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CTLA4Ig Inhibits Effector T Cells through Regulatory T Cells and TGF- β

Christine M. Deppong,* Traci L. Bricker,* Brandy D. Rannals,* Nico Van Rooijen,[†] Chyi-Song Hsieh,*[‡] and Jonathan M. Green*[‡]

The CD28 costimulatory receptor is a critical regulator of T cell function, making it an attractive therapeutic target for the treatment of immune-mediated diseases. CTLA4Ig, now approved for use in humans, prevents naive T cell activation by binding to B7 proteins and blocking engagement of CD28. However, CTLA4Ig suppresses inflammation even if administered when disease is established, suggesting alternative mechanisms. We identified a novel, CD28-independent mechanism by which CTLA4Ig inhibits activated T cells. We show that in vitro, CTLA4Ig synergizes with NO from bone marrow-derived macrophages to inhibit T cell proliferation. Depletion of regulatory T cells (Tregs) or interference with TGF- β signaling abrogated the inhibitory effect of CTLA4Ig. Parallel in vivo experiments using an allergic airway inflammation model demonstrated that this novel mechanism required both macrophages and regulatory T cells. Furthermore, CTLA4Ig was ineffective in SMAD3-deficient mice, supporting a requirement for TGF- β signaling. Thus, in addition to preventing naive T cells from being fully activated, CTLA4Ig can turn off already activated effector T cells by an NO/regulatory T cell/TGF- β -dependent pathway. This mechanism is similar to cell-extrinsic effects of endogenous CTLA4 and may be particularly important in the ability of CTLA4Ig to treat chronic inflammatory disease. *The Journal of Immunology*, 2013, 191: 000–000.

Approaches to augment or interfere with immune cell function may be of benefit in many diseases. Members of the CD28 receptor family both activate and inhibit T cell responses, making them attractive therapeutic targets. CD28 is one of the best studied and was the first to be targeted with the development of CTLA4Ig. CTLA4Ig has been shown to be effective both in vitro as well as in numerous animal models of disease (reviewed in Ref. 1). These studies led to the development of the humanized version, abatacept, and the related protein, belatacept, which are approved for use in humans to treat rheumatoid arthritis and prevent renal transplant rejection, respectively (2, 3). Biologics directed against additional members of the CD28 family have also been developed including anti-CTLA4 Abs (ipilimumab) to treat malignant melanoma, and promising results have been reported with anti-programmed death-1 therapy in early cancer trials (4–6).

CTLA4Ig is a fusion protein of the extracellular domain of CTLA4 and IgG1 that binds to both CD80 and CD86 (also referred to as B7-1 and B7-2, or collectively as B7 proteins) and prevents interaction of B7 proteins with their counterreceptors CD28 and CTLA4 expressed on T cells (7). Additionally, CD80 has been shown to

bind programmed death ligand-1 (PD-L1) and inhibit T cell activation and proliferation through this interaction (8). The primary mechanism of action for CTLA4Ig has been thought to be blockade of CD28 and therefore prevention of initial T cell activation. However, we previously demonstrated that CTLA4Ig was effective when administered after initial Ag activation of T cells and that this was independent of CD28 (9). In the present study, we report the mechanism for this novel mode of action for CTLA4Ig. We demonstrate that the effects of CTLA4Ig are mediated by regulatory T cells (Tregs) and TGF- β and require macrophage-derived NO. These data provide an entirely new insight into how treatment with CTLA4Ig suppresses inflammation and may provide information relevant to how endogenous CTLA4/B7 interactions inhibit T cell responses.

Materials and Methods

Mice

C57BL/6J and NOS2-deficient mice were purchased from The Jackson Laboratory (Bar Harbor, ME). STAT1-deficient mice were gifts of Dr. M. Holtzman and Dr. H. Virgin (Washington University School of Medicine, St. Louis, MO). CD80/86-deficient mice and Foxp3-diphtheria toxin receptor (DTR) mice were provided by Alexander Rudensky (Memorial Sloan-Kettering Cancer Center, New York, NY). SMAD3-deficient mice were provided by Dr. David Beebe (Washington University School of Medicine). IDO-deficient mice were provided by Dr. Matthew Ciorba (Washington University School of Medicine). Foxp3-IRES-GFP (B6.Cg-Foxp3^{tm2Tch}/J) mice were purchased from The Jackson Laboratory and crossed to OT-II OVA transgenic (Tg) mice on a RAG1-deficient background to generate OT-II Tg/Foxp3-GFP Tg/Rag1-knockout (KO) mice. All mice were bred and housed in specific pathogen-free facilities at Washington University School of Medicine. All animal studies have been approved by the Washington University Animal Studies Committee.

Abs

Anti-IFN- γ (clone H22, provided by R. Schreiber, Washington University, St. Louis, MO) and anti-CD4 were purchased from BioLegend (San Diego, CA). Anti-TGF- β (clone 1D11) was purchased from R&D Systems (Minneapolis, MN). Murine CTLA4Ig was provided by Bristol-Myers Squibb (Princeton, NJ).

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The online version of this article contains supplemental material.

Abbreviations used in this article: BAL, bronchoalveolar lavage; BMDM, bone marrow-derived macrophage; DT, diphtheria toxin; DTR, diphtheria toxin receptor; HEL, hen egg lysozyme; KO, knockout; L-NMMA, N^G-methyl-L-arginine acetate; NOS2, NO synthase 2; PD-L1, programmed death ligand-1; Tg, transgenic; Treg, regulatory T cell.

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Experimental allergic airway inflammation

Mice were immunized and challenged with OVA (Sigma-Aldrich, St. Louis, MO) as previously described (10). When indicated, clodronate liposomes were prepared as described (11) and administered (100 μ l i.p. and 50 μ l intranasally) 1 d prior to inhaled challenge. In some experiments, as indicated, groups of mice were given 100 μ g CTLA4Ig i.p. on the day of challenge. Neutralizing Ab against IFN- γ (250 μ g/mouse) was administered 24 h prior to inhaled challenge. For depletion of Tregs, Foxp3-DTR mice were administered 1 μ g DT i.p. (Sigma-Aldrich) 1 d prior to and again on the day of challenge, and an additional 0.2 μ g was administered 2 d after challenge. For bone marrow chimeras, recipient mice were lethally irradiated with 1000 rad and the following day injected i.v. with bone marrow harvested from femurs of donor mice. The mice were allowed to reconstitute for 8 wk and then used in the allergic airway inflammation model as described above. Unless otherwise indicated, all in vivo experiments used four to five mice per experimental group and have been repeated three times, with one representative experiment presented. Cell counts and differentials are presented as the means $\pm$ SD.

