

ORIGINAL PAPER

CD137 ligand prevents the development of T-helper type 2 cell-mediated allergic asthma by interferon- γ -producing CD8⁺ T cells

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Clinical and Experimental Allergy

Summary

Background Allergic asthma is a T-helper type 2 (Th2) cell-mediated chronic disease that is characterized by airway hyperreactivity (AHR) and chronic eosinophilic airway inflammation. Several studies suggest co-stimulatory molecules like CD137 as potential targets for therapeutic interventions in allergic airway disease. Recently, we could show in a murine asthma model that administration of an agonistic antibody against the receptor of the co-stimulatory molecule CD137 prevented and even reversed an already-established asthma phenotype.

Objective The purpose of this study was to analyse the effect of stimulation of the CD137 ligand by a monoclonal antibody (CD137L mAb).

Methods To induce an asthma-like phenotype, BALB/c mice were sensitized to ovalbumin (OVA), followed by an intrapulmonary allergen challenge. Anti-CD137L or control mAb were applied 1 day before OVA immunization or after the asthma phenotype was already established.

Results Stimulation of the CD137L instead of the receptor by CD137L mAb prevents the development of an asthma-like phenotype but does not reverse established disease. While the receptor-mediated effect is partly mediated by anergy of CD4⁺ T cells and partly by induction of IFN- γ -producing CD8⁺ T cells, the effect of the CD137L mAb is completely dependent on IFN- γ -producing CD8⁺ T cells: blockade of IFN- γ and depletion of CD8⁺ T cells fully abrogated the observed protective effect. *In vitro* experiments showed that the anti-CD137L mAb ligates directly to CD8⁺ T cells and induces the generation of IFN- γ by this cell population.

Conclusion Our results demonstrate that anti-CD137L mAb prevents disease development via IFN- γ -producing CD8⁺ T cells but is inferior to stimulation of the receptor that reverses established disease by a mechanism including CD4⁺ T cell anergy.

Keywords allergy, co-stimulation, inflammation, lung, T cells

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Introduction

Allergic asthma is a chronic inflammatory condition of the airways characterized by airway hyperreactivity (AHR), chronic eosinophilic airway inflammation and elevated serum IgE levels [1]. T-helper type 2 (Th2) lymphocytes play a key role in the initiation and perpetuation of airway inflammation. The direct modification of T cell

activation by targeting co-stimulatory interactions that are involved in this process could be a causal strategy to control asthma. Successful activation of T cells requires engagement of the T cell receptor (TCR) with peptide-major histocompatibility complex (MHC) proteins as well as engagement of T cell co-stimulatory receptors with their ligands on antigen-presenting cells (APCs). The best-characterized co-stimulatory molecule CD28, which interacts with its ligands B7-1 and B7-2, is important for the initial activation and essential for naïve T cell survival and differentiation. However, a number of additional

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co-stimulatory receptor–ligand pairs are up-regulated on T cells and APCs and may be involved in sustaining, diversifying or amplifying the immune response. In particular, members of the TNF receptor/TNF ligand family, including the CD137/CD137 ligand (CD137L), CD27/CD70, CD30/CD30 ligand and OX40/OX40 ligand, appear to be important in enhancing the T cell response subsequent to initial activation [2, 3].

CD137L (4-1BBL) has been detected on mature dendritic cells, activated macrophages and activated B cells [4]. It has been shown that CD137L can co-stimulate T cell responses independently of signalling through the CD28 molecule [5, 6] and stimulate both primary and secondary immune responses [7–9]. Ligation of CD137 using CD137L gene transfection [10], agonistic monoclonal antibodies (mAb) against the co-stimulatory molecule CD137 mAb or soluble CD137L [11] promotes T cell activation and proliferation [12, 13], enhances the survival of activated T cells [9] and suppresses CD4⁺ T cell help during T cell-dependent humoral immune responses [14]. Moreover, previous animal studies have shown that short-term administration of anti-CD137 mAb induces rejection of established tumours [15, 16], enhances the CD8⁺ T cell responses to viruses [17], blocks autoimmune disease progression [18, 19] and suppresses rheumatoid arthritis [20, 21]. The interaction of CD137 with its ligand was proposed to preferentially co-stimulate CD8⁺ T cells and to induce Th1 immune responses [22]. However, recently we demonstrated in a murine model of asthma that CD137 is critical in determining the outcome of this Th2 cell-mediated disease. A single injection of anti-CD137 mAb could prevent and even reverse an established asthma phenotype. Although anti-CD137 mAb stimulated IFN- γ -producing CD8⁺ T cells, the protective effect was mainly mediated by the induction of CD4⁺ T cell anergy [23].

In the present study, we used a different approach to investigate the role of CD137 signalling in allergic asthma by stimulating not the receptor but the ligand of the CD137/CD137L co-stimulatory pathway. Our findings reported here show that administration of an anti-CD137L mAb prevents BALB/c mice from the development of an asthma-like phenotype, while – in contrast to CD137 mAb – it does not reverse already-established disease. The protective effect of CD137L mAb did not involve CD4⁺ T cell anergy as seen with CD137 mAb but was completely mediated by INF- γ -producing CD8⁺ T cells.

Materials and methods

Mice and CD137L mAb

Female BALB/cByJ mice (6–8 weeks of age) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and bred and maintained in our animal facility at the Martin

Luther University in Halle/Saale (Germany). IL-12p40^{−/−} mice (129Sv/Ev mice on a BALB/c background) [24] were a kind gift from G. Alber, Leipzig, Germany. All animal experiments involved groups of four to six mice and were performed according to institutional and state guidelines. The Committee on Animal Welfare of Saxony-Anhalt approved the animal protocols used in this study.

Monoclonal anti-mouse CD137L mAbs were generated by immunizing Lewis rats with CD137L fusion proteins following the protocols and methods described earlier [22, 25]. To analyse the stimulatory properties of anti-CD137L mAb on APCs, we cultured bone marrow dendritic cells (DCs) and incubated them with ovalbumin (OVA, 200 μ L/mL) or OVA+anti-CD137L (10 μ g) for 3 days. Anti-CD137L mAb induced the production of IL-10 (OVA: 127 $\pm$ 4.7 pg/mL; OVA+anti-CD137L mAb: 340 $\pm$ 11.5 pg/mL) and IL-18 (OVA: 31 $\pm$ 0.8 pg/mL; OVA+anti-CD137L: 49 $\pm$ 0.7 pg/mL) as measured by ELISA.

