Published in final edited form as:

Clin Exp Allergy. 2016 February 1; 46(2): 354–364. doi:10.1111/cea.12661.

Anti-OX40L alone or in combination with anti-CD40L and CTLA4Ig does not inhibit the humoral and cellular response to a major grass pollen allergen

M. Gattringer^{#1}, U. Baranyi^{#1}, N. Pilat¹, K. Hock¹, C. Klaus¹, H. E. Ramsey¹, F. Wrba², R. Valenta³, and T. Wekerle¹

¹Department of Surgery, Division of Transplantation, Medical University of Vienna, Vienna, Austria

²Institute of Clinical Pathology, Medical University of Vienna, Vienna, Austria

³Department of Pathophysiology and Allergy Research, Division of Immunopathology, Center of Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Vienna, Austria

[#] These authors contributed equally to this work.

Summary

Background—IgE-mediated allergy is a common disease characterized by a harmful immune response towards otherwise harmless environmental antigens. Induction of specific immunological non-responsiveness towards allergens would be a desirable goal. Blockade of costimulatory pathways is a promising strategy to modulate the immune response in an antigen-specific manner. Recently, OX40 (CD134) was identified as a costimulatory receptor important in Th2-mediated immune responses. Moreover, synergy between OX40 blockade and ‘conventional’ costimulation blockade (anti-CD40L, CTLA4Ig) was observed in models of alloimmunity.

Objective—We investigated the potential of interfering with OX40 alone or in combination with CD40/CD28 signals to influence the allergic immune response.

Methods—The OX40 pathway was investigated in an established murine model of IgE-mediated allergy where BALB/c mice are repeatedly immunized with the clinically relevant grass pollen allergen Phl p 5. Groups were treated with combinations of anti-OX40L, CTLA4Ig and anti-CD40L. In selected mice, Tregs were depleted with anti-CD25.

Results—Blockade of OX40L alone at the time of first or second immunization did not modulate the allergic response on the humoral or effector cell levels but slightly on T cell responses. Administration of a combination of anti-CD40L/CTLA4Ig delayed the allergic immune response, but antibody production could not be inhibited after repeated immunization even though the allergen-specific T cell response was suppressed in the long run. Notably, additional blockade of OX40L had no detectable supplementary effect. Immunomodulation partly involved regulatory T cells as depletion of CD25⁺ cells led to restored T cell proliferation.

Correspondence: T. Wekerle, Department of Surgery, Division of Transplantation, Vienna General Hospital, Waehringer Guertel 18; 1090 Vienna, Austria. thomas.wekerle@meduniwien.ac.at.

Conflict of interest

The authors declare no financial or commercial conflict of interests.

Conclusions and Clinical Relevance—Collectively, our data provide evidence that the allergic immune response towards Phl p 5 is independent of OX40L, although reduction on T cell responses and slightly on the asthmatic phenotype was detectable. Besides, no relevant synergistic effect of OX40L blockade in addition to CD40L/CD28 blockade could be detected. Thus, the therapeutic potential of OX40L blockade for IgE-mediated allergy appears to be ineffective in this setting.

Keywords

allergy; costimulation; costimulation blockade; OX40L; suppression

Introduction

IgE-mediated allergy is a hypersensitivity disorder whose prevalence is still increasing [1]. The main feature of type I allergy is the development of IgE which bound to mast cells leads to the release of cytokines and vasoactive substances upon crosslinking by an allergen [2, 3]. A desirable but still unmet goal would be the prophylactic induction of immunological non-reactivity towards allergens.

Costimulatory molecules are a promising therapeutic target as their engagement is required for a productive immune response. Numerous costimulation pathways have been identified building a complex network modulating both T cell activation and T cell inhibition [4, 5]. CD28 is arguably the most important costimulation receptor for the activation of naïve T cells [6, 7] while CD40 regulates APC activation and is critical for isotype switching of B cells [8, 9]. Combined blockade of CD28 and CD40 has synergistic effects in numerous experimental models [10].

