

Interruption of CD28-mediated costimulation during allergen challenge protects mice from allergic airway disease

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Background: Allergic asthma is a T_H2-promoted hyperreactivity with an immediate, IgE, and mast cell-dependent response followed by eosinophil-dominated inflammation and airway obstruction.

Objective: Because costimulation by CD28 is essential for T_H2 but not T_H1 responses, we investigated the effect of selective interference with this pathway in mice using the models of ovalbumin and house dust mite-induced airway inflammation.

Methods: To study the role of CD28 in the effector phase of allergic airway inflammation, we developed an inducibly CD28-deleting mouse strain or alternatively used a CD28 ligand-binding site-specific mouse anti-mouse mAb blocking CD28 engagement.

Results: We show that even after systemic sensitization to the allergen, interruption of CD28-mediated costimulation is highly

effective in preventing airway inflammation during challenge. In addition to improving airway resistance and histopathologic presentation and reducing inflammatory infiltrates, antibody treatment during allergen challenge resulted in a marked relative increase in regulatory T-cell numbers among the CD4 T-cell subset of the challenged lung.

Conclusion: Selective interference with CD28-mediated costimulation during allergen exposure might be an attractive therapeutic concept for allergic asthma. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)

Key words: Allergic asthma, costimulation, conditional CD28 knockout mice, ovalbumin, CD28-specific mAb

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Allergic asthma is characterized by airway obstruction and airway hyperresponsiveness (AHR). Activated CD4⁺ T_H2 cells and their cytokine products play a central role in the development of allergic immune responses in the lung.¹ CD4⁺ T cells infiltrating the airways of patients with atopic asthma predominantly produce cytokines of the T_H2 type, including IL-4, IL-5, and IL-13.² These cytokines promote differentiation, migration, and activation of other effector cells, such as eosinophils, B cells, and mast cells, thus triggering the progression of the early and late asthmatic reaction.³

In mice costimulatory signals delivered to T cells through CD28 are of key importance for T_H2 cell differentiation^{4,5} and hence for allergy development. Thus CD28-deficient mice often mount normal cell-mediated immunity^{6,7} but show impaired T_H2 polarization and antibody responses.^{6,8,9} This is reflected in decreased susceptibility of CD28-deficient mice for the induction of AHR after allergen sensitization and challenge.¹⁰ Accordingly, CD28 and its ligands on antigen-presenting cells, CD80 and CD86, represent interesting targets for therapeutic interference with the development of type I allergies. However, the marked CD28 dependence of regulatory T (Treg) cells,¹¹ which are known to counteract allergic airway inflammation,^{12,13} needs to be taken into account when considering such strategies.

In mouse models of allergic airway inflammation, blockade of CD80/CD86 with a soluble Fc fusion protein of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4; CD152), the negative regulator of T-cell activation, which shares its ligands with CD28, is indeed therapeutically effective.¹⁴⁻¹⁶ Moreover, allergen-induced secretion of IL-5 and IL-13 in PBMC cultures from patients with atopic asthma is inhibited by a fusion protein composed of an immunoglobulin fused to the extracellular domain of CTLA-4 (CTLA4Ig).¹⁷ Indeed, a clinical study with this fusion protein (abatacept) has been initiated in patients with allergic asthma (ClinicalTrials.gov identifier NCT00784459).

Abbreviations used

AHR:	Airway hyperresponsiveness
BAL:	Bronchoalveolar lavage
CTLA-4:	Cytotoxic T lymphocyte-associated antigen 4
CTLA4Ig:	Immunoglobulin fused to the extracellular domain of CTLA-4
HDM:	House dust mite
MLN:	Mediastinal lymph node
OVA:	Ovalbumin
Treg:	Regulatory T

Although CTLA4Ig is therapeutically successful, its efficacy cannot be clearly attributed to the blockade of costimulation, as opposed to inhibitory signaling in dendritic cells through CD80/CD86 ligation. This mechanism was recently shown to be operative in allergic airway disease of mice.¹⁸ In addition, blockade of CD80/CD86 by CTLA4Ig also prevents ligation of endogenous CTLA-4 expressed by regulatory and activated T cells, which might partially counterbalance the desired effect. The same considerations hold true for mAb-mediated blockade of CD80/CD86, which also showed therapeutic efficacy in mice.^{14,19}

The most direct approach addressing the role of CD28 to allergic airway inflammation is the use of CD28-deficient mice, which have been extensively studied in allergic models.^{14,16,20} However, the requirements for CD28 during the effector as opposed to the priming phase, which is the therapeutically more relevant option, cannot be studied in this setting.

Therefore in the present study we have used inducible gene deletion in allergen-sensitized conditional CD28 knockout mice to study the importance of CD28-mediated costimulation for the secondary allergic T-cell response during challenge.

A therapeutically more relevant approach to manipulating the CD28 costimulatory pathway, which leaves the interaction of CTLA-4 with its ligands intact, is the use of CD28-specific mAb. To mimic potential therapeutic approaches in human subjects, which, for prolonged treatment, rely on humanized mAbs, we have developed several mouse anti-mouse CD28 mAbs.²¹ One of these, called E18, is the first such mAb that fully blocks interaction with CD80.²² *In vivo* E18 effectively prevents superantigen-driven expansion of effector but not of Treg cells and protects mice from graft-versus-host disease mediated by alloreactive T cells.²² In the present study we therefore investigated the effects of the E18 mAb in 2 mouse models of allergic airway inflammation.

We report that in allergen-sensitized mice both conditional deletion of CD28 and treatment with E18 effectively interfere with the induction of airway inflammation during subsequent challenge. Importantly, anti-CD28 treatment preferentially reduced lung infiltration by CD4 effector cells over Treg cells, resulting in an improved Treg cell/effector T-cell balance that might contribute to the therapeutic effect.

METHODS**Animals**

Female BALB/c mice (6–8 weeks old) from Harlan Winkelmann (Borchen, Germany) were used for experiments with ovalbumin (OVA). For experiments with house dust mite (HDM), mice were bred and maintained in specific pathogen-free facilities at the University of Cape Town. The generation of inducibly CD28-deleting mice on the C57BL/6 background is described in Fig E1 in this article's Online Repository at www.jacionline.org. Mice were kept

in a specific pathogen-free environment. Experiments were performed with government permits.

Induction of OVA-specific allergic airway inflammation

Mice were immunized by means of intraperitoneal injection of 10 µg of OVA (grade V; Sigma, Deisenhofen, Germany) absorbed to 200 µL of 2% aluminum hydroxide (Serva, Heidelberg, Germany) on days 0 and 14. On days 28 to 29, anaesthetized mice (ketamine [Pfizer, New York, NY] and xylazine [Bayer, Leverkusen, Germany]) were challenged intranasally with 100 µg of OVA in 50 µL of PBS.²³ Control mice received PBS instead of OVA (PBS/Alum).

HDM-specific allergic airway disease

Anaesthetized BALB/c mice were challenged intranasally with 100 µg of HDM at day 0 and 10 µg of HDM (Greer Laboratories, Lenoir, NC) at days 7 to 11, as previously described.²⁴ Antibody treatment and analyses were performed according to setting D, as shown in Table I.

mAb treatment

Anti-CD28 mAb E18 (IgG_{2b}; Serotec, Oxford, United Kingdom) was administered intraperitoneally, as detailed in Table I.

Inducible gene deletion in conditional knockout mice

CD28^{-flox} Cre^{+/-} (*rosa*[SACreER^{T2}])²⁵ mice were treated for 4 consecutive days with 2.5 mg of tamoxifen (Sigma, Deisenhofen, Germany) in vegetable oil administered by means of forced feeding.

Bronchoalveolar lavage and differential cell count

After lethal anesthesia, the trachea was cannulated and bronchoalveolar lavage (BAL) was performed by flushing the lungs and airways 3 to 4 times with 1 mL of cold PBS. BAL fluid cells were counted, spun onto glass slides, and stained with Diff-Quick (Dady, Marburg, Germany). Differential cell counts were determined microscopically, according to standard cytological criteria.

Serum antibody titers

OVA-specific IgE levels were assessed by using ELISA, as described previously.²⁶ Titers were calculated from a dilution that was 1.5-fold over the baseline (OD obtained with nonimmunized mice).

***In vitro* stimulation of lymphocytes**

Single-cell suspensions (1×10^6 cells/mL) from spleens and draining lymph nodes (mediastinal lymph nodes [MLNs]) were cultured in enriched RPMI 1640 supplemented with 10% FCS at 37°C in a humidified atmosphere containing 5% CO₂. Stimulants were either OVA (10 µg/mL) or plate-bound anti-CD3 antibody (145-2C11, 5 µg/mL; BD/PharMingen, San Diego, Calif) together with recombinant human IL-2 (200 U/mL) (Novartis, Basel, Switzerland). After 48 hours, cell-culture supernatants were harvested.