Immunization and antibody treatments

Mice were immunized with OVA (20 μ g, Sigma-Aldrich, Steinheim, Germany) adsorbed to 2 mg of an aqueous solution of aluminium hydroxide and magnesium hydroxide (Alum, Perbio Science, Bonn, Germany) intraperitoneally (i.p.) on days 1 and 14, followed by 20 μ g OVA in 40 μ L normal saline given intranasally (i.n.) on days 14–16 and 21–23. In the chronic asthma model, mice were challenged with OVA i.n. additionally on days 28–30 and 42–44. Control mice received i.p. injections of alum alone and normal saline i.n. Anti-CD137L mAb (19H3, rat IgG2a anti-mouse CD137L mAb, 200 μ g) or control mAb (rat IgG2a, 200 μ g) were applied i.p. on days 0 and 13. AHR was measured at day 24 or 45 and mice were killed on day 25 or 46. For CD8⁺ T cell depletion, anti-CD8 mAb (YTS 169.4, rat IgG2b anti-mouse CD8 mAb, 100 μ g) was injected on days −2, −1, 11, 12 and 17. Flow cytometric analysis on days 2 and 15 showed 99% depletion of CD8⁺ T cells. Anti-IFN- γ mAb (XMG1.2, rat IgG1 anti-mouse IFN- γ mAb, 10 μ g, eBioscience, San Diego, CA, USA) was injected on days −1, 6, 12 and 18 [26]. In a reversal protocol, mice were immunized with OVA on day 1, followed by OVA in normal saline i.n. on days 8–10. Control mice received alum i.p. and normal saline i.n. To confirm the induction of AHR by OVA sensitization, AHR was measured the following day. Anti-CD137L mAb or control mAb were injected i.p. 12 h after AHR measurement on day 11. I.n. OVA challenge was repeated on days 15–17 and AHR was measured on day 18. Mice were killed the following day.

Measurement of airway responsiveness

Airway responsiveness was assessed by methacholine-induced airflow obstruction from conscious mice placed

in a whole-body plethysmograph (model PLT UNR MS, EMKA TECHNOLOGIES, Paris, France) as described previously [27].

Collection of bronchoalveolar lavage (BAL) fluid

Cells in the lavage fluid were counted using a haemocytometer, and BAL cell differentials were determined as described previously [23].

Lung histology and computer-based quantification of inflammation and mucus production

The left lung was fixed in 10% formalin and stained with haematoxylin & eosin (H&E, MERCK, Darmstadt, Germany) or with PAS (Sigma, Taufkirchen, Germany). For objective evaluation and quantification of the degree of pulmonary inflammation and mucus production, lung sections were scanned with a digital camera (Visitron Systems, Puchheim, Germany, five shots per lung) and analysed with HistoClick-Software based on morphometric image analysis developed in our laboratory. The degree of inflammation and mucus production is expressed by the number of pixels.

OVA-specific IgE and IgG1 assay

OVA-specific IgE and IgG1 serum levels were measured by sandwich ELISA according to a standard protocol as described previously [23].

In vitro proliferation and cytokine production

One day after the airway function test, splenocytes or lung cells (5×10^6 cells/well) were restimulated *in vitro* with OVA (200 µg/mL) in culture medium (RPMI medium supplemented with 10% fetal calf serum (FCS), 100 U/mL Penicillin and 100 µg/mL Streptomycin). For proliferation measurement, spleen cells were restimulated with 0.2–200 µg/mL OVA in 96-well tissue culture plates in 200 µL culture medium or not stimulated for controls. After 48 h, the cultures were pulsed with 0.25 µCi of [^{3} H] TdR thymidine for 18 h, and the incorporated activity was measured in a Betaplate scintillation counter (MicroBeta Trilux, Wallac, Turku, Finland). Cytokines were measured in the supernatants from restimulated splenocytes or lung cells after 3 days using DuoSet[®] ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. For measurement of IFN- γ in the supernatants of isolated T cells, CD4⁺ and CD8⁺ T cells were separated by magnetic cell sorting (T cell isolation kits, Milteny Biotek, Bergisch-Gladbach, Germany) and stimulated with anti-CD3 mAb/anti-CD28 mAb (R&D Systems). For cytokine measurement, CD8⁺ T cells were given to anti-CD137L mAb pre-coated plates and stimulated for 3 days.

Culture and treatment of bone marrow DCs

Bone marrow cells were collected from naïve mice, depleted of red blood cells using ammonium chloride, and grown in RPMI medium containing 10% FCS and 40 ng/mL rmGM-CSF (40 ng/mL) for 7 days. DCs were analysed (CD11c⁺) and 1×10^6 cells were given to 12-well plates, which were pre-coated overnight with anti-CD137L mAb (10 µg) and then pulsed with 100 µg/mL OVA. Forty-eight hours later, the supernatant was collected for cytokine measurement.

Flow cytometric analysis

Flow cytometric analysis was performed using FITC-, PE- or PerCP-Cy5.5-conjugated mAb to mouse CD3, CD4 and CD8 α (BD Biosciences Pharmingen, Heidelberg, Germany). 1×10^6 cells were pre-incubated with 2.4G2 mAb (10 µg/mL) to block Fc γ R, and then incubated with the relevant mAbs for 45 min at 4 °C. Cells were washed twice with 1% bovine serum albumin (BSA) in PBS and analysed by FACScan (BD Biosciences Pharmingen). For intracellular FACS analysis, cells were stimulated with phorbol ester (0.1 µg/mL) and ionomycin (0.5 µg/mL, both Sigma-Aldrich) for 6 h. Brefeldin A (GolgiPlug, BD Bioscience) was added for the last 4 h. Cells were washed and stained with anti-CD4 mAb and anti-CD8 mAb, permeabilized with Cytofix/Cytoperm-buffer (BD Bioscience) and then incubated with PE-labelled anti-IFN- γ mAb. To analyse CD137L ligation, separated CD8⁺ T cells from OVA-immunized BALB/c mice were stimulated with anti-CD3 mAb/anti-CD28 mAb, incubated with anti-CD137L mAb or control mAb and stained with PE-labelled anti-rat IgG.

Statistical analysis

Student's *t*-test was used to determine the statistical significance of differences between groups.

Results

Anti-CD137L mAb prevents the development of airway hyperreactivity, eosinophilic airway inflammation and mucus production

To investigate the effect of manipulation of CD137L in the Th2 cell-mediated immune response of allergic asthma, we tested an anti-CD137L mAb in our well-established murine asthma model [23, 27, 28]. In this model, an asthma-like phenotype with AHR, eosinophilic airway inflammation, mucus hypersecretion and high-serum IgE levels is induced by sensitization of mice to OVA by systemic and intrapulmonary application of this model allergen. Injection of anti-CD137L mAb 1 day before OVA priming completely prevented the development of AHR in

BALB/c (Fig. 1a). This effect was long-lasting as AHR was still reduced 31 and 45 days after anti-CD137L mAb application (Fig. 1a).

Moreover, anti-CD137L mAb application significantly inhibited the total cell number in the BAL fluid of OVA-immunized BALB/c mice. One day after the last i.n. OVA challenge, the total cell number in the BAL fluid was reduced almost four-fold by CD137L mAb (33.91 ± 6.09 vs. 8.48 ± 2.19 cells/mL $\times 10^4$, OVA+control mAb vs. OVA+anti-CD137L mAb, $P < 0.005$); in particular, the number of eosinophils, and notably also lymphocytes, were abrogated (Fig. 1b). Similar to the observations made with AHR, the inhibitory effect of anti-CD137L mAb application on the cell number in the BAL fluid was still significant more than 6 weeks after the first OVA immunization (total cell numbers on day 46: 20.07 ± 2.61 vs. 11.33 ± 1.52 cells/mL $\times 10^4$, OVA+control mAb vs. OVA+anti-CD137L mAb, $P < 0.05$, Fig. 1b).