OX40 (CD134) is expressed on activated T cells (including regulatory T cells, i.e., Tregs) and its only ligand OX40L (CD134L) mainly on APC. The OX40/OX40L pathway plays a role both in T cell and B cell responses [11–13]. In experimental transplantation models blocking the OX40 pathway was shown to delay allograft rejection in a TCR transgenic model [14]. Moreover anti-OX40L prolongs graft survival in CD28/CD40L double knock out recipients [15] and synergizes with conventional costimulation blockade [16]. Notably, OX40 modulates particular memory T cell responses and is of value in costimulation blockade-resistant allograft rejection mediated by memory T cells [17]. An important role of OX40 for Tregs has emerged where it decreases FoxP3 expression and inhibits suppressor function [18]. Recently, exhaustion of OX40 expanded Tregs was shown to be caused by IL2 deficiency [19]. In contrast to its co-*stimulatory* function in effector T cells, the OX40 pathway has a co-*inhibitory* function in Tregs. Thus, OX40 signals promote effector cells and inhibit Tregs.

OX40 (CD134) prominently participates in Th2-mediated immune responses [20, 21]. Stuber and Strober observed decreased production of IgG1, IgG2a, IgG2b and IgG3 when anti-OX40 antibodies were administered together with TNP-KHL immunization, provoking a T cell-dependent immune response. T cell-*independent* immune responses were not influenced [12]. In OVA-induced lung inflammation, blockade of OX40L was able to attenuate disease with suppressed production of IgE, IgG and decreased Th2 cytokines after

anti-OX40L treatment [22]. Moreover, anti-OX40L therapy is currently under clinical development [23].

Previously, we assessed ‘conventional’ blockade of CD28 and CD40L during allergic sensitization and ongoing disease in a murine model of IgE-mediated allergy. Blocking CD40L (alone or together with CTLA4Ig) prevented allergic sensitization when given at the time of first allergen encounter, but the effect lasted only until re-exposure to the same allergen. In contrast, no influence was observed when anti-CD40L was administered to already sensitized mice at the time of second allergen encounter. Blocking CD28 (with CTLA4Ig) when used alone early or late had no detectable influence on the humoral response but only diminished allergen-specific T cell proliferation [24]. Thus, while ‘conventional’ costimulation blockade prevents sensitization when given at the time of allergen exposure, it does not lead to lasting tolerance withstanding repeated allergen encounters.

Therefore, the aim of this study was to investigate whether blocking OX40 alone or in addition to ‘conventional’ costimulation blockade tolerizes an IgE-mediated allergic response. We found that OX40 is not required for an IgE-mediated allergic response towards Phl p 5, a major timothy grass pollen allergen. Neither blockade of OX40L alone nor on top of ‘conventional’ costimulation blockade led to lasting tolerance.

Materials and methods

Animals

Female BALB/c mice were obtained from Charles River (Sulzfeld, Germany). All mice were housed under specific pathogen-free conditions and were used between 6 and 12 weeks of age. All experiments were approved by the local review board of the Medical University of Vienna and approved by the Austrian Federal Ministry of Science, Research and Economy, BMWF (GZ:BMWF-66.009/0295-II/3b/2011), and were performed in accordance with national and international guidelines of laboratory animal care.

Recombinant allergens and immunization

Purified recombinant (r) timothy grass pollen allergen (rPhl p 5) and birch pollen allergen (rBet v 1) were obtained from Biomay (Vienna, Austria). Mice were immunized subcutaneously with 5 µg rPhl p 5 (major grass pollen allergen) and in selected groups with 5 µg rBet v 1 (major birch pollen allergen), adsorbed to Al(OH)₃ (Alu-Gel-S, Serva, Ingelheim, Germany). Immunization started at day 0 and was repeated every 3 weeks (days 0, 21, 42, 63 and 84 as indicated in Table 1) till the end of follow-up.