Cytokine ELISA

Sandwich ELISAs (OptEIA-SET; BD PharMingen, San Diego, Calif) were used for detection of IL-4, IL-5, IFN-γ, and IL-10 in BAL fluid and cell-culture supernatants.

Lung histology

Lung tissues were fixed in 4% phosphate-buffered formalin and embedded in paraffin wax. Sections (2–3 µm) were cut and stained with hematoxylin and eosin.

TABLE I. Sensitization and therapy scheme

	Sensitization OVA/Alum	Challenge OVA	Treatment Anti-CD28 (E18)	Analysis
Setting A				
Prophylactic	Days 0 + 14	Days 28-29	Days 0, 7, 14, 28, and 29	Day 34
Setting B				
Therapeutic	Days 0 + 14	Days 28-29	Days 20, 22, 24, 28, and 29	Day 34
Setting C				
Therapeutic	Days 0 + 14	Days 28-29	Day 26	Day 37
	HDM	HDM	Anti-CD28 (E18)	
Setting D	Day 0	Days 7-11	Day 6	Day 13

In settings A through C groups of BALB/c mice were sensitized with intraperitoneal injections of 10 μ g of OVA adsorbed to 200 μ L of Al(OH)₃ and intranasally challenged with 100 μ g of OVA. In setting D BALB/c mice were intranasally sensitized with 100 μ g of HDM and challenged with 10 μ g of HDM. The treatment was performed with 100 μ g of anti-CD28 for each indicated time point.

Measurement of AHR

Measurements of airway resistance were performed on anesthetized, intubated, and mechanically ventilated mice (FlexiVent; Scireq, Montreal, Quebec, Canada) in response to increased doses of inhaled methacholine (3.125, 6.25, 12.5, 425, 50, and 100 mg/mL). Airway resistance measurements were performed every 15 seconds after each nebulization step until a plateau phase was reached.²⁷

Flow cytometry

Single-cell suspensions were incubated with anti-CD16/CD32 mAb (2.4G2; Fc block, BD PharMingen), followed by staining of extracellular CD4 (clone L3T4 or RM4-5, BD PharMingen) and CD28 (clone E18, Serotec). Forkhead box protein 3 analysis was performed according to the manufacturer's instructions (eBioscience, San Diego, Calif). For intracellular cytokine staining, whole lungs were digested for 1 hour at 37°C in Iscove modified Dulbecco medium (Invitrogen, Carlsbad, Calif) containing 13 mg/mL DNase I (Roche, Mannheim, Germany) and 50 U/mL collagenase IV (Sigma-Aldrich, St Louis, Mo). Lung cells were stimulated for 4 hours at 37°C with phorbol 12-myristate 13-acetate (50 ng/mL), ionomycin (250 ng/mL), and monensin (200 mmol/L). Intracellular cytokines were stained with anti-IL-4, anti-IL-5, anti-IFN- γ (BD Bioscience), anti-IL-13 (eBioscience), and rat anti-mouse IgG₁ isotype control (BD Biosciences) antibodies. Additionally, BAL fluid cells were stained with CCR3 PE (R&D Systems, Minneapolis, Minn) and GR-1 fluorescein isothiocyanate (clone RB68C5) to identify neutrophils (GR-1^{high}CCR3⁻) and eosinophils (CCR3⁺GR-1^{int}). Data were acquired on a BD LSR II and analyzed with FlowJo software (TreeStar, Ashland, Ore).

Statistical analysis

Data were calculated as means $\pm$ SEMs. Statistical significance for normally distributed samples was analyzed by using an unpaired *t* test. Non-normal data or data with unequal variances were tested for significance by using the Mann-Whitney rank sum test. *P* values of less than .05 were considered statistically significant.

RESULTS

Effects of induced CD28 deletion on the effector phase of allergic airway disease

To investigate a requirement of CD28 for the effector phase of allergic asthma, we developed C57BL/6 mice with a tamoxifen-inducible interruption of the CD28 gene (see Fig E1, A and B). Consistent and complete deletion was achieved

by heterozygous expression on a CD28-deficient background (CD28^{-flox}, *rosa*(*CreER*^{T2}) or CD28^{-flox} *Cre*^{+/-} mice for short; see Fig E1, C).

To induce an antigen-specific T_H2 response, CD28^{-flox} *Cre*^{+/-} mice were systemically sensitized to OVA/Alum, followed by intranasal exposure to OVA (Fig 1, A). CD28 deletion was performed after the second OVA/Alum application. Analysis of BAL fluid revealed a prominent (5-fold) reduction in cellular infiltration after CD28 gene deletion, which was most obvious for eosinophils and lymphocytes (Fig 1, B). Improved airway pathology was also observed by means of histologic examination of lung tissue sections (Fig 1, C). An improvement in asthmatic symptoms was directly shown by reduced AHR to inhaled methacholine (Fig 1, D).

In contrast to these clear-cut effects on cellular infiltration and airway resistance of the lung but in keeping with the relatively low T_H2 response of the C57BL/6 strain, cytokine levels in BAL fluid were low, even without CD28 deletion (see Fig E2, A, in this article's Online Repository at www.jacionline.org).

Deletion of CD28 between systemic sensitization and allergen challenge led only to a slight reduction in OVA-specific IgE levels (data not shown), indicating that the contribution of CD28-mediated costimulation to airway pathology in response to intranasal allergen challenge was independent of allergen-specific IgE.

Induction of airway inflammation and treatment with CD28-specific mAb during priming and effector phases

To test whether the decisive contribution of CD28-mediated costimulation revealed by induced gene deletion could be translated into therapeutic interference with CD28-ligand interaction, we used a mouse anti-mouse CD28 antibody (E18) that blocks the CD28 binding site²² in the BALB/c mouse model of allergic airway inflammation. Treatment protocols are shown in Table I.

As shown in Fig 2, A, the strong peribronchial eosinophil-rich inflammatory infiltrate observed in sensitized and challenged mice was largely absent in the prophylactically treated group. This is in keeping with the demonstration that constitutively CD28-deficient mice have only a minimal recruitment of inflammatory cells to the lungs²⁸ and illustrates that interference with the

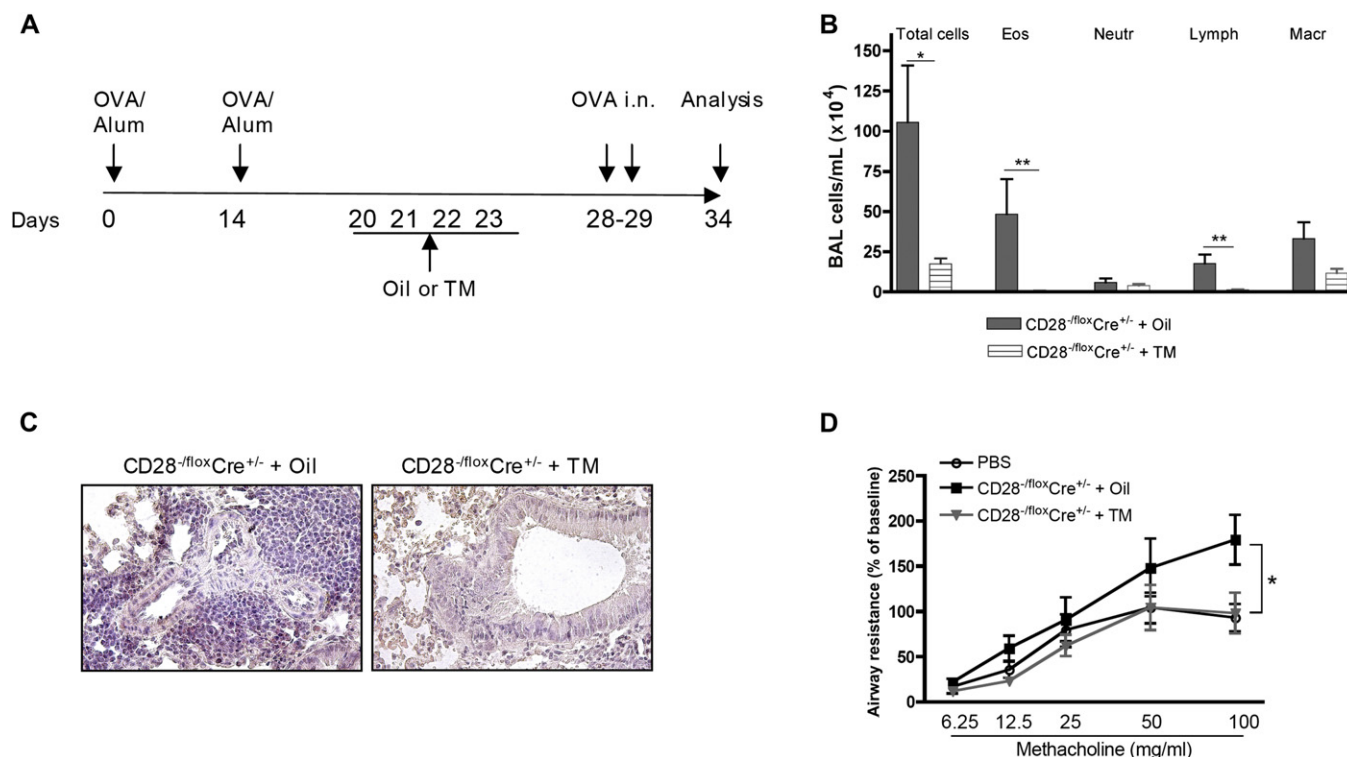


FIG 1. Induced CD28 deletion before allergen challenge prevents the development of airway pathology. **A**, Induction of OVA-specific airway inflammation and CD28 deletion. **B**, CD28 deletion before challenge inhibits the recruitment of eosinophils to the airways. **C**, Hematoxylin and eosin-stained lung tissues (magnification $\times 200$). **D**, Invasive measurement of airway resistance. Data are shown as means $\pm$ SEMs ($n = 5$) from one of 3 experiments. *Eos*, Eosinophils; *i.n.*, intranasal; *Lymph*, lymphocytes; *Macr*, macrophages; *Neutr*, neutrophils; *TM*, tamoxifen. * $P < .05$ and ** $P < .01$.

