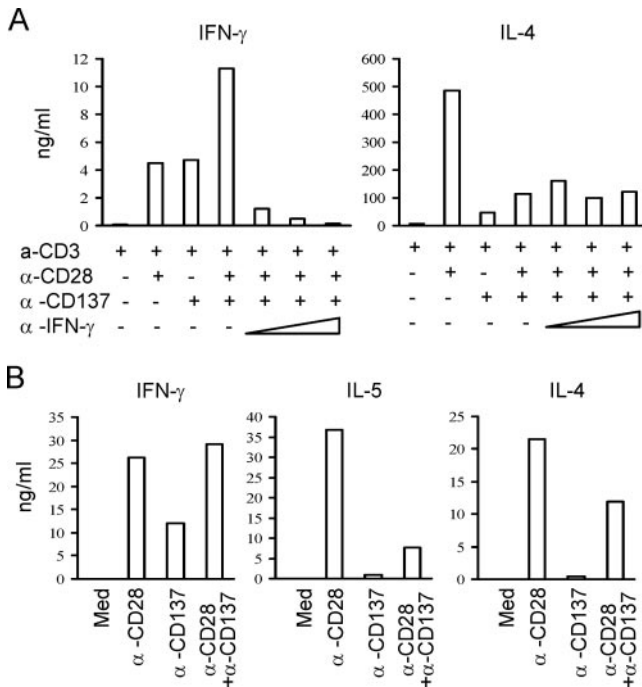


FIGURE 6. CD137 costimulation inhibits Th2 cell responses in vitro. *A*, LN cells were cultured with 0.5 μ g/ml anti-CD3 alone or in the presence of anti-CD28 (1 μ g/ml) and/or 2A (30 μ g/ml). Anti-IFN- γ ascites (1/5000, 1/500, and 1/50) were added to the cultures to neutralize IFN- γ . Supernatants were collected on day 3 and triplicate wells were pooled for cytokine analysis. *B*, LN cells were cultured with 0.5 μ g/ml anti-CD3 in the presence of anti-CD28 (1 μ g/ml) or 2A (30 μ g/ml) for 7 days, then live cells were isolated and restimulated with anti-CD3 (1 μ g/ml) for 2 days. Supernatants were collected and triplicate wells were pooled. IFN- γ , IL-5, and IL-4 levels were analyzed by cytokine bead array. The results are representative of three independent experiments.

cells could then potentially reduce Th2 responses. However, our data suggest that the mechanism of CD137-mediated Th2 inhibition is not due to increased IFN- γ . First, we observed that the inhibition of Th2 responses in vitro by CD137 engagement is not reversed by neutralization of IFN- γ . Second, shortly after treatment we did not observe an increase in Ag-specific IFN- γ production with the observed lack of IL-5 production. Lastly, in vivo IFN- γ blockade did not reverse the CD137-mediated amelioration of Th2 responses. Therefore, CD137-mediated inhibition of Th2 responses is IFN- γ independent. This is further supported by the studies of Fukushima et al. (49), which have shown that CD137 engagement by a different agonistic Ab can also inhibit both priming and effector phases of Th2-mediated experimental allergic conjunctivitis in vivo in the absence of IFN- γ .

Previous studies by Zheng et al. (15) have shown that CD137 costimulation induces proliferation of CD4⁺CD25⁺ Treg both in vitro and in vivo. Using the *S. mansoni* model of AAD, we also observed an increase of the CD4⁺CD25⁺Foxp3⁺ population in anti-CD137-treated mice 7 days after immunization and treatment (data not shown). However, the depletion of CD25-positive cells did not reverse the suppressed Th2 cytokine production, suggesting Treg does not play an essential role in CD137-mediated inhibition of Th2 responses. GATA3 is a key transcription factor involved in Th2 cell differentiation (44). It promotes Th2 responses through multiple mechanisms, which include induction of Th2 cytokine production, selective growth of Th2 cells, and inhibition of Th1 cell-specific factors (50). We detected reduced GATA3 expression in spleen of anti-CD137-treated mice. Thus, it will be

interesting to determine whether and how CD137 signaling can directly down-regulate GATA3 and other transcription factors involved in Th2 cell development.

In summary, these studies suggest that the engagement of CD137 on different subsets of cells can result in distinctive roles including a strong promotion of CD8 responses, a dual effect on Th1 responses, and, now, we show that CD137 engagement can strongly inhibit Th2 cell priming at early phases of differentiation. Strong engagement of CD137 can even inhibit CD28-mediated costimulation of Th2 cytokines but not Th1 cytokine production. Therefore, CD137 may work as a coinhibitory molecule for the Th2 response. Consistently, agonistic anti-CD137 treatment in vivo inhibited Th2-mediated allergic airway disease. Future studies will need to dissect how CD137 signaling exhibits different effects on various T cell subsets and explore its potential application in the treatment of various T cell-mediated diseases. Our demonstration of both costimulatory and coinhibitory roles for the same molecule, CD137, by an agonistic Ab in both in vitro and in vivo conditions also raises the possibility of a dual role for other costimulatory molecules. It may be important to determine whether this dual role depends on the type of T cells, activation status, and possible coordination with other ligands or cytokines which may be a critical guide for the design of future immunotherapies.

Disclosures

The authors have no financial conflict of interest.

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