In parallel, histological examination of H&E- and PAS-stained lung sections isolated from OVA-immunized mice on day 25 revealed significantly decreased airway inflammation and mucus production in the anti-CD137L mAb-treated group compared with the control group (Fig. 1c). Inhibition of airway inflammation and mucus production by anti-CD137L mAb could be verified by an objective, investigator-independent computer program based on morphometric image analysis of lung sections from at least nine animals per group (Fig. 1d).

Our data demonstrate that CD137L mAb is able to prevent the development of AHR and airway inflammation in OVA-immunized BALB/c mice.

Anti-CD137L mAb reduces OVA-specific serum IgE and IgG1 levels, reduces Th2-cytokine production from lung cells and increases Th1 cytokine production from spleen cells

Another hallmark of allergic asthma is the production of allergen-specific IgE. In murine models of asthma, IgG1 production is often increased while IgG2a production is decreased. To determine whether the application of anti-CD137L mAb leads to differences in the production of OVA-

specific IgE, IgG1 and IgG2a, serum was taken on day 25 and Ig levels were measured by ELISA. Anti-CD137L mAb significantly inhibited OVA-specific IgE as well as OVA-specific IgG1 serum levels on days 25 and 46 (Fig. 2a) while IgG2a serum levels were not affected (data not shown).

To investigate the effect of anti-CD137L mAb on cytokine production in OVA-immunized BALB/c mice, lung and spleen cells were isolated on day 25 and restimulated *in vitro* with OVA for 3 days. Analysis of cytokine levels in cell culture supernatants showed that anti-CD137L mAb significantly decreased the production of the Th2-cytokines IL-4, IL-13, IL-5 and IL-10 from lung cells, while IFN- γ production from lung cells was not affected. In contrast, anti-CD137L mAb application before OVA immunization significantly increased IFN- γ production from spleen cells, while the production of Th2 cytokines of spleen cells was not changed (Fig. 2b). IFN- γ production of splenocytes was already remarkably increased 5 days after anti-CD137L mAb injection (data not shown). Our data demonstrate different effects of anti-CD137L mAb on cytokine production locally and systemically. In the lung, where the disease is manifested, the immune response is dominated by inhibition of Th2 cytokine production, while in the spleen the immune response is dominated by increased IFN- γ production whereas Th2 cytokines remain unchanged.

Ex vivo proliferation of splenocytes isolated from anti-CD137L mAb-treated animals was slightly increased compared with the OVA group after restimulation with OVA (Fig. 2c).

To investigate whether anti-CD137L mAb may have a therapeutical potential, we administered the mAb after the asthma phenotype was already established. However, anti-CD137L mAb did not reduce airway inflammation, OVA-specific IgE levels or the production of Th2 cytokines (data not shown).

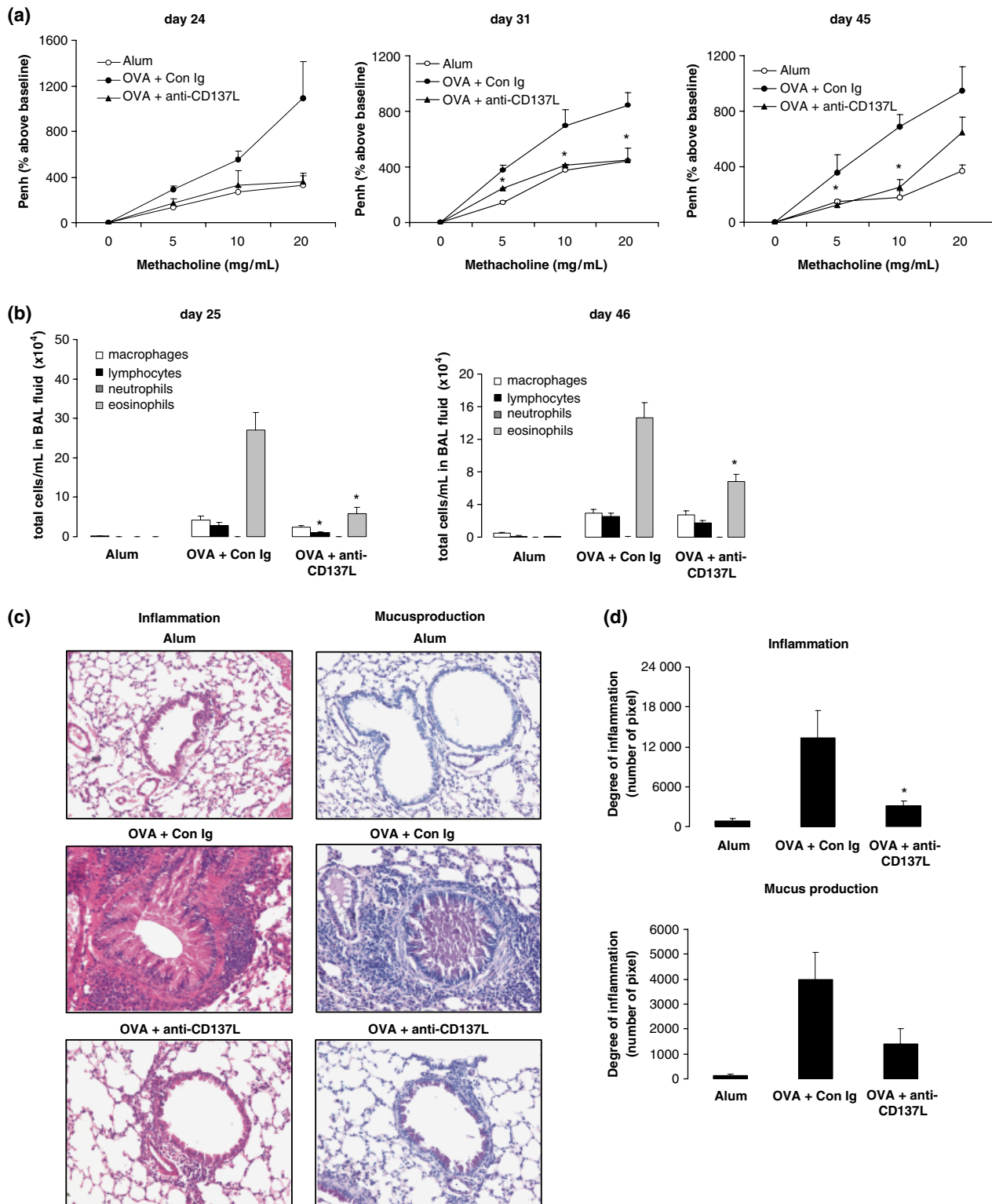
Anti-CD137L mAb-driven protective effects depend on IFN- γ -producing CD8⁺ T cells

Anti-CD137L mAb application to BALB/c mice before first OVA immunization increased the production of IFN- γ