CD28 pathway in an otherwise healthy animal suffices to prevent lung pathology. Supporting our observations in CD28 gene-deleted mice, anti-CD28 therapy at the challenge phase also remarkably improved lung histopathology (Fig 2, A) and AHR (Fig 2, B), supporting our conclusions on the importance of CD28 costimulation during the recall phase and demonstrating the feasibility of CD28 blockade as a therapeutic principle in this setting.

To closely investigate the preventive effect of anti-CD28 therapy, we examined the cellular composition of BAL fluid. In line with the improved lung histopathology, there was a marked reduction in the total number of recruited cells to the airways in mice that received anti-CD28 in both prophylactic and therapeutic settings (Fig 3, A and B). In addition, analysis of the cellular composition of BAL specimens revealed the virtual absence of the major types of inflammatory cells, such as eosinophils, neutrophils, and macrophages, in treated mice.

Modulation of the cytokine profile in BAL fluid by anti-CD28 treatment

Direct examination of cytokine levels in BAL fluid revealed that costimulation blockade during sensitization significantly reduced IL-4 levels in BAL fluid, whereas therapeutic treatment with anti-CD28 was less effective (Fig 3, C and D). More importantly, however, levels of IL-5, which promotes eosinophil differentiation, were significantly reduced in both settings, which is in line with the effects of anti-CD28 treatment on peribronchial infiltration of the lungs and inflammatory cells in the BAL fluid

(Fig 3). In contrast to IL-5, no obvious changes were found for IL-10 and IFN- γ levels in the BAL fluid after administration of anti-CD28 (Fig 3, C and D). Taken together, our results demonstrate that interference with CD28-mediated costimulation during sensitization suppresses the generation of a local T_H2 -type environment in the lung. Therapeutic interference is less effective with regard to IL-4 production but still able to reduce levels of the eosinophil-promoting cytokine IL-5 to a significant and apparently therapeutically meaningful degree.

Improved BAL Treg cell/effector T-cell ratio after CD28 blockade

The marked reduction in infiltrating CD4 T-lymphocyte numbers in therapeutically treated mice was more pronounced for forkhead box protein 3-negative (ie, effector) cells than for Treg cells, resulting in an increased frequency of Treg cells in the BAL CD4 T-cell compartment (Fig 4), which might contribute to the protective effect of anti-CD28 treatment. This potentially beneficial shift in the balance between CD4 effector and Treg cells is restricted to the site of inflammation.

Therapeutic CD28 blockade acts independently of allergen-specific IgE levels

As expected from the known importance of CD28 signals for the induction of T_H2 responses,²⁹⁻³² costimulation blockade in the prophylactic setting was associated with a profound reduction in OVA-specific serum IgE levels (see Fig E3, A, in this article's

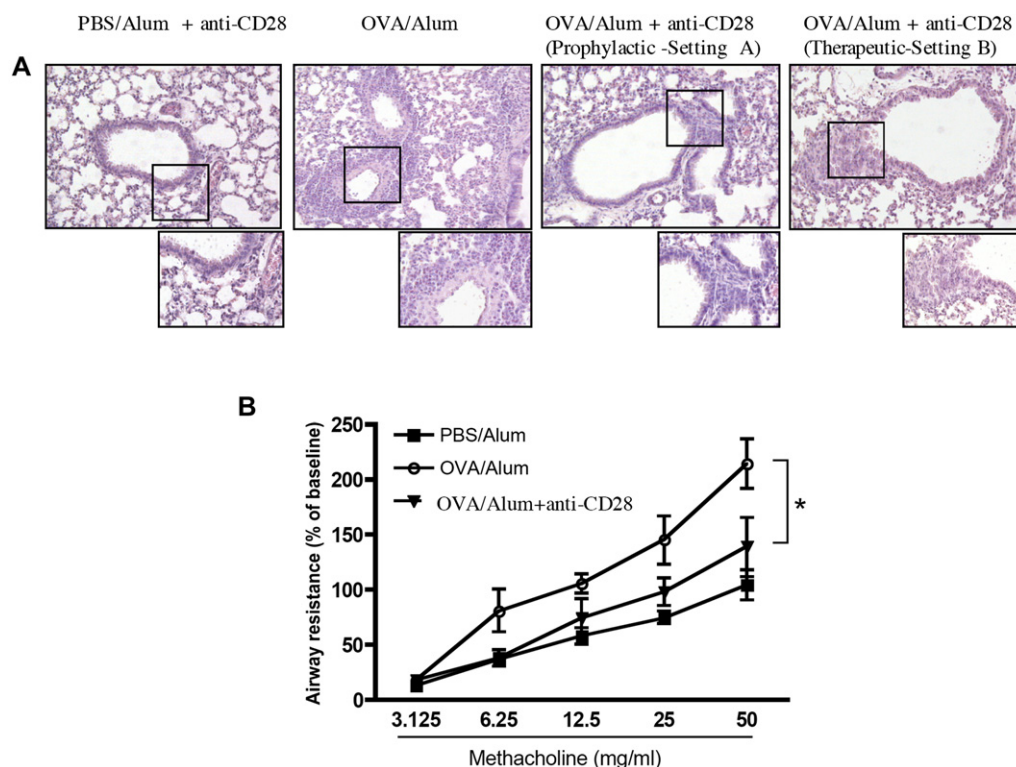


FIG 2. Treatment with anti-CD28 impairs lung histopathology and improves airway resistance. Representative hematoxylin and eosin staining of lung tissues. Data are shown from one of 3 independent experiments. **A**, Magnification $\times 200$ and $\times 400$. **B**, Invasive measurement of airway resistance for the anti-CD28 therapeutically treated group (setting B) performed with increasing doses of methacholine. $*P < .05$.

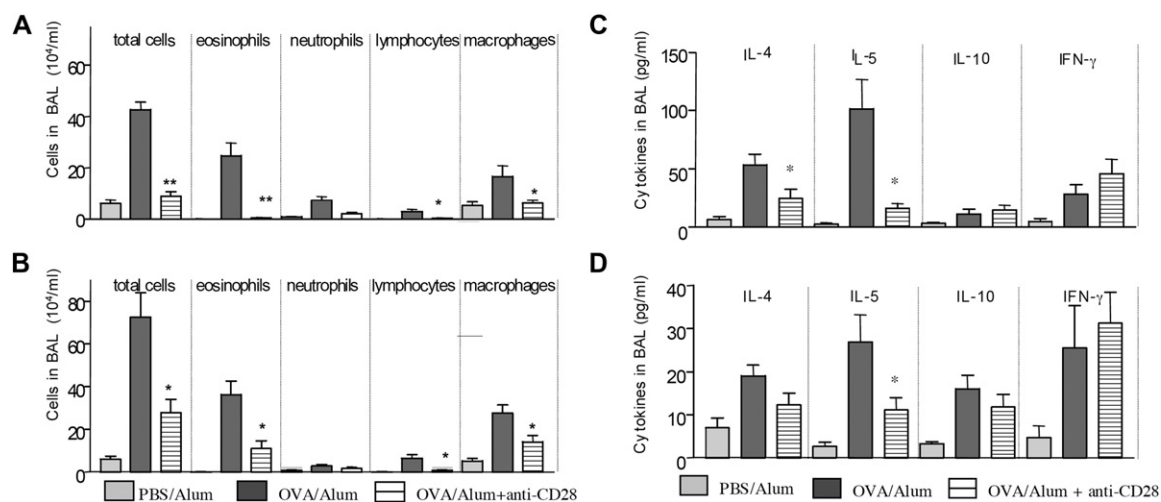


FIG 3. CD28 blockade reduces inflammatory cell infiltration and cytokine production in the airways. Cellular composition of BAL fluid: **A**, prophylactic, setting A; **B**, therapeutic, setting B. Cytokine levels in BAL fluids: **C**, prophylactic, setting A; **D**, therapeutic, setting B. Data are presented as means $\pm$ SEMs from one of 3 independent experiments ($n = 5$). $*P < .05$ and $**P < .01$.