Adoptive transfer studies

Splenocytes were isolated from OT-II Tg/FoxP3-GFP Tg/Rag1 KO mice (which are CD45.2⁺) and 3×10^6 cells injected i.v. into CD45.1⁺ C57BL/6 recipients. The mice were rested for 1 d and then primed and challenged with OVA/aluminum hydroxide as described for the allergic airway inflammation studies. Spleen and lung draining lymph nodes were isolated 48 h after inhaled challenge and isolated cells were stained for CD4 and CD45.2. The samples were then analyzed by flow cytometry and the percentages of total CD45.2 cells that are positive for CD4 and Foxp3-GFP expression are presented. Each group contains five mice, and the means $\pm$ SEM are presented.

In vitro cocultures

Bone marrow was isolated from mice of various genotypes as indicated and cultured in non-tissue culture-treated Petri dishes in DMEM supplemented with 10% FBS, 15% L cell supernatant, and 5% horse serum (12). Macrophages were harvested 6 d later using cold PBS and 5×10^5 macro-

phages cocultured in 96-well plates for 5 d with 1×10^5 splenocytes labeled with CFSE (Molecular Probes/Invitrogen, Grand Island, NY) and activated with PMA (5 ng/ml, Sigma-Aldrich) and ionomycin (0.4 μ g/ml, Sigma-Aldrich). When indicated, the following reagents were included: CTLA4Ig (10 μ g/ml), *N*^G-methyl-L-arginine acetate (L-NMMA; 100 μ M, Sigma-Aldrich), anti-IFN- γ (10 μ g/ml), anti-TGF- β Ab, or SB431542 (Calbiochem, Billerica, MA). Nonadherent cells were harvested, stained with anti-CD4-allophycocyanin, and subsequently analyzed on a FACS-Calibur flow cytometer (BD Biosciences, San Jose, CA) to measure cell proliferation. Flow cytometry data were analyzed using Winlist 6.0 (Verity Software, Topsham, ME). Cell death was determined by staining with annexin V and 7-aminoactinomycin D (BD Biosciences) and analyzed by flow cytometry. Cytokine concentrations were measured on culture supernatants using a Th1/Th2/Th17 cytokine bead array (BD Biosciences). Pooled results from multiple independent experiments are shown. The number of repeats included is indicated in each figure legend. Unless otherwise indicated, all experiments have been repeated at least three times and data from one representative experiment are presented.

Statistical analysis

For the in vitro coculture experiments, the results from independent experiments were pooled and statistical comparisons performed using a paired *t* test using Microsoft Excel (Microsoft, Redmond, WA). For the bronchoalveolar lavage (BAL) cell counts and differentials obtained in the allergic airway inflammation model, *p* values were obtained using the Kruskal-Wallis one-way ANOVA and a Dunn posttest comparison or Mann-Whitney *U* test as indicated in the figure legends (GraphPad Prism, GraphPad Software, San Diego, CA).

Results

Inhibition of CD28-independent induction of T cell proliferation in vitro requires macrophage-derived NO and expression of B7 proteins

Administration of CTLA4Ig at the time of allergen challenge can suppress airway inflammation in wild-type mice but is ineffective

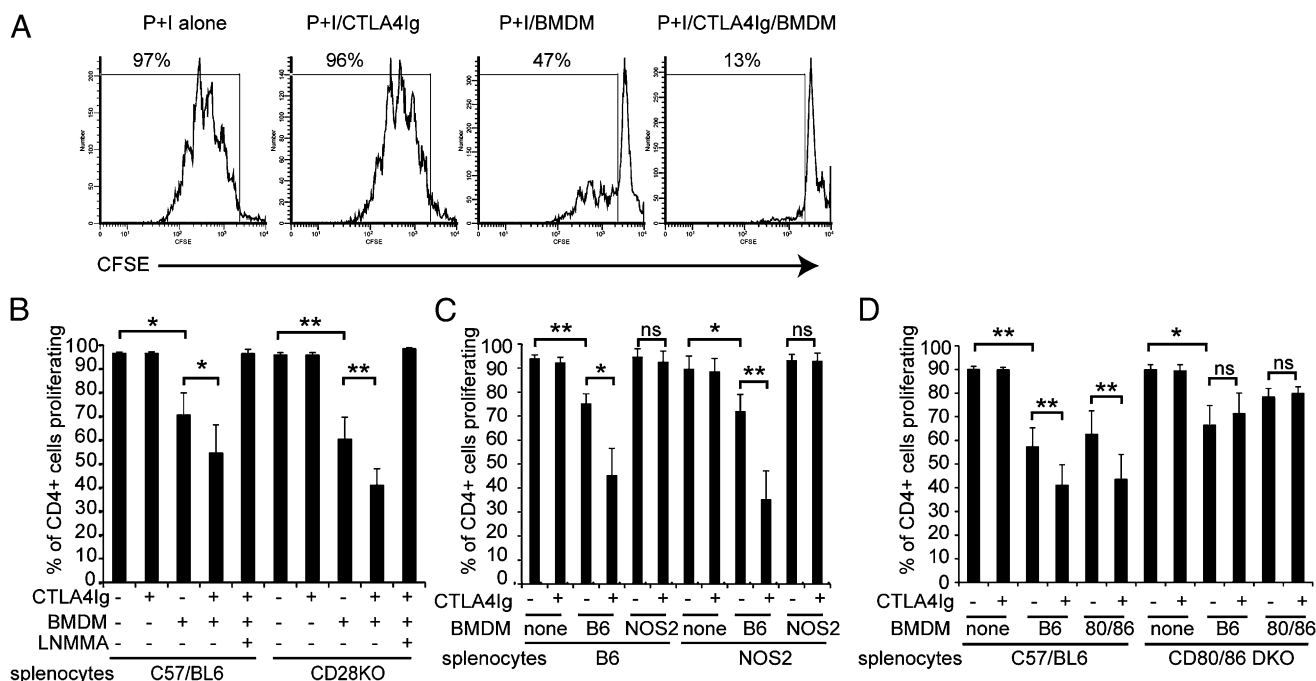


FIGURE 1. CD28-independent inhibition of T cell proliferation by CTLA4Ig in vitro. CFSE-labeled splenocytes were cocultured with BMDMs and activated with PMA and ionomycin. Cells were harvested and stained with anti-CD4 and proliferation was determined by flow cytometry after 96 h of culture. Where indicated, CTLA4Ig (10 μ g/ml) and/or L-NMMA (100 μ M) were included at the start of the culture. **(A)** Representative CFSE dye dilution histograms of wild-type splenocytes stimulated with PMA and ionomycin alone or in combination with BMDMs and/or CTLA4Ig as indicated. The percentage of cells that have undergone at least one cell division is indicated. **(B)** Splenocytes from wild-type or CD28-deficient mice were cocultured with wild-type BMDMs. Presented are the combined results from six independent experiments. **(C)** Splenocytes from wild-type or NOS2-deficient mice were cocultured with BMDMs from wild-type or NOS2-deficient mice. Presented are the combined results from three independent experiments. **(D)** Splenocytes from wild-type or CD80/CD86 double-deficient mice were cocultured with BMDMs from wild-type or CD80/CD86 double-deficient mice. Presented are the combined results from seven independent experiments. **p* < 0.05, ***p* < 0.01 by two-tailed paired *t* test.