Costimulation blockade

Treatment with anti-CD40L (MR1, 1 mg/mouse) and human CTLA4Ig (abatacept, 0.5 mg/mouse) was given by intraperitoneal (i.p.) injection early (days 0, 2 and 4) or late (days 21, 23 and 25) [24]. OX40L was blocked by i.p. injection of the anti-OX40L mAb RM134 (0.5 mg/mouse) early (days 0, 2, 4 and 8) or late (days 21, 23, 25 and 29) [15]. For depletion of CD25⁺ cells, a cytotoxic anti-CD25 mAb (PC61) was given i.p. at days 0 (0.5 mg/mouse), 4

and 8 (0.25 mg/mouse) or days 21, 25 and 29. In combination with anti-CD40L/CTLA4Ig/anti-OX40L treatment, CD25⁺ cells were depleted at day 16 (PC61, 1 mg/mouse). Anti-CD40L, anti-CD25 and anti-OX40L were purchased from BioXCell (West Lebanon, NH, USA); hCTLA4Ig (abatacept) was generously provided by Bristol-Myers, Squibb Pharmaceuticals (Princeton, NJ, USA).

Flow cytometry and antibodies

For analysis of Tregs, mAbs with specificity against CD4 (RM4-4) and CD25 (7D4, non-cross-reactive with PC61) were used. Antibodies were conjugated to FITC and PE and detected in FL1 and FL2. Surface staining was performed according to standard procedures, and flow cytometric analysis was performed on a Coulter Cytomics FC500. CXP software (Coulter, Vienna, Austria) was used for acquisition and analysis.

ELISA

To measure antigen-specific antibodies in the sera of immunized mice, ELISAs were performed as described previously [24]. Blood samples were taken from the tail vein, and serum was stored at – 20 °C until analysis. Plates were coated with rBet v 1 or rPhl p 5 (5 µg/mL); sera were diluted 1 : 20 for IgE, 1 : 100 for IgM, IgA, IgG2a, IgG3 and 1 : 500 for IgG1; and bound antibodies were detected with monoclonal rat anti-mouse IgE, IgG1, IgG2a, IgG3, IgA and IgM antibodies (BD Pharmingen, San Jose, CA, USA) diluted 1 : 1000 and a HRP-coupled goat anti-rat antiserum (Biosciences, Cambridge, UK) diluted 1 : 2000. The substrate for HRP was ABTS (60 mM/L citric acid, 77 mM/L Na₂HPO₄ × 2H₂O, 1.7 mM/L ABTS (Sigma-Aldrich, MO, St. Lois, USA), 3 mM/L H₂O₂). Absorbance was measured at 405 nm (0.1 s) and 490 nm (0.1 s). For calculation, values obtained at 490 nm were abstracted from 405 nm values.

Lymphocyte proliferation assay

Spleens were removed under sterile conditions and homogenized. Single-cell suspension was filtered through a 70 µm Nylon cell strainer to remove remaining tissue. Erythrocytes were removed by adding cold lysing buffer (Red Blood Cell Lysing Buffer, Sigma-Aldrich). Cells were diluted to a final concentration of 5 × 10⁵ cells/well; 2 µg/well allergen stimulant was added, and 0.5 µg/well concanavalin A (Sigma-Aldrich) was added as control. The 96-well plates were incubated at 37 °C, 5% CO₂. On day 4, 0.5 µCi H³ thymidine ([methyl-3H] thymidine, Amersham, UK) per well was added. Sixteen hours later, cells were harvested and thymidine uptake was measured in a beta counter (Beta scintillation liquid, Wallac) [24].