Online Repository at www.jacionline.org). Furthermore, consistent with the effects of induced *CD28* deletion, *CD28* blockade during challenge resulted only in a tendency toward reduced OVA-specific IgE levels (see Fig E3, B). These results indicate that the observed therapeutic effect of *CD28* blockade is not explained by reduced allergen-specific IgE production.

Anti-CD28 therapy modulates the allergen-specific cytokine response in draining lymph nodes and the spleen

To test the effect of therapeutic anti-CD28 treatment on the responsiveness of the peripheral immune system in established allergy, we analyzed cytokine production by cells from the

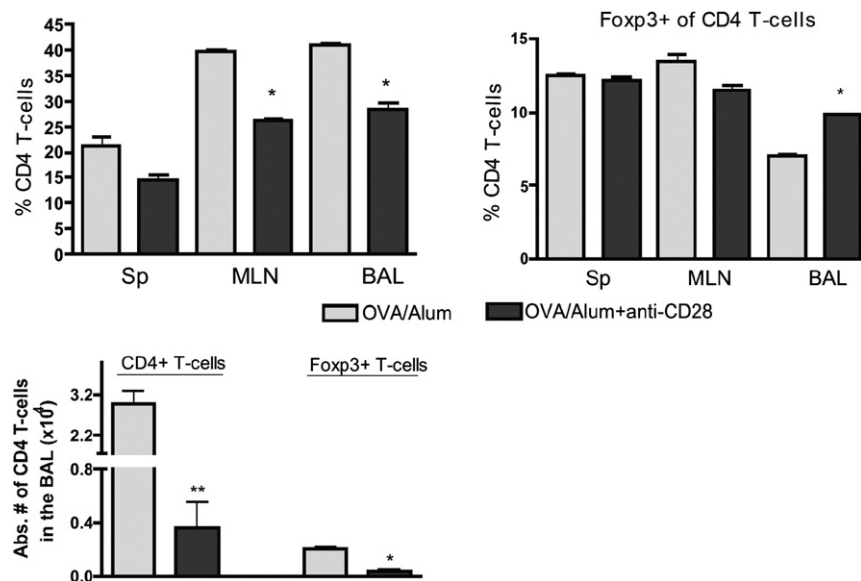


FIG 4. Anti-CD28 therapy improves the balance of infiltrating Treg cells/effector T cells at the site of inflammation. Cells from spleens (Sp), MLNs, and BAL fluid were analyzed by using flow cytometry for expression of CD4 and forkhead box protein 3 (Foxp3). Means $\pm$ SEMs are shown (n = 5). *P < .05 and **P < .01.

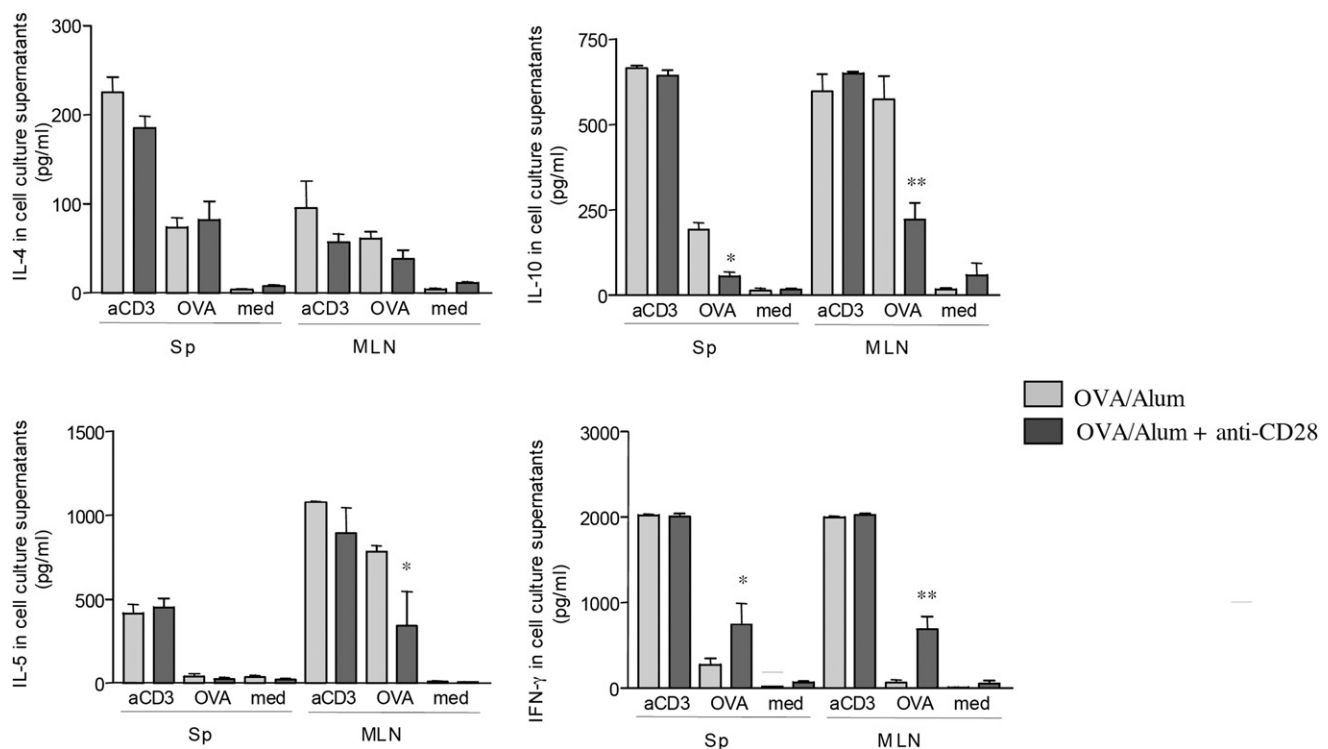


FIG 5. Anti-CD28 therapy (setting 2) changes the profile of the allergen-specific cytokine response. Single-cell suspensions from spleens (Sp) and MLNs of therapeutically treated mice were cultured for 48 hours in the presence of anti-CD3 or with OVA. Means $\pm$ SEMs of cell-culture triplicates are shown from one of 3 independent experiments. *P < .05; **P < .01; and ***P < .001.

spleen and MLNs. Cells were stimulated *in vitro* either polyclonally with anti-CD3 or specifically with OVA, and the culture supernatants were tested for secreted cytokines (Fig 5). Anti-CD28 treatment did not alter cytokine production induced by anti-CD3 stimulation in the spleen and MLNs, indicating that the therapy had not resulted in a generalized functional skewing

of the T-cell compartment. In contrast, anti-CD28 treatment clearly affected the antigen-specific cytokine response. Particularly in the MLNs, production of IL-5 and IL-10 was reduced (a 2- to 3-fold reduction) and IFN- γ production was increased (a 3- to 5-fold increase) as a result of *in vivo* treatment with anti-CD28. Although this result reflects the *ex vivo* measurement