in mice lacking NO synthase 2 (NOS2) (9). One of the major sources of NO is the macrophage, and previous work had demonstrated that macrophage-derived NO can suppress T cell proliferation (13). Therefore, we tested whether CTLA4Ig synergized with macrophage-derived NO in an in vitro coculture system. Splenocytes were labeled with CFSE, activated with the costimulation independent agonists PMA and ionomycin, and cocultured with bone marrow-derived macrophages (BMDMs). Cell division was measured by flow cytometry. Macrophages partially suppressed T cell proliferation, an effect that was enhanced by addition of CTLA4Ig, whereas CTLA4Ig alone had no effect on proliferation. Representative CFSE histograms are shown in Fig. 1A. This was independent of CD28, as splenocytes isolated from CD28-deficient mice were suppressed to the same extent as those from wild-type mice (Fig. 1B). Proliferation was restored by the addition of the NOS2 inhibitor L-NMMA, demonstrating that CTLA4Ig can inhibit T cell proliferation in a CD28-independent, NO-dependent manner.

To determine the cellular source of NO, we isolated splenocytes and macrophages isolated from wild-type and NOS2-deficient mice and tested for T cell suppression with and without CTLA4Ig (Fig. 1C). Whereas splenocytes from either genotype were suppressed by

wild-type macrophages and further suppressed by CTLA4Ig, macrophages derived from NOS2-deficient mice were unable to suppress T cell proliferation either alone or in combination with CTLA4Ig, demonstrating that NO is derived from the macrophage.

We next tested whether expression of CD80 or CD86 was required on the splenocyte or the BMDM (Fig. 1D). Macrophages from either wild-type or CD80/CD86 double-deficient mice were capable of suppressing T cell proliferation, and this was further augmented by CTLA4Ig. However, CTLA4Ig was ineffective in cocultures containing splenocytes from CD80/CD86-deficient mice. Thus, in contrast to the requirement for NOS2, CD80/CD86 expression was dispensable on the macrophage but required on the splenocyte. These data demonstrate that B7 proteins and NOS2 must be expressed in different cell subsets for CTLA4Ig to be effective. Thus, the mechanism cannot be a direct modulation of NOS2 activity by CTLA4Ig binding to B7 on the macrophage.

Inhibition of allergic airway inflammation by CTLA4Ig requires macrophages, NOS2, and B7 expression

To establish that the observations made in vitro were relevant to an in vivo response, we tested whether the requirements for CTLA4Ig