Rat basophile leukaemia (RBL) cell degranulation assay

RBL-2H3 cell subline was cultured as described previously [25] in RPMI 1640 medium (Biochrome AG, Berlin, Germany) containing 10% foetal calf serum. 6 × 10⁴ cells were plated in 96-well tissue culture plates (Greiner, Bio-One frickenhaus, Germany), loaded with 1 : 30 diluted mouse sera and incubated for 2 h at 37 °C and 5% CO₂. Supernatants were removed and the cell layer was washed 2× with Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 0.5 mM MgCl₂, 1.8 mM CaCl₂, 0.4 mM NaH₂PO₄, 5.6 mM D-glucose, 12 mM NaHCO₃, 10 mM HEPES and 0.1% w/v BSA, pH 7.2). Preloaded cells were stimulated with rPhl p 5

or rBet v 1 (0.03 µg per well) for 30 min at 37 °C. The supernatants were analysed for β-hexosaminidase activity by incubation with the substrate 80 µM 4-methylumbelliferyl-N-acetyl-β-D-glucosamide (Sigma-Aldrich) in citrate buffer (0.1 M, pH 4.5) for 1 h at 37 °C. The reaction was stopped by addition of 100 µL glycine buffer (0.2 M glycine, 0.2 M NaCl, pH 10.7), and the fluorescence was measured at λ_{ex} : 360/ λ_{em} : 465 nm using a fluorescence microplate reader (Wallac, Perkin Elmer, Vienna, Austria). Results are reported as percentage of total β-hexosaminidase released after the addition of 1% Triton X-100. Determinations were performed in triplicates.

Airway hyperresponsiveness

Mice were treated with OX40L and immunized with r Phl p 5 like described above. Additionally, mice were treated intranasally (10 µg r Phl p 5 per dose/mouse) at days 46, 47 and 48, and whole body plethysmography (WBP) (Buxco Research Systems, Wilmington, NYC, USA) was performed. Mice were challenged with different concentrations of methacholine (8, 16, 32 and 64 mg/mL) (Sigma-Aldrich Darmstadt, Germany) and PBS as baseline. After methacholine provocation, mice were killed and lungs were stored in 4.5% phosphate-buffered formaldehyde. Lung sections were stained for hematoxylin and eosin (H&E) and periodic acid Schiff stain (PAS).

Statistical analysis

Means, standard deviations (SD) and standard error of the mean (SEM) were calculated for statistical documentation. The reported *P*-values are results of the Mann–Whitney *U*-test.

Values of *P* < 0.05 were considered as statistically significant. GraphPad prism statistical software (version 5.01) (Graph pad, la Jolla, CA, USA) was used for statistical calculations. For box blots, the median and interquartile range in the box with min and max range between bars is shown.

Results

Blockade of OX40L has no relevant effect on the humoral and cellular response towards Phl p 5

To investigate the role of OX40, a well-characterized model of IgE-mediated allergy was employed in which BALB/c mice are repeatedly immunized with recombinant Phl p 5 (plus aluminium hydroxide; on days 0 and 21) (Table 1: group A, untreated control group). Groups of mice (*n* = 6/group) received anti-OX40L mAb early, at the time of first immunization (group B, anti-Ox40L early) or late, at the time of second immunization (group C, anti-Ox40L late). Consistent with previous reports [24], untreated immunized mice (control group) produced high levels of allergen-specific IgE, IgG1, IgG2a, IgG3, IgA and IgM (Fig. 1a–f). Treatment with anti-OX40L early or late had no detectable effect on the levels of allergen-specific antibody production (Fig. 1a–f). The impact of anti-OX40L treatment on effector cell function was assessed in RBL cell degranulation assays. Anti-OX40L treatment did not significantly reduce mediator release in comparison with untreated controls (Fig. 1g). *In vitro* T cell proliferation towards Phl p 5 was also not significantly reduced in mice treated with anti-OX40L early or late although T cell responses were

modestly diminished (Fig. 1h). Additionally, the asthmatic phenotype (as assessed by whole body plethysmography and histology) was slightly but not significantly diminished in mice after early or late treatment with OX40L (Fig. S1). Thus, blockade of OX40L does not significantly alter the primary or secondary immune response towards Phl p 5.