Fig. 1. Anti-CD137 ligand (CD137L) monoclonal antibody (mAb) prevents the development of airway hyperreactivity, eosinophilic airway inflammation and mucus production. BALB/c mice were immunized with ovalbumin (OVA) on days 1 and 14, followed by several i.n. OVA challenges as described in 'Materials and methods'. (a) Anti-CD137L mAb or isotype-matched control mAb were injected the days before OVA immunization. Anti-CD137L mAb inhibits airway hyperreactivity (AHR) in OVA-immunized BALB/c mice measured on days 24, 31 and 45 in a whole-body plethysmograph. (b) Application of anti-CD137L mAb before immunization significantly reduces the total cell number in the bronchoalveolar lavage (BAL) fluid of OVA-immunized BALB/c mice. BAL was performed 1 day after measurement of AHR (day 25 or 46). (c) Lung tissues from BALB/c mice killed on day 46 (H&E, $\times 100$) revealed dense peribronchiolar mononuclear cell infiltrates consisting mainly of lymphocytes and eosinophils in the OVA-immunized group. Inflammation was strongly reduced by anti-CD137L mAb. PAS-stained lung sections ($\times 100$) revealed mucus hypersecretion in OVA-immunized mice compared with controls, which was almost abrogated by anti-CD137 mAb. (d) Quantification of lung inflammation and mucus production by a computer-based image-analysing program clearly demonstrated the significant anti-inflammatory effect of anti-CD137 mAb and verified the inhibition of mucus production. * $P < 0.05$, OVA+anti-CD137L mAb vs. OVA+control Ab by Student's *t*-test. $n \geq 9$ animals per group and data point; data are expressed as mean $\pm$ SEM from three independent experiments.

from spleen cells, suggesting a possible role of this cytokine in the observed effects. To investigate the mechanisms mediating the effect of anti-CD137L mAb in the murine asthma model, we blocked IFN- γ using a specific

anti-IFN- γ mAb. Anti-IFN- γ mAb but not control mAb abolished the increase of IFN- γ production in splenocytes and reversed the reduction of Th2 cytokines from lung cells derived from mice that had received anti-CD137L



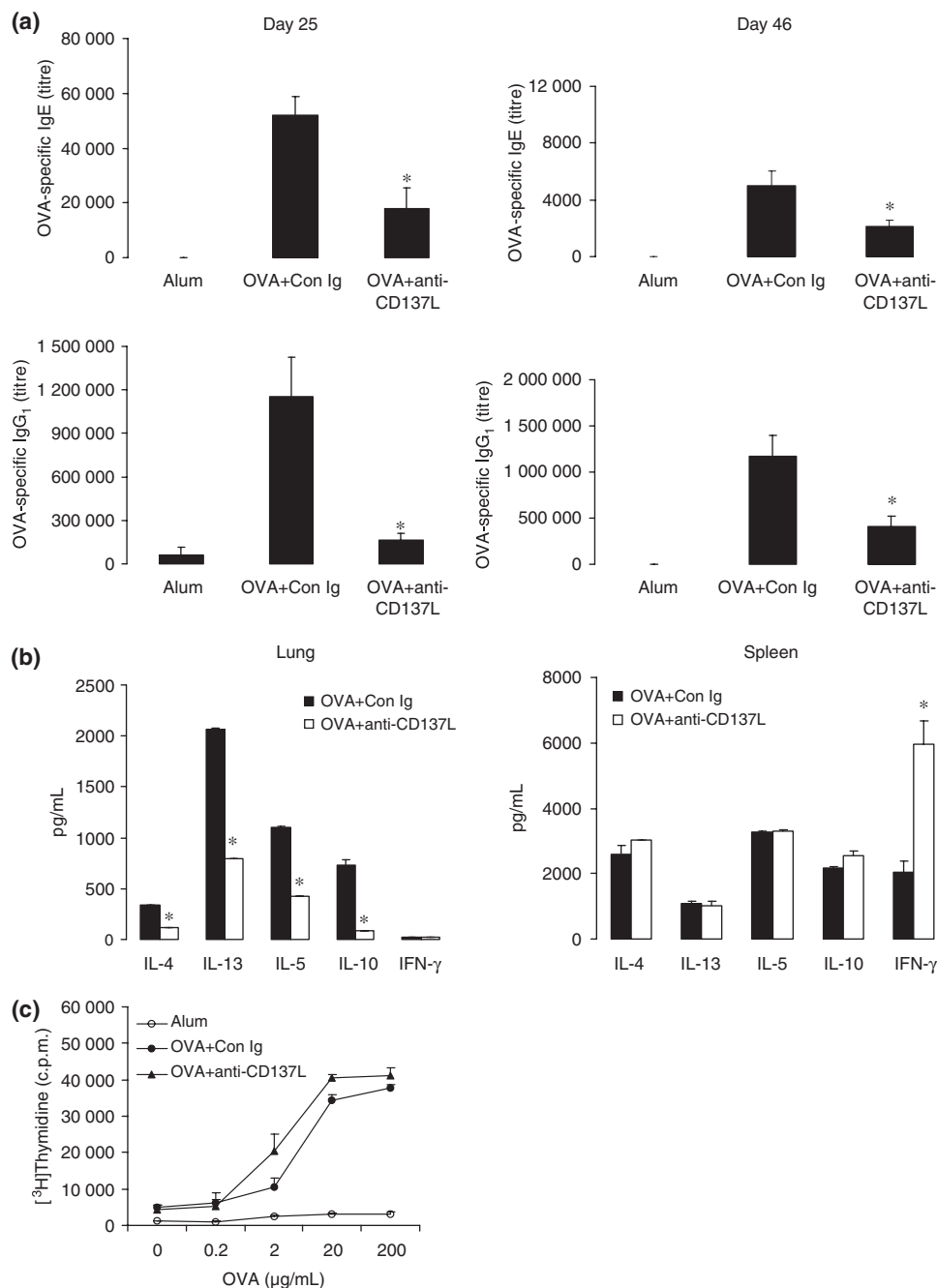


Fig. 2. Anti-CD137 ligand (CD137L) monoclonal antibody (mAb) reduces ovalbumin (OVA)-specific serum IgE and IgG1 levels, T-helper type 2 (Th2)-cytokine production from lung cells and increases T-helper type 1 (Th1) cytokine production from spleen cells. (a) Anti-CD137L mAb significantly reduces OVA-specific IgE and IgG1 serum levels in OVA-immunized mice. (b) Splenocytes and lung cells from OVA-immunized BALB/c mice were isolated and restimulated *in vitro* with OVA in culture medium 1 day after an airway function test. Anti-CD137L mAb inhibited Th2-cytokine production in lung cells but did not affect Th2 cytokine production from spleen cells. In contrast, IFN- γ was strongly induced by anti-CD137L mAb in spleen cell culture already on day 2, 1 day after OVA immunization. (c) One day after an airway function test, splenocytes from OVA-immunized BALB/c mice were isolated and restimulated with OVA (0.2–200 μ g/mL). Forty-eight hours later, cell cultures were pulsed with [³H] TdR thymidine for 18 h, and incorporated activity was measured in a scintillation counter. Anti-CD137L mAb had no effect on the proliferation of spleen cells of OVA-immunized BALB/c mice. * $P < 0.05$, OVA+anti-CD137L mAb vs. OVA+control Ab by Student's *t*-test. $n \geq 8$ animals per group and data point; data are expressed as mean $\pm$ SEM from three independent experiments.

mAb before immunization (Fig. 3a). In parallel, it almost completely abrogated the observed protective effect of CD137L mAb on AHR (Fig. 3b), eosinophilic lung inflammation (Fig. 3c) and OVA-specific IgE serum levels (Fig. 3d).