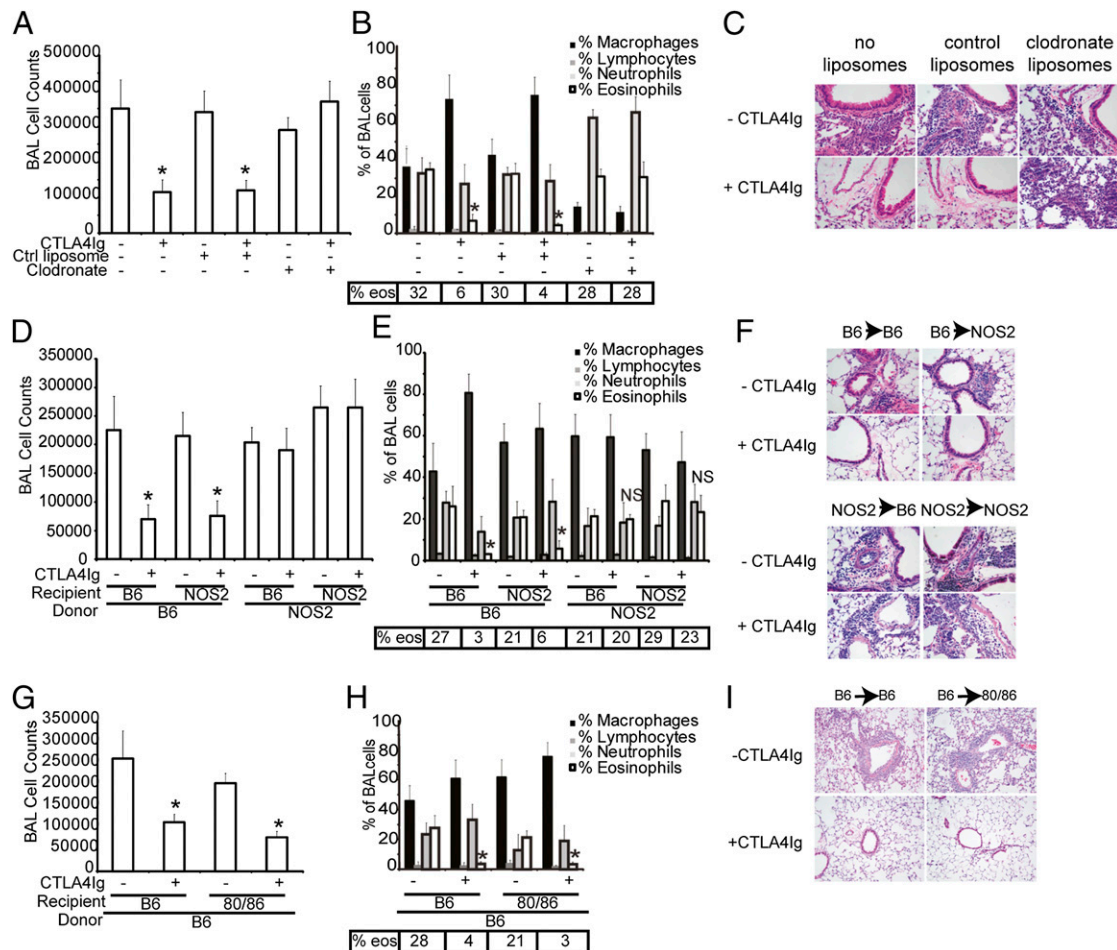


FIGURE 2. Inhibition of allergic airway inflammation by CTLA4Ig in vivo requires macrophages as well as expression of CD80/CD86 and NOS2. (A–C) C57BL/6J mice were sensitized and challenged with OVA as described. Some mice received nothing, control liposomes, or clodronate liposomes to deplete macrophages as indicated. Where indicated, CTLA4Ig was administered at the time of inhaled challenge only. (D–F) Bone marrow chimeras established from C57BL/6J or NOS2-deficient mice reconstituted with either C57BL/6J or NOS2-deficient marrow mice. The mice were sensitized and challenged with OVA and treated with CTLA4Ig at the time of inhaled challenge. (G–I) Bone marrow chimeras established with C57BL/6J or CD80/CD86 double-deficient mice reconstituted with bone marrow cells from C57BL/6J mice were sensitized and challenged with OVA and treated with CTLA4Ig at the time of inhaled challenge. (C, F, I) Lung sections were stained with H&E (original magnification $\times 20$). For all experiments, tissue was collected for analysis 72 h after inhaled challenge. Each experimental group consisted of five mice and each experiment has been repeated three times. Shown are data from one representative experiment. * $p < 0.005$ as compared with condition without CTLA4Ig as determined by Kruskal–Wallis with Dunn posttest comparisons.

to modulate allergic airway inflammation paralleled the results obtained with the coculture system. Mice were primed and challenged with OVA as described (10). CTLA4Ig was injected i.p. at the time of inhaled challenge. In all cases, priming was performed in the absence of administration of CTLA4Ig or any additional drugs, reagents, or experimental manipulations. We first tested whether macrophages were necessary by depleting them via systemic administration of clodronate liposomes (11). Flow cytometry of whole blood obtained at the time of harvest confirmed an absence of circulating macrophages (data not shown), although there were a few macrophages detected in the BAL fluid (Fig. 2B). Mice treated with clodronate or control liposomes developed an eosinophilic inflammatory infiltrate in the lung, as determined by BAL cell counts, differentials, and histology (Fig. 2A–C). However, CTLA4Ig failed to abrogate the inflammatory response in mice that had been treated with clodronate liposomes, whereas it was effective in control mice, demonstrating a requirement for macrophages and consistent with the *in vitro* data.

We had previously shown that administration of CTLA4Ig did not suppress airway inflammation when administered to NOS2-deficient mice at the time of inhaled challenge (9). However, many cell types express NOS2, including both hematopoietic and non-hematopoietic cells. To determine in which compartment NOS2 expression is required, we established mixed bone marrow chimeras with wild-type and NOS2-deficient mice. CTLA4Ig was ef-

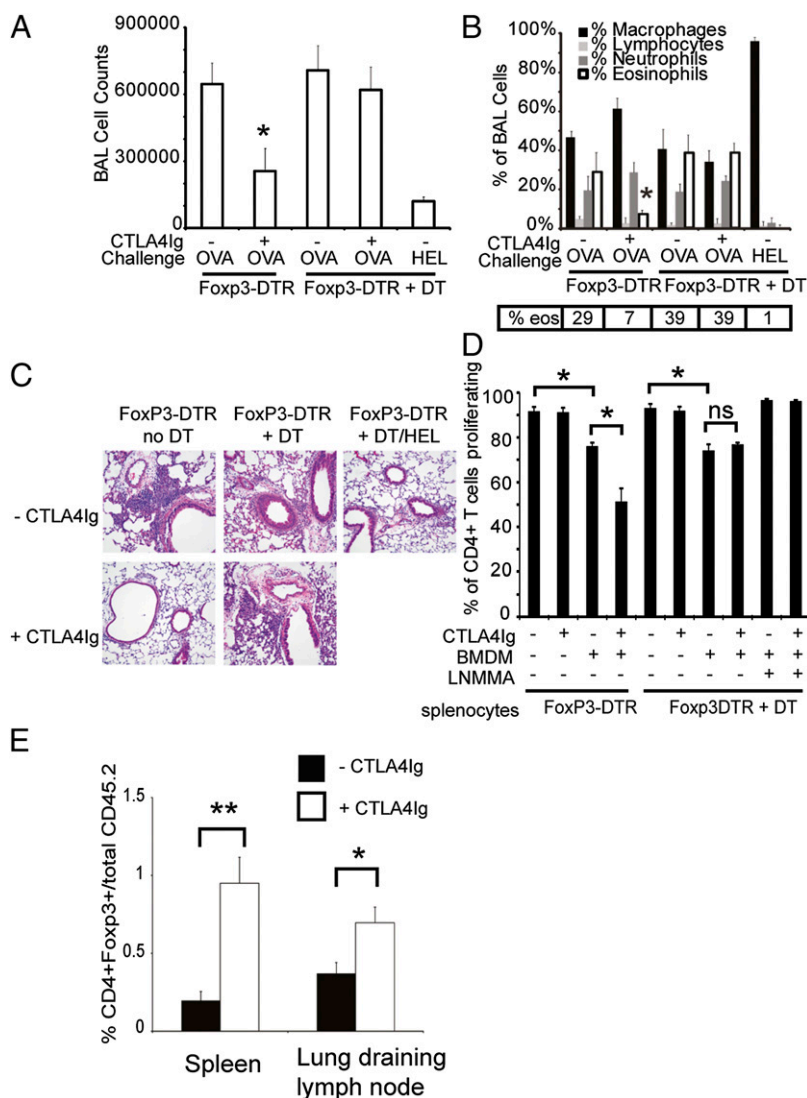
fective only when NOS2 was expressed in the hematopoietic cell compartment, consistent with the requirement for macrophages as a source of NO (Fig. 2D–F).

CD80 and CD86 are expressed primarily on professional APCs; however, they are inducible in other cell types (14–16). To test which cell compartment expression was required, we reconstituted wild-type mice with bone marrow from CD80/CD86 double-deficient mice and vice versa. We found that CD80/CD86 expression in the bone marrow-derived compartment is sufficient for CTLA4Ig to suppress airway inflammation, as shown by the results presented in Fig. 2G–I. In the complementary chimera, in which CD80/CD86-deficient marrow was used to reconstitute wild-type or CD80/CD86-deficient mice, we were unable to induce airway inflammation owing to the necessity of B7/CD28 interactions for T cell priming (data not shown). Taken together, these data are highly consistent with the results obtained from the *in vitro* coculture system.