Blockade of OX40L has no relevant additive effect when combined with anti-CD40L and CTLA4Ig

As blocking OX40L has been reported to act synergistically with CD40 and CD28 blockade in several models [11, 15, 26], we investigated the effect of blocking OX40L by treating groups of mice before immunization with anti-CD40L/CTLA4Ig (group D) with or without additional anti-OX40L (group E). Mice receiving anti-CD40L/CTLA4Ig showed significantly delayed allergen-specific antibody production compared to the control mice (as previously shown [24]) with allergen-specific IgE levels becoming detectable after the third immunization (by day 63, Fig. 2a). Other Ig isotypes (IgG1, IgG2a, IgG3, IgA and IgM) were also significantly suppressed for at least that long (Fig. 2b–f) IgG2a and IgG3 at least until day 84 after immunization in case of IgG2a and IgG3 even longer (day 84, Fig. 2c–d). Unexpectedly, additional treatment with anti-OX40L had no detectable effect on the course or levels of allergen-specific antibody production. Likewise, no significant difference was observed in RBL assays (Fig. 2g) and in T cell proliferation assays (Fig. 2h) with or without additional anti-OX40L.

As anti-OX40L is effective in modulating a primed response in models of allotransplantation [16], we tested the effect of anti-OX40L in addition to anti-CD40L/CTLA4Ig (late) in an already primed allergic response (group F, Table 1). Allergen-specific IgG1, IgG2a, IgG3 and IgM levels stayed constant after second immunization and late treatment (d21 vs. d42) (Fig. 3b, c, d and f). Further Phl p 5-specific IgG1 and IgG3 were significantly reduced compared to untreated immunized mice on d42 suggesting a slight effect on secondary Ab responses. IgE and IgA were unaffected by late administration of anti-CD40L/CTLA4Ig/anti-OX40L (Fig 3). Phl p 5-specific T cell proliferation was substantially (albeit not significantly reduced) after treatment with combined antibodies in contrast to untreated immunized mice (Fig. 3g).

Hence, treatment with anti-OX40L administered as preventive or curative protocol at the time of first or second immunization had no detectable effect on the transient antigen-specific non-responsiveness achieved with anti-CD40L/CTLA4Ig, although the initial tolerizing effect was prolonged in some isotypes.

To investigate the possibility that therapeutic levels of anti-CD40L/CTLA4Ig/anti-OX40L administered at the time of first immunization persist at the time of repeated immunization and contribute to the observed immunomodulation, anti-CD40L/CTLA4Ig/anti-OX40L were injected into naïve mice 3 weeks (i.e. day -21) before the first immunization with rPhl p 5 (day 0) (group G). Anti-Phl p 5 IgE production was absent on day 21 but high on day 42 following the second immunization, while it emerged only on day 63 following the third immunization when antibodies were injected at day 0. Thus, persisting anti-CD40L/CTLA4Ig/anti-OX40L treatment indeed delayed humoral responses, with anti-Phl p 5 IgE levels becoming detectable 63 days after anti-CD40L/CTLA4Ig/anti-OX40L administration

with both treatment regimens (Fig. 4a). Critically, however, T cell proliferation in splenocytes was only suppressed when mice received anti-CD40L/CTLA4Ig/anti-OX40L at the time of first immunization indicating that higher drug levels are necessary to induce a lasting T cell immunomodulatory effect (Fig. 4b).