To determine the source for IFN- γ in our model, we measured this cytokine by intracellular FACS staining and directly showed that IFN- γ is produced by CD8⁺ T cells after treatment with anti-CD137L mAb (Fig. 3e).

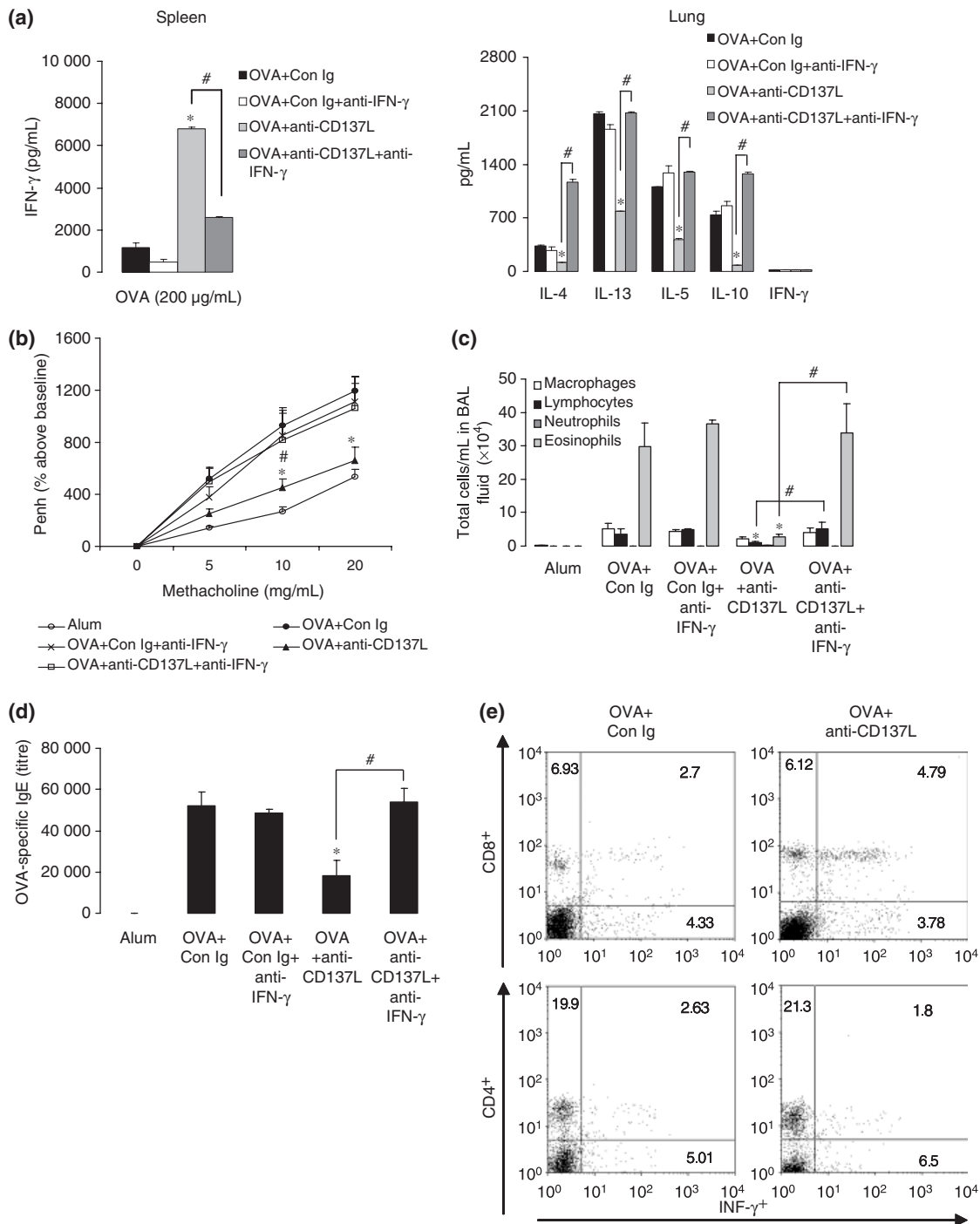


Fig. 3. Anti-CD137 ligand (CD137L) monoclonal antibody (mAb)-driven protective effects depend on IFN- γ . (a) BALB/c mice were immunized as described in 'Materials and methods'. For inhibition of IFN- γ , anti-IFN- γ mAb was injected on days -1, 6, 12 and 18. Inhibition of IFN- γ abrogated the inhibitory effect of anti-CD137L mAb. (b) On IFN- γ production from spleen cells and T-helper type 2 (Th2) cytokine production from lung cells, (c) on airway hyperreactivity (AHR) of ovalbumin (OVA)-immunized BALB/c mice compared with control mice, (d) on the total cell number in the bronchoalveolar lavage (BAL) fluid of OVA-immunized mice compared with control animals and (e) on OVA-specific IgE serum levels of OVA-immunized mice compared with control animals. Anti-CD137L mAb increased IFN- γ produced by CD8 $^{+}$ T cells detected with intracellular FACS staining. * $P < 0.05$, OVA+anti-CD137L mAb vs. OVA+control Ab, # $P < 0.05$, OVA+anti-CD137L vs. OVA+anti-CD137L mAb+anti-IFN- γ mAb by Student's t -test. (a) and (b), Data are expressed as mean $\pm$ SEM; $n \geq 9$ animals per group and data point of three independent experiments. (c) and (d), Data are expressed as mean $\pm$ SEM; $n \geq 4$ animals for each group and representative results from one of two experiments are shown.