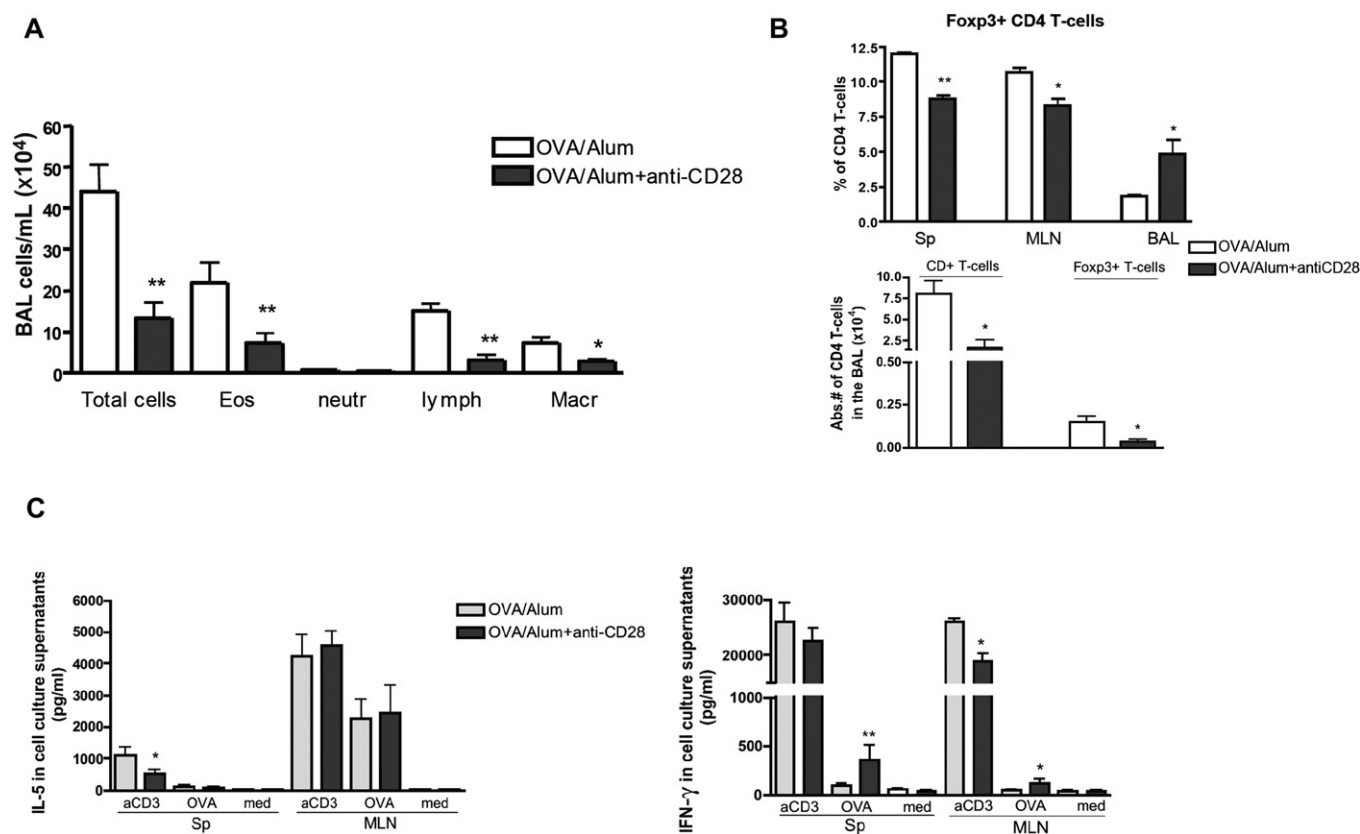


FIG 6. Transient blockade (therapeutic, setting C) of CD28 reduces inflammatory cell infiltration and increases the frequency of forkhead box protein 3 (Foxp3)-positive Treg cells in the airways. **A**, Cells in BAL fluid. **B**, Frequency and absolute numbers of Foxp3⁺ CD4 T cells. **C**, Cytokines in *in vitro*-stimulated cell cultures. Values represent means $\pm$ SEMs ($n = 5$). *Eos*, Eosinophils; *lymph*, lymphocytes; *Macr*, macrophages; *neutr*, neutrophils; *Sp*, spleen. * $P < .05$ and ** $P < .01$.

of IL-5 seen in Fig 3, the shift in IL-10 and IFN- γ production was not apparent in BAL fluid. Regardless of whether this discrepancy is due to the site examined or a need for acute antigenic restimulation to become apparent, it might contribute to the observed reduction in airway pathology. IL-4 itself, which is important for the isotype switch to IgE, was readily detected in both polyclonally and OVA-stimulated spleen and lymph node cultures but remained unaffected by anti-CD28 therapy (Fig 5).

Protective and immunoregulatory effects after transient therapeutic CD28 blockade

In the above experiments a total of 500 μ g of the E18 mAb was applied to achieve full and long-lasting CD28 blockade (Table I, setting B). To limit the interruption of CD28 costimulation to the phase of acute challenge, we next used a single dose of 100 μ g of E18 two days before challenge and analyzed leukocyte composition of the BAL fluid and cytokine responses of the spleen and draining lymph nodes another 10 days later (ie, when CD28 had become available again for ligand interaction; Table I, setting C). As shown in Fig 6, A, infiltration by lymphocytes, macrophages, and eosinophils was still markedly reduced at this time point. Importantly, we again observed a BAL fluid-specific increase in the frequency of Treg cells among the residual infiltrating CD4 T cells (Fig 6, B).

As had been seen under continuous CD28 blockade, antigen-specific but not polyclonal stimulation of spleen and MLN cells resulted in a marked increase in IFN- γ production (Fig 6, C). However, removal of CD8 T cells and natural killer cells, 2 major sources of IFN- γ , abolished this increase (data not shown), arguing against a T_H2 to T_H1 deviation.

CD28 blockade improves airway inflammation in response to HDM

To extend our observations to a clinically more relevant allergen and to use a model in which priming is achieved by airway exposure, we sensitized BALB/c mice with an HDM preparation intranasally, followed by intranasal challenges on days 7 to 11. CD28 blockade was performed with 100 μ g of E18 one day before the first challenge, and mice were analyzed on day 13 (Table I, setting D). As shown in Fig 7, airway resistance, inflammatory infiltrates, and histopathologic presentation were greatly diminished if challenge was performed under CD28 blockade. Of note, infiltrating total (see Fig E4 in this article's Online Repository at www.jacionline.org) and effector memory (Fig 7, D) CD4 T-cell numbers of anti-CD28-treated mice remained at PBS control values, indicating a blockade of CD4 T-cell activation in these mice. Numbers of lung CD4 T cells capable of cytokine production, either type 1 (IFN- γ) or type 2 (IL-4, IL-5, IL-10 or IL-13), in response to polyclonal activation were also greatly

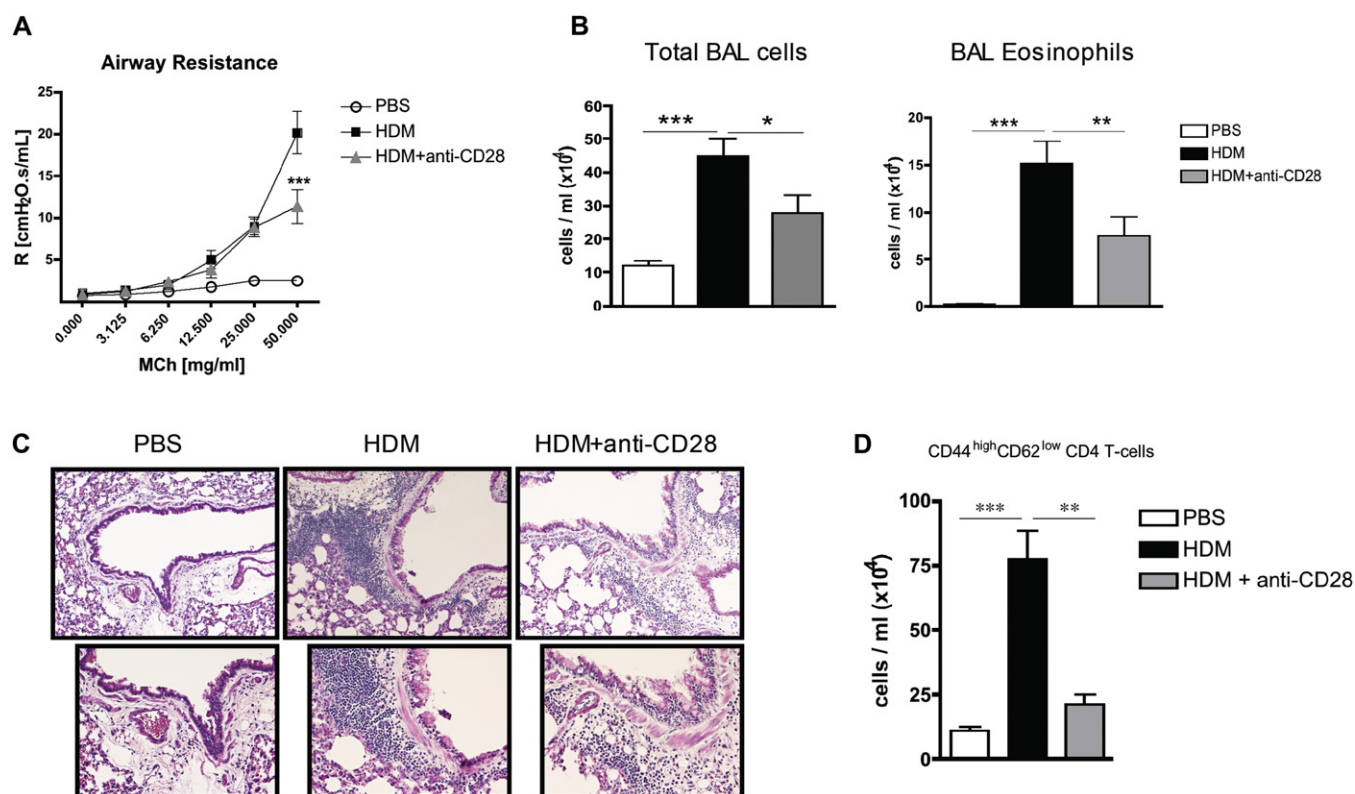


FIG 7. CD28 blockade reduces airway resistance and inflammatory cell infiltration in the airways. **A**, Airway resistance to methacholine of HDM- or PBS-sensitized/challenged and anti-CD28-treated mice. **B**, Cells in BAL fluid. BAL fluid eosinophils were analyzed by using fluorescence-activated cell sorting. **C**, Hematoxylin and eosin-stained lung tissues. **D**, Effector memory CD4 T cells in the lung. Values represent means $\pm$ SEMs from one of 3 experiments ($n = 6$). * $P < .05$; ** $P < .01$; and *** $P < .001$.

reduced (see Fig E4). Thus even if sensitization occurred intranasally, systemic anti-CD28 treatment was able to blunt a subsequent allergic response to a second intranasal antigen application.