Tregs are required for the suppressive effects of CTLA4Ig

The data thus far suggests that a complex interplay of multiple cell types is necessary for the inhibitory effects of CTLA4Ig. Both CD28 and CTLA4 are expressed on Tregs and have important roles in their development and function, leading us to hypothesize that they might be involved in modulation of inflammation by CTLA4Ig (17–20). Using mice in which the DTR had been knocked into the Foxp3 locus we were able to specifically deplete Tregs by

FIGURE 3. Foxp3⁺ cells are required for CTLA4Ig-mediated inhibition of T cell proliferation and allergic airway inflammation. (A–C) Foxp3-DTR mice were sensitized with OVA and challenged with either OVA or HEL. Mice were either untreated or administered DT to deplete Foxp3⁺ cells at the time of challenge. CTLA4Ig was administered to the indicated groups at the time of inhaled challenge. Specimens were collected 72 h after inhaled challenge. Each experimental group consisted of five mice and experiments were repeated three times. Shown are data from one representative experiment. * $p < 0.05$ as compared with condition without CTLA4Ig. (C) Lung sections were stained with H&E (original magnification $\times 20$). (D) Foxp3-DTR mice were untreated or administered DT and splenocytes harvested 24 h later, labeled with CFSE, and cocultured with BMDM alone, with CTLA4Ig, or with L-NMMA. Proliferation was determined by flow cytometry after 96 h. Presented are the combined results from four independent experiments. * $p < 0.05$ by two-tailed paired t test. (E) Splenocytes from OT-II/Foxp3-GFP/Rag1 KO mice (CD45.2⁺) were adoptively transferred into CD45.1⁺ C57BL/6 recipients. The mice were then primed and challenged with OVA. One group received CTLA4Ig at the time of challenge. Spleen and lung draining lymph node were harvested 48 h later. Presented is the percentage of CD45.2⁺ cells that are Foxp3-GFP⁺. Each experimental group consisted of five mice and experiments were performed twice. Shown are representative data from one independent experiment. * $p < 0.05$, ** $p < 0.01$ as determined by Mann-Whitney U test.



administration of DT (21). Successful depletion was confirmed in all experiments by flow cytometry (data not shown). As shown in Fig. 3D, Treg-depleted splenocytes were not suppressed by CTLA4Ig, although proliferation was still inhibited by coculture with BMDMs in an NO-dependent manner. Thus, whereas Tregs are required for inhibition by CTLA4Ig, NO can suppress independent of Tregs.

To test whether Tregs were required for CTLA4Ig to suppress inflammation *in vivo*, we depleted mice by injecting them with DT 1 d prior to challenge, assuring that priming occurred in the presence of Tregs. As shown in Fig. 3A–C, mice depleted of Foxp3⁺ cells mounted an inflammatory response that was similar in quality and intensity to mice that did not receive DT. However, depletion of Tregs prevented CTLA4Ig from suppressing inflammation in the OVA-challenged mice. Challenge with a different protein Ag, hen egg lysozyme (HEL), did not induce inflammation, confirming that depletion of Tregs did not result in nonspecific inflammation. When we analyzed the percentage and number of Foxp3⁺ cells recovered from the spleen, lung, or lymph nodes of mice treated with CTLA4Ig in comparison with controls, we were unable to detect an increase in Treg number (data not shown).

The number of Ag-specific Tregs might be too small to detect a change using the above model system. To further examine this, we adoptively transferred cells isolated from OT-II-Tg/FoxP3-GFP-Tg/Rag1-KO mice into wild-type C57BL/6 mice. The donor cells were CD45.2⁺ and the recipient mice expressed CD45.1. Additionally, the donor Tregs expressed GFP, allowing for detection and quantitation of the OVA-specific Tregs. Following transfer, the mice were primed and challenged and either left untreated or treated with CTLA4Ig. Tissue was harvested 48 h after challenge and the percentage of transferred cells that express Foxp3 was determined by flow cytometry. As shown in Fig. 3E, we detected a significant increase in Foxp3⁺ cells in the spleen and lung draining lymph nodes of mice that were treated with CTLA4Ig. Taken together, these data establish that Foxp3⁺ Tregs are required for the suppressive effect of CTLA4Ig, and they suggest that the mechanism may be promoting the expansion or induction of Ag-specific Tregs. Additionally, CTLA4Ig may also be enhancing Treg function or the susceptibility of the effector T cell to Treg-mediated suppression.

CTLA4Ig inhibits by a TGF- β -dependent mechanism

Tregs use a number of mechanisms to inhibit effector T cells, including through TGF- β (17, 22). TGF- β itself inhibits T cells directly as well as indirectly by promoting the differentiation of CD4⁺ T cells into Tregs (22–25). Therefore, we tested whether TGF- β might be involved in the inhibitory effects of CTLA4Ig. Inclusion of a neutralizing Ab against TGF- β prevented CTLA4Ig from inhibiting T cell proliferation *in vitro* (Fig. 4A), as did treatment with SB431542, an inhibitor of the TGF- β receptor (Fig. 4B). Thus, the effects of CTLA4Ig *in vitro* require TGF- β /TGF- β R signaling. However, inhibition by the BMDMs alone was un-

affected by neutralization of TGF- β , indicating that these are separate pathways.

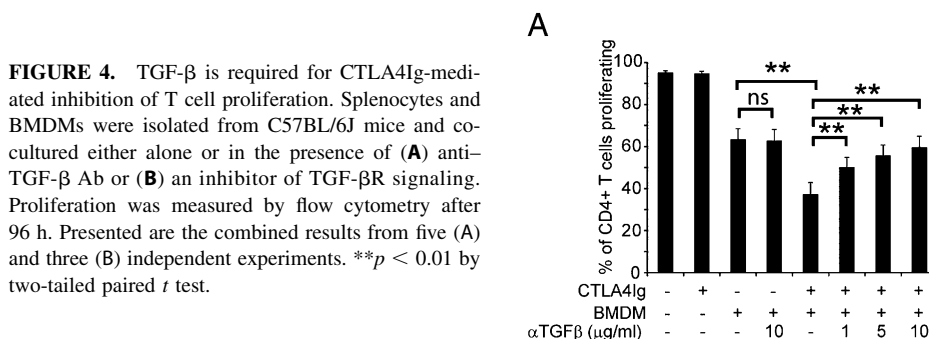
To confirm a role for TGF- β signaling *in vivo*, we tested the effect of CTLA4Ig in SMAD3-deficient mice (Fig. 5). SMAD3-deficient mice develop spontaneous inflammation, which may complicate the analysis of an *in vivo* Ag challenge model; however, consistent with a previous report, we found they responded to inhaled allergen challenge comparably to wild-type mice and that the response was Ag specific (26, 27). Following priming and challenge with OVA, the SMAD3-deficient mice developed an eosinophilic inflammatory cell infiltrate in the lung that was indistinguishable from wild-type mice. In contrast to wild-type, however, this was not blocked by administration of CTLA4Ig, indicating that SMAD3-dependent signaling is required *in vivo*, consistent with the *in vitro* findings of a requirement for TGF- β .