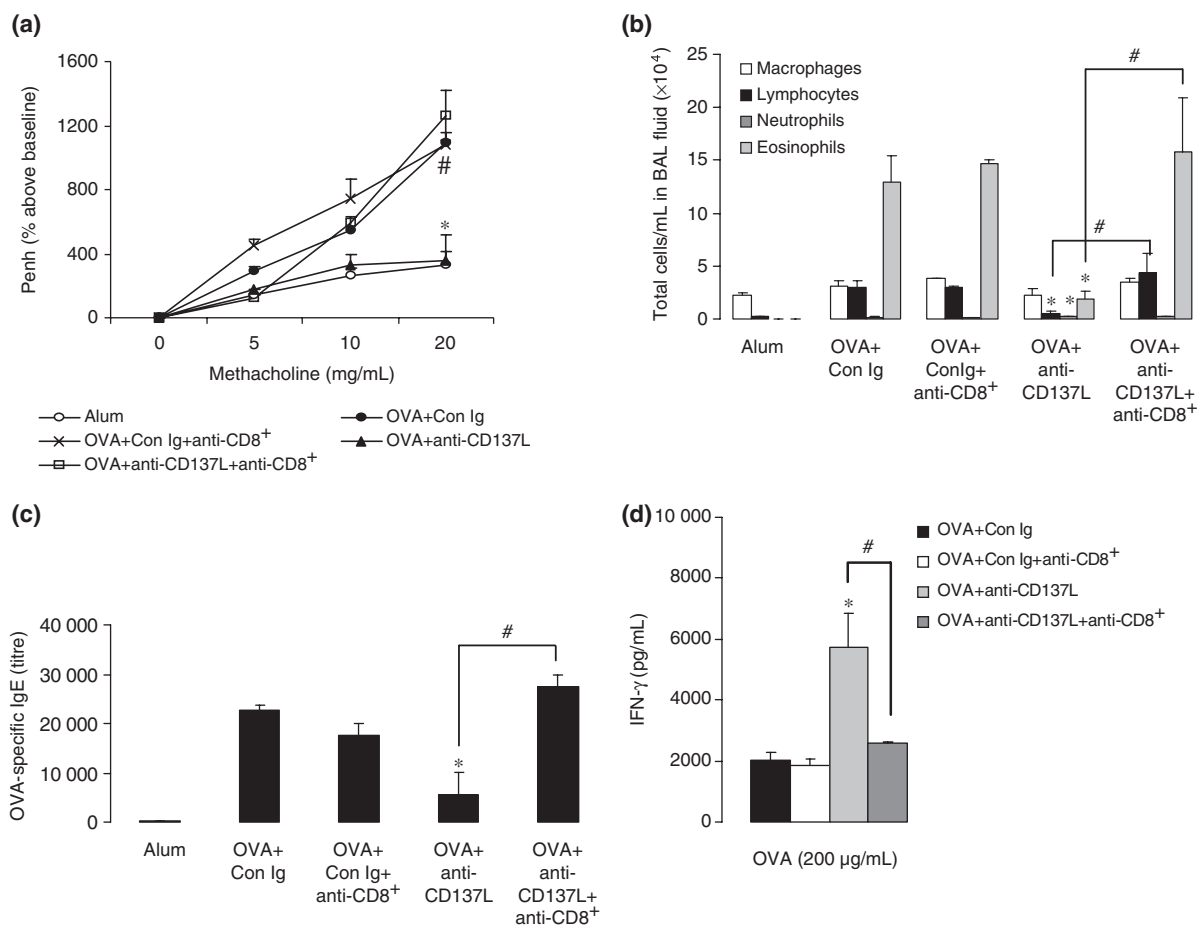


Fig. 4. Anti-CD137 ligand (CD137L) monoclonal antibody (mAb)-driven protective effects depend on CD8 T cells. BALB/c mice were immunized as described in 'Materials and methods'. For CD8⁺ T cell depletion, anti-CD8 mAb was injected on days -2, -1, 11, 12 and 17. CD8⁺ T cell depletion abrogated the inhibitory effect of anti-CD137L mAb. (a) On airway hyperreactivity (AHR) of ovalbumin (OVA)-immunized BALB/c mice compared with control mice, (b) on the total cell number in the bronchoalveolar lavage (BAL) fluid of OVA-immunized mice compared with control animals, (c) on OVA-specific IgE serum levels of OVA-immunized mice compared with control animals and (d) on IFN- γ production from spleen cells. * $P < 0.05$, OVA+anti-CD137L mAb vs. OVA+control Ab, # $P < 0.05$, OVA+anti-CD137L vs. OVA+anti-CD137L mAb+anti-CD8 mAb by Student's *t*-test. (a) and (b) Data are expressed as mean $\pm$ SEM; $n \geq 9$ animals per group and data point of three independent experiments. (c) and (d) Data are expressed as mean $\pm$ SEM; $n \geq 4$ animals for each group and representative results from one of two experiments are shown.

To further investigate a possible role of IFN- γ -producing CD8⁺ T cells in the CD137L-mediated effects, we depleted CD8⁺ T cells using an anti-CD8 mAb. Injection of anti-CD8 mAb resulted in 99% depletion of the CD8⁺ T cells compared with the control animals (data not shown). Depletion of CD8⁺ T cells completely reversed the observed protective effects of CD137L mAb on AHR, eosinophilic lung inflammation and IgE serum levels (Fig. 4a–c) similar to anti-IFN- γ mAb treatment. CD8 T cell depletion also abrogated the increased IFN- γ production of spleen cells isolated from OVA-immunized mice that had received anti-CD137L mAb (Fig. 4d), indicating indirectly that CD8⁺ T cells are the main source of IFN- γ .

Our data demonstrate a crucial role of IFN- γ -producing CD8⁺ T cells in the anti-CD137L mAb-mediated effects.

Anti-CD137L mAb-mediated increase of IFN- γ is independent of IL-12

CD137L has been detected on mature DCs, activated macrophages and activated B cells [4]. It has been shown that macrophage/DC-derived IL-12 or IL-23 may induce Th1 cells to produce IFN- γ [29, 30]. To investigate the role of IL-12 in mediating the protective effect of CD137L mAb, we treated IL-12p40^{-/-} mice with anti-CD137L mAb in our murine asthma model. OVA immunization resulted in an asthma-like phenotype in IL-12p40^{-/-} mice that was comparable with the one in wild type (WT) animals. Moreover, the protective effect of anti-CD137L mAb was comparable in IL-12p40^{-/-} and BALB/c mice (Fig. 5a–c), demonstrating that the anti-CD137L-mediated increase of IFN- γ is independent of