DISCUSSION

CD28 is one component of a costimulatory/coinhibitory system that also contains the negative Treg cell-surface receptor CTLA-4 (CD152) and their shared ligands, CD80 and CD86.³³ Similar to what had been described earlier for the inhibitory receptor CTLA-4,³⁴ we recently demonstrated that it is feasible to selectively interfere with the costimulatory receptor CD28 by using a ligand-binding site-specific mAb.²² Here we apply this strategy to 2 mouse models of allergic asthma and report that therapeutic mAb treatment after systemic sensitization with the model antigen OVA in the T_H2-polarizing adjuvant alum or after intranasal sensitization with HDM antigens effectively curtails the development of lung pathology in response to intranasal antigen challenge. In addition, we obtained independent support for this conclusion using inducible deletion of CD28 between sensitization and challenge in the OVA model. We show that both mAb treatment and induced gene deletion after allergen sensitization are highly effective in ameliorating airway obstruction and inflammation during the recall response without affecting pre-established allergen-specific IgE levels.

In the more allergy-prone BALB/c strain (Fig 3, C, and see Fig E2, A), which was used for CD28-directed antibody therapy, allergen-induced cytokines in the lung and cytokine responses

of spleen and MLN cells were readily observed with HDM (data not shown) and OVA as allergens. Of note, therapeutic antibody interference markedly suppressed OVA-induced production of the T_H2 cytokines but left IFN- γ production intact in BAL fluid and even led to its increased production after antigen-specific *in vitro* restimulation of spleen or MLN cells with the allergen. This effect was not due to reprogramming of the T_H2 response toward a T_H1 response because the increase in IFN- γ production was abolished after removal of CD8⁺ and natural killer cells. The mechanistic basis of this unexpected effect awaits further analysis.

In patients with HDM-induced allergy, anti-CD28 treatment before challenge reduced total numbers of cytokine-producing and CD44^{high}CD62^{low} CD4 T-cell numbers in the lung. This reduction in activated CD4⁺ T-cell numbers at the site of allergic airway inflammation provides a convincing explanation for the observed reduction of asthmatic symptoms after therapeutic CD28 treatment.

The particular dependency of Treg cells on CD28-mediated costimulation for their generation, homeostasis, and function begged the question of their role in the present scenarios. In keeping with the importance of CD28 for Treg cells, their frequencies were reduced both in MLNs and in BAL fluid of C57BL/6 mice in the model of induced CD28 deletion before allergen challenge. This result suggests that impairment of CD4 effector cell function by CD28 ablation is beneficial for airway inflammation, even if Treg cell numbers are disproportionately reduced after the loss of CD28. However, in the BALB/c model of

E18 mAb therapy, reduced Treg cell frequencies were only observed in spleens and MLNs (Fig 6, B), whereas they were even increased in the challenged lung. We have previously observed a similar effect in superantigen-stimulated mice, in which CD4 effector, but not Treg cell, proliferation was impaired under E18 application, suggesting that under conditions of antigen stimulation in an inflamed environment, stimulatory properties of the mAb might override its blocking effect on Treg cells, which leads to the reduction of their steady-state compartment.²² Taken together, the beneficial effect of treatment with a ligand-binding site-specific antibody to CD28 is likely to consist of 2 components: the inhibition of allergen-driven restimulation of memory T_H2 cells, which is the mechanism also operative after induced CD28 deletion, and an improved effector to regulatory CD4 T-cell balance at the site of inflammation, which is specific to the antibody therapy.

In summary, our study with the CD28-specific mAb E18 shows that mAbs to the ligand-binding site of this key costimulatory receptor and decision maker in T_H2 development do harbor therapeutic potential for allergic disease. Furthermore, in mimicking the situation with humanized mAbs in patients, this mouse anti-mouse mAb will allow us to extend observations to therapy of chronic disease in mouse models. In a different setting (that of preventing allograft rejection) a binding site-specific monovalent single-chain fusion antibody specific for human CD28 has recently been found to be effective in primate models,³⁵ suggesting that selective CD28 blockade is indeed feasible. Of note, these authors report that under conditions of antigenic challenge (mixed lymphocyte reaction *in vitro* or organ transplantation *in vivo*), this treatment blocks expansion of effector cells while preserving that of Treg cells, which is quite similar to our present observations in allergen-challenged lungs.

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Key messages

- Using 2 types of allergens and 2 experimental approaches for selective interference with CD28-mediated costimulation (induced gene targeting and mAb blockade), we show reduced airway inflammation, leukocyte infiltration, and airway hyperreactivity if CD28 is targeted before allergen challenge.
- Although effector cell numbers were reduced in the challenged lung in all settings tested, mAb therapy also led to an improved Treg cell/effector T-cell balance at the site of inflammation.
- Interference with the CD28 pathway might be an effective means to treat allergic asthma, even after its establishment.

REFERENCES

- Maddox L, Schwartz DA. The pathophysiology of asthma. *Annu Rev Med* 2002; 53:477-98.
- Robinson DS, Hamid Q, Ying S, Tsicopoulos A, Barkans J, Bentley AM, et al. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N Engl J Med* 1992;326:298-304.
- Durham SR, Ying S, Varney VA, Jacobson MR, Sudderick RM, Mackay IS, et al. Cytokine messenger RNA expression for IL-3, IL-4, IL-5, and

- granulocyte/macrophage-colony-stimulating factor in the nasal mucosa after local allergen provocation: relationship to tissue eosinophilia. *J Immunol* 1992; 148:2390-4.
- Padrid PA, Mathur M, Li X, Herrmann K, Qin Y, Cattamanchi A, et al. CTLA4Ig inhibits airway eosinophilia and hyperresponsiveness by regulating the development of Th1/Th2 subsets in a murine model of asthma. *Am J Respir Cell Mol Biol* 1998;18:453-62.
- Andres PG, Howland KC, Nirula A, Kane LP, Barron L, Dresnek D, et al. Distinct regions in the CD28 cytoplasmic domain are required for T helper type 2 differentiation. *Nat Immunol* 2004;5:435-42.
- Shahinian A, Pfeffer K, Lee KP, Kundig TM, Kishihara K, Wakeham A, et al. Differential T cell costimulatory requirements in CD28-deficient mice. *Science* 1993; 261:609-12.
- Honstetter A, Meghari S, Nunes JA, Lepidi H, Raoult D, Olive D, et al. Role for the CD28 molecule in the control of *Coxiella burnetii* infection. *Infect Immun* 2006; 74:1800-8.
- Webb LM, Feldmann M. Critical role of CD28/B7 costimulation in the development of human Th2 cytokine-producing cells. *Blood* 1995;86:3479-86.
- Lenschow DJ, Herold KC, Rhee L, Patel B, Koons A, Qin HY, et al. CD28/B7 regulation of Th1 and Th2 subsets in the development of autoimmune diabetes. *Immunity* 1996;5:285-93.
- Kimzey SL, Liu P, Green JM. Requirement for CD28 in the effector phase of allergic airway inflammation. *J Immunol* 2004;173:632-40.
- Bour-Jordan H, Esensten JH, Martinez-Llordella M, Penaranda C, Stumpf M, Bluestone JA. Intrinsic and extrinsic control of peripheral T-cell tolerance by costimulatory molecules of the CD28/ B7 family. *Immunol Rev* 2011;241: 180-205.
- Robinson DS, Larche M, Durham SR. Tregs and allergic disease. *J Clin Invest* 2004;114:1389-97.
- Akdis CA, Blesken T, Akdis M, Wuthrich B, Blaser K. Role of interleukin 10 in specific immunotherapy. *J Clin Invest* 1998;102:98-106.
- Keane-Myers A, Gause WC, Linsley PS, Chen SJ, Wills-Karp M. B7-CD28/CTLA-4 costimulatory pathways are required for the development of T helper cell 2-mediated allergic airway responses to inhaled antigens. *J Immunol* 1997; 158:2042-9.
- Van Oosterhout AJ, Hofstra CL, Shields R, Chan B, Van Ark I, Jardiue PM, et al. Murine CTLA4-IgG treatment inhibits airway eosinophilia and hyperresponsiveness and attenuates IgE upregulation in a murine model of allergic asthma. *Am J Respir Cell Mol Biol* 1997;17:386-92.
- Krinzman SJ, De Sanctis GT, Cernadas M, Mark D, Wang Y, Listman J, et al. Inhibition of T cell costimulation abrogates airway hyperresponsiveness in a murine model. *J Clin Invest* 1996;98:2693-9.
- Jaffar Z, Roberts K, Pandit A, Linsley P, Djukanovic R, Holgate S. B7 costimulation is required for IL-5 and IL-13 secretion by bronchial biopsy tissue of atopic asthmatic subjects in response to allergen stimulation. *Am J Respir Cell Mol Biol* 1999;20:153-62.
- Deppong CM, Parulekar A, Boomer JS, Bricker TL, Green JM. CTLA4-Ig inhibits allergic airway inflammation by a novel CD28-independent, nitric oxide synthase-dependent mechanism. *Eur J Immunol* 2010 [Epub ahead of print].
- Ji W, Chen X, Zhengrong C, Yumin H, Huang L, Qiu Y. Therapeutic effects of anti-B7-1 antibody in an ovalbumin-induced mouse asthma model. *Int Immunopharmacol* 2008;8:1190-5.
- Harris N, Campbell C, Le Gros G, Ronchese F. Blockade of CD28/B7 costimulation by mCTLA4-Hgamma1 inhibits antigen-induced lung eosinophilia but not Th2 cell development or recruitment in the lung. *Eur J Immunol* 1997; 27:155-61.
- Dennehy KM, Elias F, Zeder-Lutz G, Ding X, Altschuld D, Luhder F, et al. Cutting edge: monovalency of CD28 maintains the antigen dependence of T cell costimulatory responses. *J Immunol* 2006;176:5725-9.
- Beyersdorf N, Ding X, Blank G, Dennehy KM, Kerkau T, Hunig T. Protection from graft-versus-host disease with a novel B7 binding site-specific mouse anti-mouse CD28 monoclonal antibody. *Blood* 2008;112:4328-36.
- Major T, Wohlleben G, Reibetanz B, Erb KJ. Application of heat killed *Mycobacterium bovis*-BCG into the lung inhibits the development of allergen-induced Th2 responses. *Vaccine* 2002;20:1532-40.
- Hammad H, Plantinga M, Deswarte K, Pouliot P, Willart MA, Kool M, et al. Inflammatory dendritic cells—not basophils—are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen. *J Exp Med* 2010;207: 2097-111.
- Seibler J, Zevnik B, Kuter-Luks B, Andreas S, Kern H, Hennek T, et al. Rapid generation of inducible mouse mutants. *Nucleic Acids Res* 2003;31:e12.
- Hahn C, Teufel M, Herz U, Renz H, Erb KJ, Wohlleben G, et al. Inhibition of the IL-4/IL-13 receptor system prevents allergic sensitization without affecting