Discussion

CD28 was initially identified as providing a costimulatory signal required for the activation of naive T cells, thus fulfilling the role of a second signal in Bretscher and Cohn's (28) two-signal model of lymphocyte activation. Subsequent work has led to an in-depth understanding of how CD28 regulates T cell function along with an expansion of the family to include additional activating and inhibitory receptors (reviewed in Refs. 29, 30). The development of reagents that manipulate costimulation has led to important insights into normal T cell function as well as new therapeutics; therefore, an accurate understanding of how these agents work is essential. The data we report in the present study detail a novel mechanism of action for CTLA4Ig, with direct implications for therapy as well as for our understanding of immune regulation.

In these studies we have used a well-established model of allergic airway inflammation. This model has been shown to be dependent on both B7 and CD28, and administration of CTLA4Ig completely abrogates OVA-induced airway inflammation, although it may be less effective when exposed to a more intense regimen of inhaled challenges (10, 31–35). Additionally, the CD28-independent effects of CTLA4Ig were first detected using this model in mice deficient for both CD28 and BTLA (9). These features make it ideal for dissecting the mechanism by which costimulatory pathways regulate inflammation *in vivo*. Although we recently reported that administration of CTLA4Ig to people with mild atopic asthma did not inhibit the inflammatory response to allergen, this does not detract from the utility of the murine model to perform mechanistic studies designed to determine how the CTLA4Ig exerts its anti-inflammatory function (36).

Although independent of CD28, this alternative mechanism remains dependent on expression of CD80/CD86. Others have shown that binding of either CD28 or CTLA4 to CD80/CD86 could induce secretion of IFN- γ (37). However, we did not detect any increase in either IFN- γ or NO concentrations in culture supernatants of CTLA4Ig-treated cells, and abundant IFN- γ was present in the



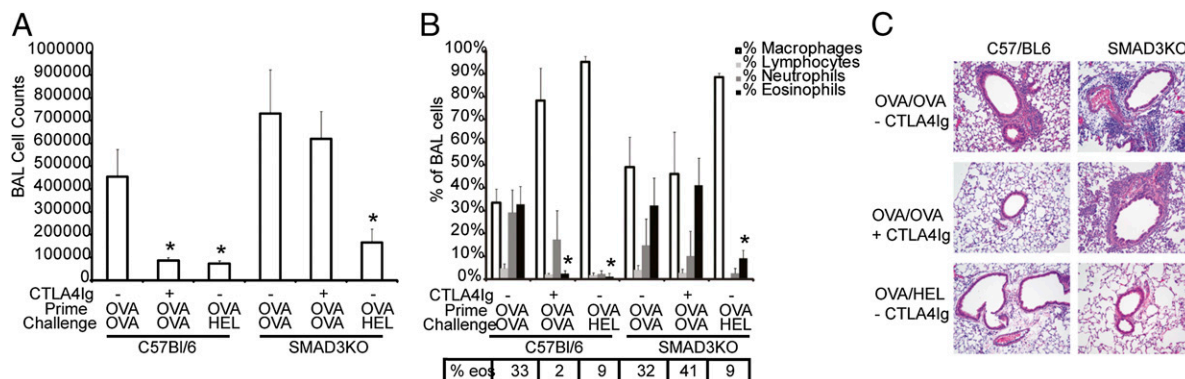


FIGURE 5. CTLA4Ig is ineffective in SMAD3-deficient mice. Wild-type or SMAD3-deficient mice were sensitized with OVA and given an inhaled challenge with either OVA or HEL. CTLA4Ig was administered to groups of mice as indicated and samples were collected 72 h after challenge. Each experimental group consisted of five mice and experiments were repeated three times. Shown are data from one representative experiment. BAL (A) cell counts, (B) differentials, and (C) H&E stained lung sections (original magnification $\times 20$) shown. * $p < 0.05$ as compared with OVA primed and challenged without CTLA4Ig as determined by Kruskal–Wallis with Dunn posttest comparisons.

cocultures irrespective of CTLA4Ig (data not shown). Nonetheless, blockade of IFN- γ signaling, either by inclusion of a neutralizing Ab or by using STAT-1 mice, prevented NO production (data not shown) and completely abrogated the effect of CTLA4Ig (Supplemental Figs. 1, 2). Although CTLA4Ig was ineffective in the absence of IFN- γ or NOS2, depletion of Tregs or interference with TGF- β did not affect NO-dependent inhibition by macrophages. These data demonstrate that NO and CTLA4Ig act synergistically yet indicate that they act through different mechanisms.

In addition to CD28 and CTLA4, CD80 also binds to PD-L1 (8). This was revealed by examination of cells deficient in both CD28 and CTLA4 and was shown to inhibit T cell activation and proliferation. Whereas this interaction is also blocked by CTLA4Ig, if interference with CD80/PD-L1 interactions were the primary mechanism operative in our system, we would expect CTLA4Ig to increase T cell proliferation by preventing the inhibitory signal.

Binding of murine CTLA4Ig to CD80 or CD86 on mouse dendritic cells can induce IDO activity, which can suppress T cell proliferation by several mechanism, including promoting Treg development and function (38, 39). IDO has also been shown to be an important regulator of allergic inflammation in the lung (40, 41). More recently, IDO has been shown to regulate TGF- β production in plasmacytoid dendritic cells by a nonenzymatic mechanism that is not inhibited by 1-methyltryptophan (42). Although we had previously shown that treatment of mice with 1-methyltryptophan had no effect, we tested IDO-deficient mice to determine whether this alternative mechanism might be operative in our system (9). However, CTLA4Ig was as effective in inhibiting allergic airway inflammation in IDO-deficient mice as in wild-type mice, demonstrating that IDO is not involved in the suppressive effects of CTLA4Ig (Supplemental Fig. 3).

We found that Tregs were absolutely required for CTLA4Ig to inhibit T cell proliferation in vitro as well as in our in vivo model of allergic airway inflammation. Although we did not detect an increase in Treg cell numbers when wild-type mice were treated with CTLA4Ig, adoptive transfer studies revealed an increase in the percentage of Ag-specific Tregs, suggesting that CTLA4Ig may facilitate the development of inducible Tregs. However, this does not exclude that CTLA4Ig might also augment their functional ability or enhance the sensitivity of the targets of Treg suppression. Tregs use a number of mechanisms to inhibit effector T cells, including through TGF- β (17, 43). In fact, neutralization of TGF- β or inhibition of TGF- β receptor signaling abrogated the effect of CTLA4Ig. Although we did not detect an increase in TGF- β con-

centration in the culture supernatants, this may be misleading, as much of the regulation of TGF- β is posttranslational (44).