CD25-positive cells partly mediate costimulation blockade-induced suppression

Our results demonstrate that blocking CD40L, CD28 and OX40L results in transient non-responsiveness towards Phl p 5. As, in allotransplant models, blocking CD40L acts in part through the induction of Tregs [27], we tested whether regulation is a mechanism of allergen-specific immune modulation induced by combined costimulation blockade. First, we assessed whether Tregs attenuate the immune response to Phl p 5 in naïve untreated mice by depleting Tregs at the time of first or second immunization (group I and J). Neither early nor late Treg depletion altered allergen-specific IgE levels (Fig. 5a) despite effective depletion of Tregs as verified by flow cytometric analysis (Fig. 5b). Therefore, effector cells are not substantially eliminated by CD25-depleting antibodies. Next, we depleted Tregs shortly before the second immunization (day 16) of mice treated with anti-CD40L/CTLA4Ig/anti-OX40L at the time of first immunization (group H). Depletion of Tregs partly abrogated the immunomodulatory effect of anti-CD40L/CTLA4Ig/anti-OX40L leading to allergen-specific IgE levels as high as in untreated controls at day 63 (Fig. 5c). The effect was most pronounced with IgE and IgG1, with the other isotypes showing a similar trend but no significant difference (data not shown). Likewise, T cell hyporesponsiveness was abrogated through Treg depletion in mice treated with anti-CD40L/CTLA4Ig/anti-OX40L (Fig. 5d). Thus, Tregs play a role in modulating the allergen-specific immune response upon treatment with costimulation blockers but do not suppress the humoral immune response in untreated immunized mice.

It has been proposed that while Tregs are activated in an antigen-specific manner, their suppressive function is antigen non-specific in some settings [28]. Therefore, we evaluated whether Tregs activated with one allergen (in the presence of costimulation blockade) suppress the immune response towards another unrelated allergen (in our experimental setting Bet v 1, a major birch pollen allergen). Mice were treated with anti-CD40L/CTLA4Ig/anti-OX40L at the time of immunization with Phl p 5. Three weeks later, mice were immunized with both Phl p 5 and Bet v 1 (group L). Double immunized control mice (group K) developed Bet v 1-specific IgE after the first immunization. Notably, IgE levels of immunized mice treated with anti-CD40L/CTLA4Ig/anti-OX40L exhibited a delayed immune response not only towards Phl p 5 but also towards Bet v 1. Bet v 1-specific IgE levels were significantly suppressed till day 63 (Fig. 5e). Based on data shown in Fig. 4a, suppressed Bet v 1-specific IgE production could be partly due to persisting anti-CD40L/CTLA4Ig/anti-OX40L levels. However, the effect was slightly prolonged in double-immunized mice as IgE levels were still reduced on day 63 and were fully restored only on day 84 (instead of day 63 in Fig. 4b) (Fig. 5e). Likewise, Bet v 1-specific β -hexosaminidase release by RBL cells (Fig. 5f) was diminished in anti-CD40L/CTLA4Ig/anti-OX40L treated mice, as was Bet v 1-specific T cell proliferation (Fig. 5g). As costimulation blocking treatment 21 days prior to immunization was not able to inhibit the T cell response (Fig. 4b), these results provide evidence that the T cell regulation induced at the time of immunization

with Phl p 5 was in part antigen non-specific. Indeed, this immunomodulatory effect on T cell reactivity was partly abrogated when Tregs were depleted at the time of Bet v 1 immunization (Fig. 5g).

Collectively, these results demonstrate that Tregs induced by anti-CD40L/CTLA4Ig (+/- anti-OX40L) treatment modulate the allergic T cell response towards Phl p 5 and that part of the regulatory activity is not antigen specific.

Discussion

The OX40-OX40L costimulatory pathway is critically involved in regulating Th2 responses and is hence investigated as potential pharmacological target for the treatment of IgE-mediated allergy [29]. Blocking OX40L potentially inhibited allergic lung inflammation in mice and non-human primates [22, 30]. Consequently, anti-OX40L has recently been evaluated in a clinical phase II trial for allergen-induced airway obstruction in adults with mild asthma (NCT00983658). Short course treatment with an anti-OX40L mAb (oxelumab) had no clinical effect on allergen-induced airway responses [23]. Total IgE levels were reduced significantly at one time point, but allergen-specific IgE was only slightly decreased. It is presently unclear whether modifications of dose and duration of anti-OX40L treatment could lead to a clinical benefit or whether anti-OX40L is clinically ineffective in treating IgE-mediated allergy.