- established allergy in a mouse model for allergic asthma. *J Allergy Clin Immunol* 2003;111:1361-9.
27. Reuter S, Heinz A, Sieren M, Wiewrodt R, Gelfand EW, Stassen M, et al. Mast cell-derived tumour necrosis factor is essential for allergic airway disease. *Eur Respir J* 2008;31:773-82.
 28. Burr JS, Kimzey SL, Randolph DR, Green JM. CD28 and CTLA4 coordinately regulate airway inflammatory cell recruitment and T-helper cell differentiation after inhaled allergen. *Am J Respir Cell Mol Biol* 2001;24:563-8.
 29. Rulifson IC, Sperling AI, Fields PE, Fitch FW, Bluestone JA. CD28 costimulation promotes the production of Th2 cytokines. *J Immunol* 1997;158:658-65.
 30. Rodriguez-Palmero M, Hara T, Thumbs A, Hunig T. Triggering of T cell proliferation through CD28 induces GATA-3 and promotes T helper type 2 differentiation in vitro and in vivo. *Eur J Immunol* 1999;29:3914-24.
 31. Tao X, Constant S, Jorritsma P, Bottomly K. Strength of TCR signal determines the costimulatory requirements for Th1 and Th2 CD4+ T cell differentiation. *J Immunol* 1997;159:5956-63.
 32. Kubo M, Yamashita M, Abe R, Tada T, Okumura K, Ransom JT, et al. CD28 costimulation accelerates IL-4 receptor sensitivity and IL-4-mediated Th2 differentiation. *J Immunol* 1999;163:2432-42.
 33. Sharpe AH, Freeman GJ. The B7-CD28 superfamily. *Nat Rev Immunol* 2002;2:116-26.
 34. Krummel MF, Allison JP. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J Exp Med* 1995;182:459-65.
 35. Poirier N, Azimzadeh AM, Zhang T, Dilek N, Mary C, Nguyen B, et al. Inducing CTLA-4-dependent immune regulation by selective CD28 blockade promotes regulatory T cells in organ transplantation. *Sci Transl Med* 2010;2:17ra0.

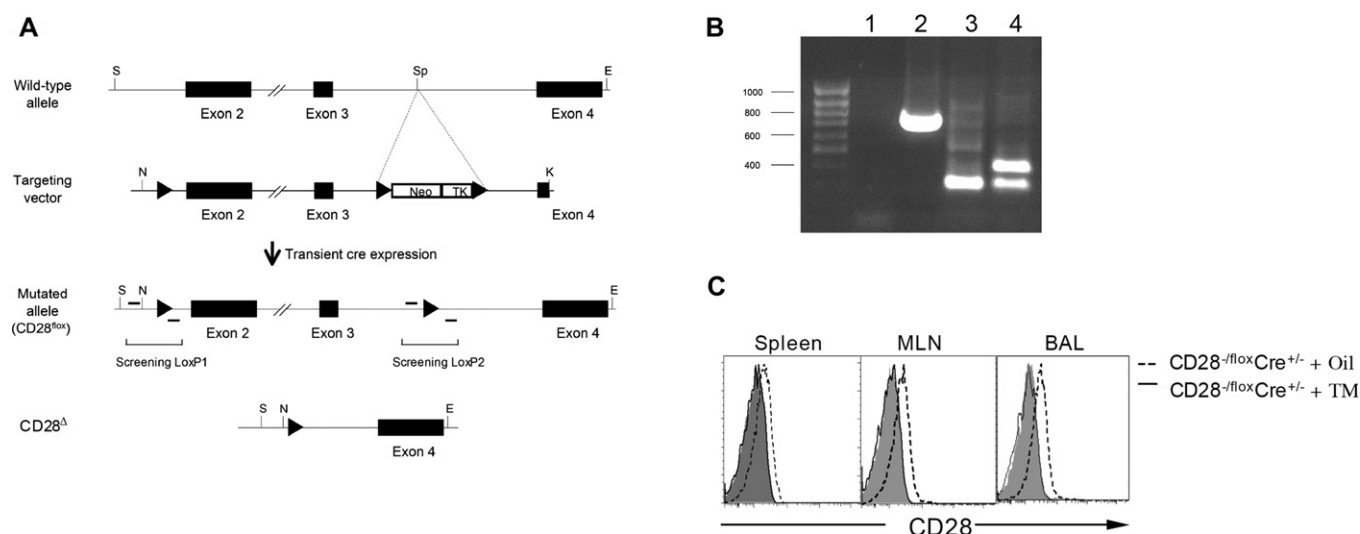


FIG E1. Generation of *CD28^{flox}* mice. **A**, A LoxP site was cloned into a genomic *CD28* clone from a 129/SvJ mouse upstream of exon 2 together with a neo/TK cassette flanked by 2 LoxP sites in the intron between exon 3 and 4, resulting in the targeting vector. This was electroporated into 129 embryonic stem cells, and after identification of positive clones, the neo/TK cassette was removed by means of transient expression of cre, resulting in the *CD28^{flox}* allele. Embryonic stem cells harboring this modification were injected into C57Bl/6 blastocysts, resulting in *CD28^{flox}* mice that were backcrossed 9 times to the C57Bl/6 background. After crossing with appropriate cre-expressing mice, both exon 2 and exon 3 of *CD28* will be deleted (*CD28^Δ*). The position of the primers for the screening strategy of the LoxP1 and LoxP2 sites are indicated by bars. Restriction sites: *E*, *EcoRV*; *K*, *KpnI*; *N*, *NotI*; *Neo*, neomycin; *S*, *SalI*; *Sp*, *SpeI*; *TK*, thymidine kinase. **B**, PCR screening strategy. The LoxP site upstream from exon 2 produced a 718-bp PCR product (lane 3), which was absent in the wild-type allele (lane 2). The LoxP site in the intron between exon 3 and exon 4 was detected by using PCR, resulting in 2 bands of equal intensity of 406 bp (floxed allele) and 297 bp (wild-type allele, lane 5); only the latter was present in wild-type embryonic stem cells (lane 4). Lane 1, Marker with the indicated molecular weights (in base pairs). **C**, Loss of cell-surface expression of *CD28* in *CD28^{-flox} Cre^{+/-}* mice after induced gene deletion. Spleen, MLN, and BAL fluid cells were stained for CD4 and *CD28*. Expression of *CD28* is shown for *CD4⁺* T cells. The solid histogram indicates the isotype control. Experiments were done 3 times with at least 5 animals per group.