Several studies have examined the interactions of TGF- β , NO, and Tregs, with conflicting results. TGF- β has been shown to downregulate macrophage production of NO, and this involves both SMAD2 and SMAD3 proteins (45–47). Others have reported that NO interacts with TGF- β or IFN- γ to either inhibit or promote Treg differentiation (48, 49). Each of these studies used widely varying systems, likely accounting for the differences in results and conclusions. Nonetheless, it appears that NO can influence Treg development and/or function.

The failure of CTLA4Ig to block inflammation in SMAD3-deficient mice is further support for the importance of a TGF- β -dependent pathway. TGF- β signals through both SMAD-dependent and independent pathways, and these may be differentially involved in T cell suppression by TGF- β and in Treg development and function (23, 26, 50). Nonregulatory CD4 T cells deficient in both SMAD2 and SMAD3 display a phenotype of spontaneous activation and are not suppressed by TGF- β . However, thymic Tregs specifically deleted for SMAD2 and SMAD 3 were essentially normal in both number and function, although peripheral conversion of Foxp3⁻ CD4 cells to Foxp3⁺ cells required SMAD3 (50–52). Thus, our data are consistent with either a lack of TGF- β signaling in the effector T cell, or a defect in inducible Treg development or function.

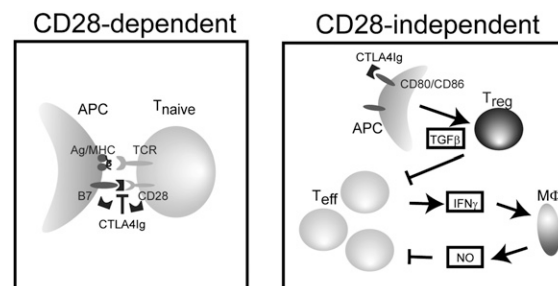


FIGURE 6. Proposed model for CTLA4Ig. Binding of CTLA4Ig to CD80/CD86 expressed on an APC can prevent naive T cell activation by preventing CD28-mediated co-stimulation (left panel). Additionally, CTLA4Ig can suppress the proliferation of activated effector T cells (T_{eff}) through a TGF- β and Treg-dependent mechanism that is independent of CD28 co-stimulation (right panel). This pathway requires and is synergistic with macrophage-derived NO.

Both CD28 and CTLA4 have been implicated in Treg development and function, with particular attention focused on the role of CTLA4 (17, 18, 53–56). Tregs express high levels of CTLA4 constitutively, as opposed to effector T cells in which it is expressed only after activation. CTLA4-deficient mice die of diffuse lymphoproliferation and autoimmunity, in large part due to defective Treg function (53, 54, 57). Recent data support that CTLA4 regulates Tregs and conventional T cells by both direct signaling (cell-intrinsic mechanism) and indirectly by acting on other cells through its binding to CD80 and CD86 on the APC (cell-extrinsic mechanism). Tai et al. (58) showed that the intracellular domain of CTLA4 was not necessary for CTLA4 to regulate the intrinsic proliferative capacity of Tregs or their ability to suppress the proliferation of naive effector T cells. Others have suggested that CTLA4 binding induces a loss of CD80 or CD86 expression on the APC as a mechanism of suppression (54, 59). Although our data were generated using CTLA4Ig and not CTLA4 expressed by T cells, it raises the possibility that binding of CTLA4Ig to CD80 and CD86 is at least partially mimicking the cell-extrinsic mechanisms of endogenously expressed CTLA4. Whereas these mechanisms are distinct from our observations in that they ultimately rely upon impaired CD28-mediated costimulation, they are consistent in that they are mediated by a signal delivered via the extracellular domain of CTLA4 to B7 proteins rather than vice versa.

In conclusion, our study has demonstrated that CTLA4Ig can suppress T cell proliferation in vitro and allergen-induced airway inflammation in vivo via a mechanism distinct from either IDO or impaired CD28-mediated costimulation. As illustrated in Fig. 6, CTLA4Ig can inhibit T cell responses by both CD28-dependent and -independent mechanisms. CTLA4Ig interferes with the activation of naive T cells by preventing B7 engagement with CD28 (Fig. 6, *left panel*). This pathway is likely most important when the drug is administered at the time of initial sensitization. Additionally, CTLA4Ig binding to B7 proteins can also induce a TGF- β - and Treg-dependent inhibition of T cell proliferation and inflammation (Fig. 6, *right panel*). This mechanism of action has not previously been described and may be the predominant mechanism when CTLA4Ig is administered clinically, which is typically at a time well after sensitization has occurred and when inflammation has already been established. Thus, this may very well be the predominant mechanism of action for the drug in the treatment of chronic inflammatory diseases such as rheumatoid arthritis. Additionally, the effects we have observed with CTLA4Ig share some similarities with the effects of endogenous CTLA4 on Tregs. Thus, in addition to illuminating how this important therapeutic agent may work in vivo, it may provide insight into how CTLA4 regulates effector T cell function.

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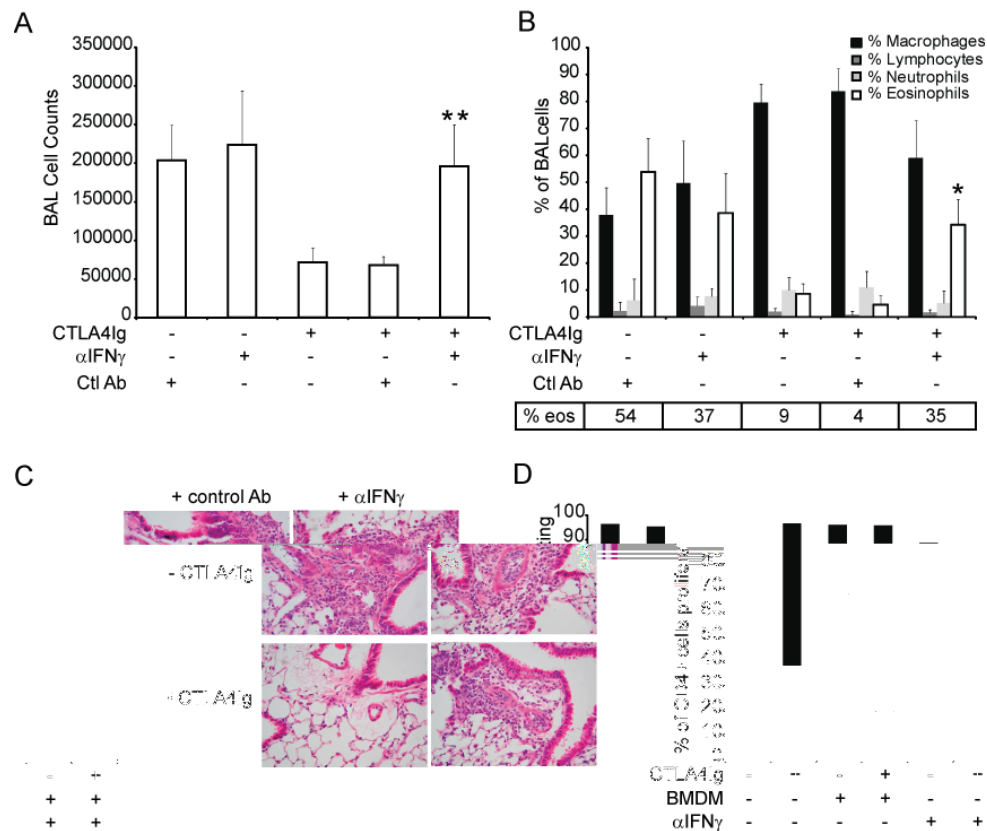
Disclosures

The authors have no financial conflicts of interest.