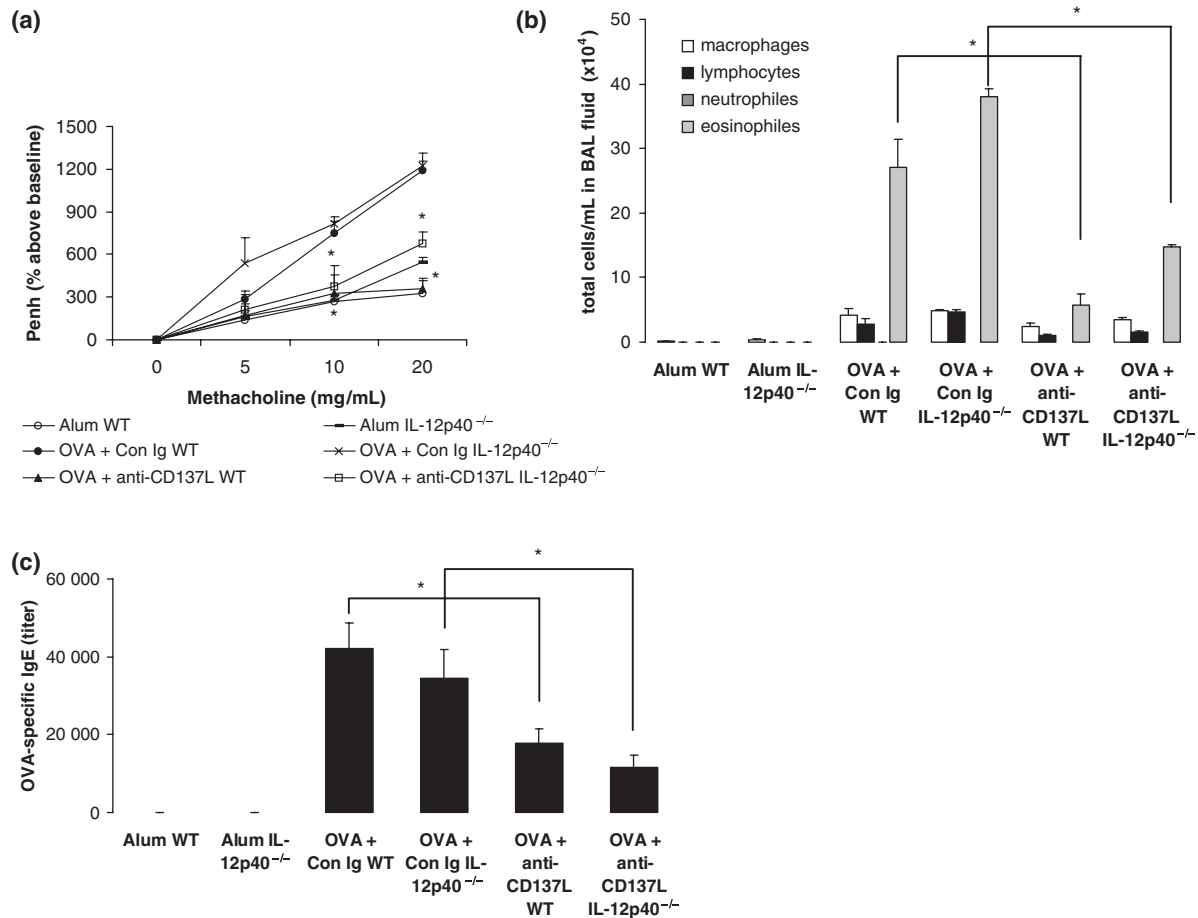


Fig. 5. Anti-CD137 ligand (CD137L) monoclonal antibody (mAb)-mediated increase of IFN- γ is independent of IL-12. IL-12p40^{-/-} mice were immunized with OVA on days 1 and 14, followed by several intranasal ovalbumin (OVA) challenges as described in 'Materials and methods'. Anti-CD137L mAb or isotype-matched control mAb were injected the day before OVA immunization. (a) Anti-CD137L mAb inhibits the airway hyperreactivity (AHR) in OVA-immunized IL-12p40^{-/-} mice measured on day 24. (b) Application of anti-CD137L mAb before immunization significantly reduces the total cell number in bronchoalveolar lavage (BAL) fluid of OVA-immunized IL-12p40^{-/-} mice. BAL was performed 1 day after measurement of AHR (day 25). (c) Anti-CD137L mAb significantly reduces OVA-specific IgE serum levels in OVA-immunized mice. * $P < 0.05$, OVA+anti-CD137L mAb vs. OVA+control Ab by Student's t -test. (a) and (b) $n \geq 9$ animals per group and data point; data are expressed as mean $\pm$ SEM from three independent experiments. (c) $n \geq 4$ animals for each group, here, representative results from one of three experiments are shown.

IL-12. To further verify these results, we investigated the cytokine production by DCs after *in vitro* treatment with anti-CD137L mAb. Bone marrow DCs were stimulated with OVA (100 μ g) or lipopolysaccharides (LPS) as control (1 μ g/mL) overnight and then treated with anti-CD137L mAb (10 μ g) for another 24 h. DCs were analysed by FACS for CD11c and MHC II expression and cytokine levels were measured in the supernatant. In contrast to stimulation with LPS, which strongly increased IL-12 and IL-23 cytokine levels, anti-CD137L mAb did not induce IL-12 or IL-23 production from mature DCs (data not shown).

Taken together, these results suggest that the protective effect of anti-CD137L mAb is independent of the release of IL-12 by macrophages/DCs.

Anti-CD137L mAb directly ligates to CD8⁺ T cells and induces IFN- γ production

It has been reported that CD137L was expressed on activated murine and human T cells [31, 32]. To investigate whether anti-CD137L mAb directly ligates to CD8⁺ T cells, we separated CD8⁺ T cells from BALB/c splenocytes and stimulated them with anti-CD3 mAb/anti-CD28 mAb for 3 days. Cells were then incubated with anti-CD137L mAb or control mAb and afterwards stained with PE-labelled anti-rat IgG. As shown in Fig. 6a, anti-CD137L specifically binds to CD8⁺ T cells. Moreover, pre-incubation of isolated CD8⁺ T cells with anti-CD137L mAb, followed by stimulation with anti-CD3/anti-CD28 mAbs significantly increased the IFN- γ production. This effect was

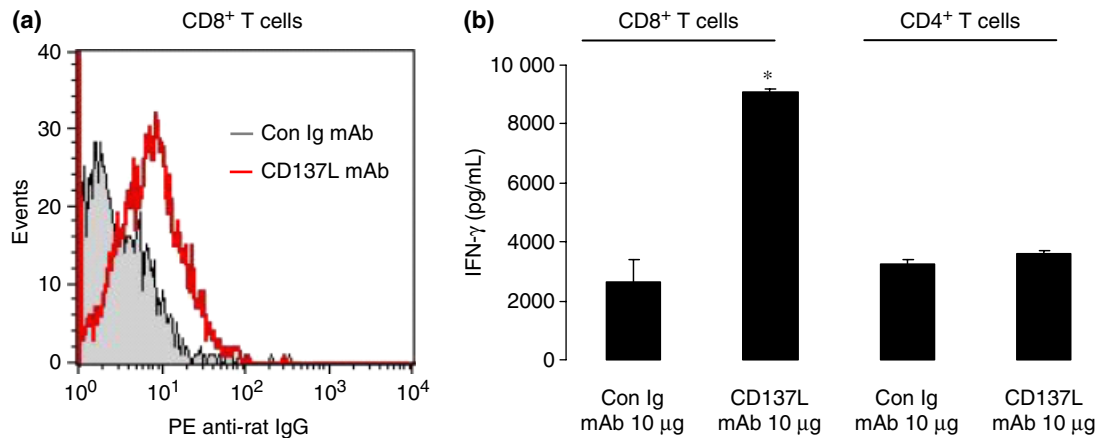


Fig. 6. Anti-CD137 ligand (CD137L) monoclonal antibody (mAb) directly ligates to CD8⁺ T cells and induces IFN- γ production. BALB/c mice were immunized as described in 'Materials and methods'. On day 7, splenocytes were isolated and CD4⁺ and CD8⁺ T cells were separated and stimulated with anti-CD3 mAb/anti-CD28 mAb. Three days later, cells were incubated with anti-CD137L mAb and stained with PE-labelled anti-rat IgG. For cytokine measurement, CD4⁺ and CD8⁺ T cells were given to anti-CD137L mAb-pre-coated plates and stimulated for 3 days. (a) Anti-CD137L mAb ligates to CD8⁺ T cells. (b) Anti-CD137L mAb induces IFN- γ production in CD8⁺ T cells. * $P < 0.05$, anti-CD137L mAb vs. control Ab by Student's t -test. Data are expressed as mean $\pm$ SEM; $n \geq 4$ animals for each group, representative results from one of three experiments are shown.

not seen when CD4⁺ T cells or control mAb were used (Fig. 6b).