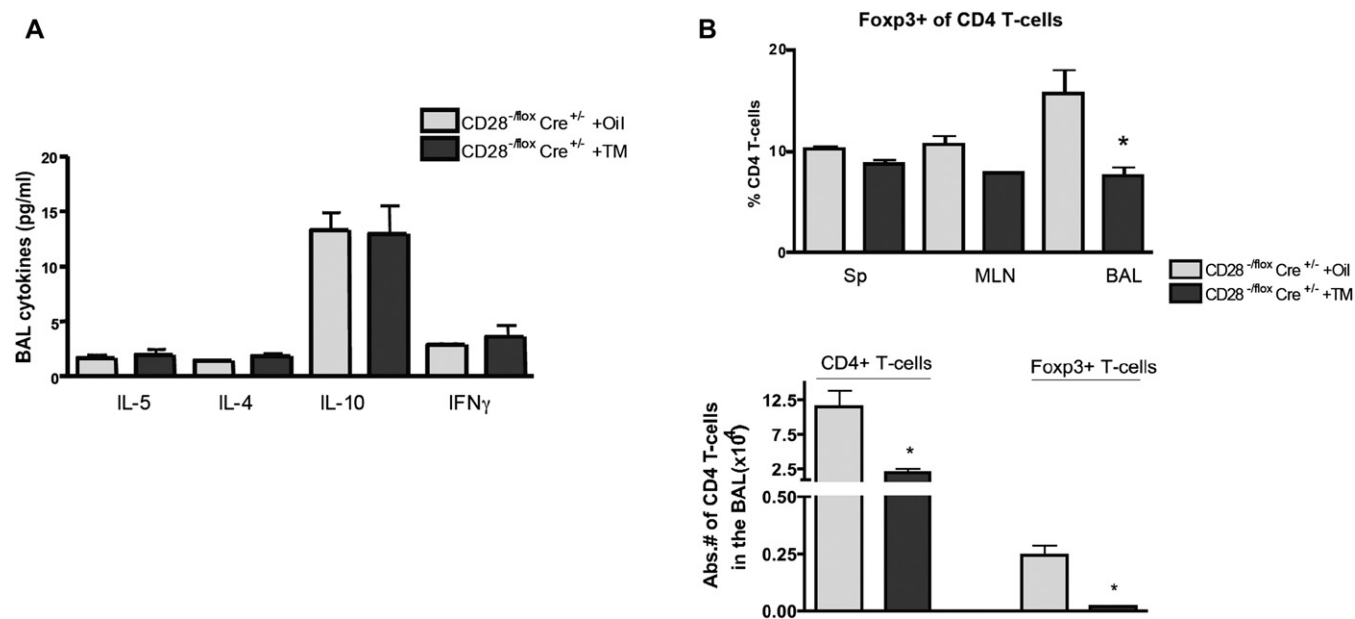


FIG E2. Inducible gene deletion in *CD28*^{-flox} mice reduces T_H2-associated cytokine secretion without changing the OVA-specific response. **A**, Cytokines in BAL fluid. **B**, Frequency and absolute cell number of Treg cells. Means $\pm$ SEMs from one of 3 experiments are shown (n = 5). Sp, Spleen. **P* < .05.

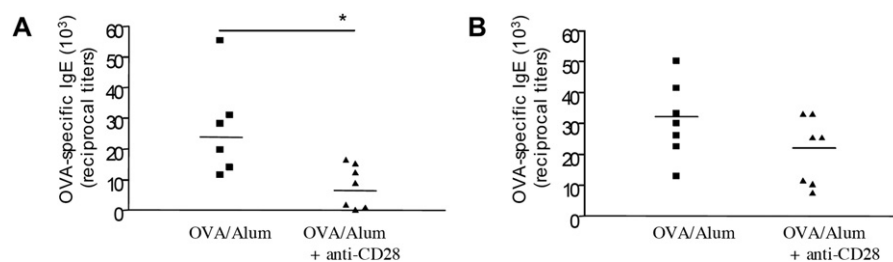


FIG E3. Blockade of CD28 during sensitization prevents production of OVA-specific IgE but has little effect in therapeutic setting. **A**, Antigen-specific IgE levels in sera of mice treated with anti-CD28 during sensitization. **B**, Levels of OVA-specific IgE in therapeutically treated mice. OVA-specific IgE levels were measured by means of ELISA 1 week after intranasal challenge. Means $\pm$ SEMs of one of 3 independent experiments are shown (n = 6-7).

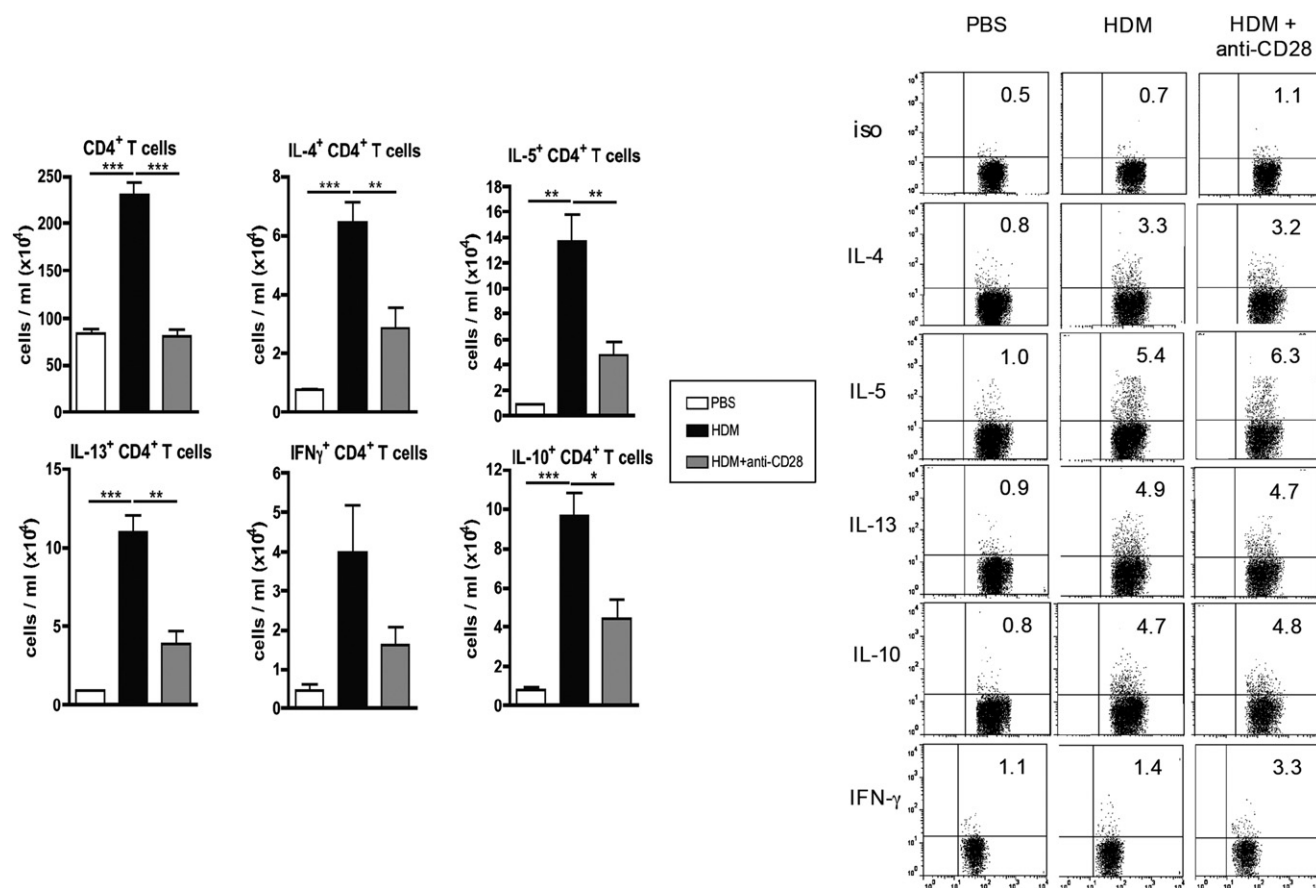


FIG E4. Anti-CD28 treatment reduces CD4 T-cell infiltration in lung tissues and numbers of cytokine-producing CD4⁺ T cells. Total CD3⁺CD4⁺ T-cell numbers and cytokine-producing CD4⁺ T-cell numbers were identified by means of fluorescence-activated cell sorting in the lung tissue. Values represent means $\pm$ SEMs from one of 3 experiments ($n = 4$). * $P < .05$; ** $P < .01$; and *** $P < .001$.