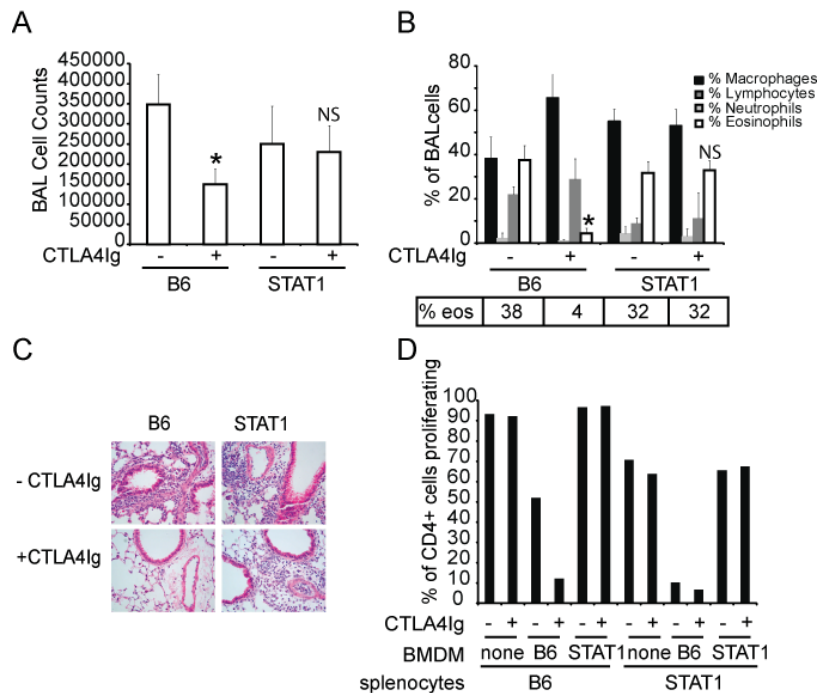
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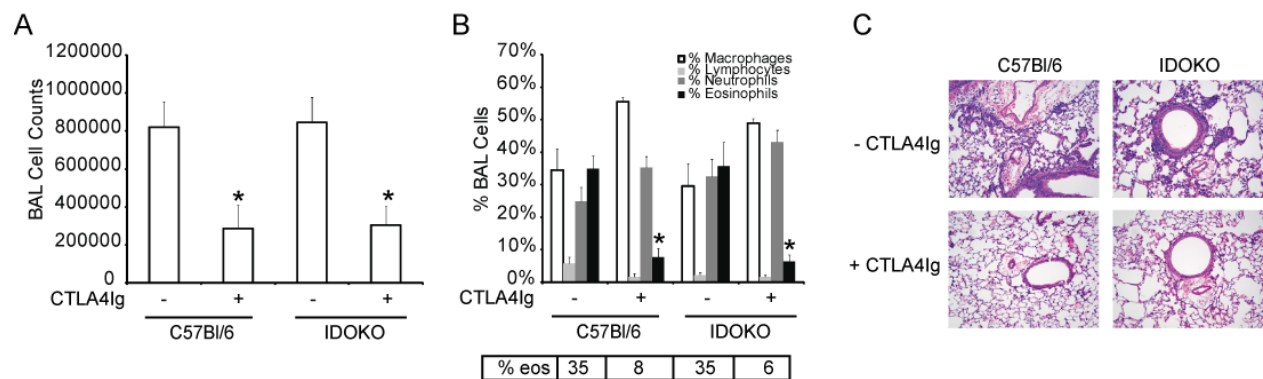
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Supplemental Figure 1: IFN γ is required for inhibition of allergic airway inflammation and inhibition of T cell proliferation by CTLA4Ig. A-C) C57Bl/6J mice were sensitized and challenged with OVA as described. CTLA4Ig was administered at the time of challenge as indicated. Neutralizing antibody against IFN γ or a control antibody was administered 24 hours prior to challenge. Samples were collected for analysis 72 hours later. Data presented is representative of 2 independent experiments with 5 mice per group. **A)** Total BAL cells, **B)** BAL cell differentials with % eosinophils for each group shown below the x-axis labels and **C)** representative lung sections stained with H&E. **D)** CFSE labeled splenocytes were activated with PMA and ionomycin and co-cultured with BMDM alone or in the presence of neutralizing IFN γ antibody and/or in the presence of CTLA4Ig. Data shown for BMDM experiments is representative of 3 independent experiments. * = $p < 0.05$. ** = $p < 0.01$ as compared to control antibody. For *in vivo* experiments, all groups consisted of 5 mice.



Supplemental Figure 2: STAT1 is required for CTLA4Ig to inhibit allergic airway inflammation and T cell proliferation. **A-C)** Wild type or STAT1 deficient mice were sensitized and challenged with OVA and treated with CTLA4Ig as indicated. Samples were collected for analysis 72 hours after inhaled challenge. Each experimental group consisted of 5 mice and has been repeated 3 times. Shown is data from one representative experiment. * = $p < 0.005$ as compared to no CTLA4Ig, **D)** CFSE labeled splenocytes from C57Bl/6J or STAT1-deficient mice were activated with PMA and ionomycin and cultured either alone or co-cultured with BMDM derived from either C57Bl/6J or STAT1-deficient mice. CTLA4Ig was added as indicated. Proliferation was determined by flow cytometry after 96 hours of culture. Shown is representative data from one of 3 independent experiments.



Supplemental Figure 3: IDO is not required for CTLA4Ig to inhibit allergic airway

inflammation. A-C) Wild type or IDO-deficient mice were sensitized and challenged with OVA and treated with CTLA4Ig as indicated. Samples were collected for analysis 72 hours after inhaled challenge. Each experimental group consisted of 5 mice and has been repeated 3 times. Shown is data from one representative experiment. * = $p < 0.005$ as compared to no CTLA4Ig