Discussion

In the present study, we investigated the role of CD137 signalling in allergic asthma by stimulating the ligand of the CD137/CD137L co-stimulatory pathway by an anti-CD137L mAb. Our results show that injection of anti-CD137L mAb before OVA priming of BALB/c mice prevented the development of an asthma-like phenotype during the complete observation period of almost 7 weeks. This effect was associated with an increase of IFN- γ and a decrease of Th2 cytokines and was completely abolished by inhibition of IFN- γ and depletion of CD8⁺ T cells.

Previous studies using an agonistic anti-CD137 mAb have shown before *in vitro* [22] as well as *in vivo* in a murine model of autoimmune disease [18] and rheumatoid arthritis [20] that IFN- γ plays a crucial role in mediating the protective effects of CD137. However, in our own previous study in an allergic asthma model, the induction of IFN- γ by a mAb targeting the CD137 receptor was only partly responsible for the observed protective and therapeutic effects. In addition, anergy of CD4⁺ T cell was crucial for the CD137 mAb-mediated response [23]. In contrast, blocking of IFN- γ in the present system, where the CD137L and not its receptor was targeted, completely abolished the preventative effect of anti-CD137L mAb on the development of an asthma-like phenotype. This suggests that here, IFN- γ is completely responsible for the anti-CD137L mAb-mediated effects. Our results indirectly implicate that the induction of T cell anergy by stimulation of the CD137 receptor is crucial for reversing an established asthma phenotype because (a) this mechanism

is not observed when the CD137L is targeted and (b) targeting the CD137L does not lead to inhibition of established asthma.

To identify the source of IFN- γ that was induced by anti-CD137L mAb application, we depleted CD8⁺ T cells. In our previous study, where we targeted the CD137 receptor, depletion of CD8⁺ T cells was not crucial for the preventative effect of anti-CD137 mAb (unpublished data). In contrast, in the current study with anti-CD137L mAb, depletion of CD8⁺ T cells completely abrogated the protective effect of CD137L mAb. This phenomenon was comparable with the abrogation of the CD137L mAb effect that we observed when IFN- γ was blocked. Furthermore, the elevated IFN- γ levels in spleen cell culture supernatants restimulated with OVA were almost completely abolished, indicating that CD8⁺ T cells are the source for IFN- γ . Our indirect finding was directly supported by intracellular FACS analysis revealing an almost twofold increase of IFN- γ produced by CD8⁺ T cells, but not by CD4⁺ T cells. Different from other studies [18, 22], where agonistic anti-CD137 mAb drastically increased the number of CD8⁺ T cells, the administration of a mAb against the CD137L did not change the number or the percentage of CD8⁺ T cells, demonstrating again that targeting the CD137L induces different effects from targeting the CD137 receptor.

Seo et al. [20] recently showed in a rheumatoid arthritis model that IFN- γ suppressed CD4⁺ T cells through an indoleamine 2,3-dioxygenase (IDO)-dependent mechanism. These authors further observed that stimulation of the receptor with anti-CD137 mAb expands regulatory CD11c⁺CD8⁺ cells expressing IFN- γ . However, in our system we could neither detect an increase of IDO nor of CD11c⁺CD8⁺ cells after stimulation of the ligand with

anti-CD137L mAb in the spleen or lung (data not shown), suggesting a different mechanism. Moreover, in the current study, the inhibitory effect of anti-CD137L mAb on Th2 cytokines was detected in the lung but – in contrast to our previous study with an antibody against the receptor [23] – not in the supernatants of splenocyte cell cultures whereas the Th1 cytokine IFN- γ was strongly induced in the spleen but not in the lung. Therefore, we speculate, similar to Marsland et al. [33], that IFN- γ might inhibit the migration of allergen-specific CD4⁺ T cells to the mediastinal lymph nodes and to the lung as seen by a reduced number of infiltrating lymphocytes in the BAL, accompanied by clearly decreased Th2 cytokine production.

We performed further experiments to analyse the mechanism by which stimulation of CD137L resulted in an increase of IFN- γ production by CD8⁺ T cells. First, we investigated the involvement of antigen-presenting cells expressing CD137L [4]. It has been shown that IL-12, generated by e.g. DCs or macrophages, induces IFN- γ production by T cells [30] and furthermore, that stimulation of CD137L induces the production of IL-12 by DCs [34]. In our murine asthma model, anti-CD137L mAb administration to IL-12p40^{-/-} mice reduced the asthma phenotype similar to WT mice, suggesting an IL-12-independent pathway. This is supported by *in vitro* incubation of DCs with anti-CD137L mAb after stimulation of DCs with OVA. CD137L mAb did not induce the production of IL-12 or the cytokine family member IL-23. However, we could see an increase of IL-10 levels after treatment with anti-CD137L mAb, supporting stimulation of DCs by anti-CD137L mAb (data not shown). We are currently investigating the role of this cytokine in CD137L-driven effects.

Because DCs seem not to be critically involved in the anti-CD137L mAb-mediated induction of IFN- γ -producing CD8⁺ T cells, we investigated the possibility of direct engagement of anti-CD137L mAb to CD8⁺ T cells. Different studies have shown before that CD137L was expressed on activated T cells [31]. Our present results demonstrate that anti-CD137L not only binds directly to isolated CD8⁺ T cells but also induces the production of IFN- γ from these cells.

While in our model, IFN- γ was crucial in mediating the preventative effects of CD137L mAb, the role of IFN- γ in the pathogenesis of allergic asthma in general seems to be complex. On the one hand, several studies have shown that this Th1 cytokine is able to counter-regulate Th2-dominated immune responses like allergic asthma [35–39]. In accordance, T cells from airways of patients with asthma showed reduced expression of the Th1 transcription factor T-bet in comparison with T cells from airways of non-asthmatic patients and mice with a targeted deletion of the T-bet gene spontaneously developed multiple physiological and inflammatory features characteristic of asthma [40].

On the other hand, several studies, including our own previous work [27], indicate pro-inflammatory contributions of IFN- γ to chronic asthma. IFN- γ levels were increased in the sputum of asthmatic patients compared with healthy controls [41]; transfer of IFN- γ -producing antigen-specific T cells into SCID mice did not inhibit but – in contrast – worsened the pro-inflammatory effect of transferred Th2 cells [27], and local expression of IFN- γ in the lung increased markers of allergic airway inflammation, but decreased airway hyper-responsiveness in a murine model of allergic asthma [42]. These data demonstrate that the role of IFN- γ in allergic asthma is not always beneficial as in the context presented here but may also be harmful.

In conclusion, the findings reported here show that application of an anti-CD137L mAb prevents the development of a Th2-biased disease like allergic asthma in our murine model. In contrast to stimulating the CD137 receptor, targeting the CD137L does not inhibit established disease and is thereby inferior to CD137 mAb. However, it may be possible that it could facilitate current standard immune therapy. Moreover, both strategies seem to follow different mechanisms, with CD137 mAb functioning via IFN- γ induction from CD8⁺ T cells and CD4⁺ T cell anergy while the CD137L mAb seems to operate completely via IFN- γ -producing CD8⁺ T cells.

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