Here, we investigated the effect of OX40: OX40L blockade alone or in combination with targeting the CD40 and CD28 pathways in a well-defined murine model of IgE-mediated allergy. Our results reveal that OX40L blockade did not significantly inhibit the allergen-specific humoral response in either a preventive or a curative approach. Besides, it reduced the allergen-specific T cell response only modestly. Nevertheless, for studying allergen-specific IgE levels in mice, sensitization with alum is necessary for IgE induction although it does not completely reflect the natural situation in human.

In previous studies, reduced responses of lung inflammation after blockade of OX40L were detectable. In both studies, mice were sensitized with OVA and alum and subsequently mice were treated intranasally with either OVA inhalation or induced by thymic stromal lymphopoietin (TSLP). OVA-specific IgE levels were reduced after OX40L blockade even in pre-sensitized mice [22, 30]. In a rhesus monkey model, however, an anti-human OX40L mAb had no significant effect on the IgG response to house dust mite (HDM) [22]. An additional study revealed an effect of OX40L neutralization on memory rather than on primary responses in an airway inflammation model with OVA [31]. Differently to those studies, the asthmatic phenotype was not prevented or ameliorated in our model. The discrepancies between our study and the studies mentioned above might be due to the differences in study design between airway inflammation and subcutaneous application of a clinically relevant aeroallergen.

Adding anti-OX40L to anti-CD40L/CTLA4Ig therapy also had no detectable benefit. Anti-OX40L did not prevent the delayed development of allergen-specific antibodies in anti-CD40L/CTLA4Ig-treated mice, and thus, lasting tolerance was not achieved. This is in

contrast to models of alloreactivity, in which anti-OX40L acted synergistically with CD40/CD28 pathway blockade [15, 16]. These studies focused primarily, however, on the effect on T cell reactivity without investigating the humoral response. More importantly, the distinct characteristics of an immune response, including the pre-existing antigen-specific T cell precursor frequency, have been shown to determine the requirement for certain costimulatory signals and hence the susceptibility to their therapeutic blockade [32]. As such, the (murine) T cell and B cell responses towards a major grass pollen allergen seem not to depend on OX40/OX40L costimulatory signals and hence are resistant to their therapeutic modulation.

We also show that depletion of Tregs does not enhance the humoral response to Phl p 5 in untreated mice, suggesting that Tregs do not physiologically control the humoral immune response towards allergens in a significant manner. In contrast, the therapeutic effect of combined costimulation blockade (anti-CD40L/CTLA4Ig/anti-OX40L) on the cellular and humoral response towards an allergen seems to involve Tregs.

Induction of Tregs by anti-CD40L leads to a tolerogenic effect involving FoxP3 Tregs and 'infectious tolerance' in organ transplantation models [33, 34]. Although Bet v 1-specific primary responses were delayed after costimulation blockade, no effect on secondary allergen-specific responses was detectable. Interestingly, the Tregs induced in such a way act in an antigen non-specific manner raising the concern of potential side effects of such therapies.

Collectively, our study provides evidence that the naïve and primed cellular and humoral immune responses towards a major grass pollen allergen do not depend on the OX40/OX40L costimulatory pathway and cannot be inhibited by anti-OX40L. Thus, the therapeutic potential of OX40L blockade for IgE-mediated allergy appears to be limited in certain settings and needs to be evaluated carefully.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by the Austrian Science Fund (FWF F230 to T.W., P21989-B11 to U.B. and FWF, F4605 to R.V.) and a research grant from Biomay (to T.W.).

References

1. Holt PG, Thomas WR. Sensitization to airborne environmental allergens: unresolved issues. *Nat Immunol.* 2005; 6:957–60. [PubMed: 16177798]
2. Valenta R. The future of antigen-specific immunotherapy of allergy. *Nat Rev Immunol.* 2002; 2:446–53. [PubMed: 12093011]
3. Geha RS, Jabara HH, Brodeur SR. The regulation of immunoglobulin E class-switch recombination. *Nat Rev Immunol.* 2003; 3:721–32. [PubMed: 12949496]
4. Pilat N, Sayegh MH, Wekerle T. Costimulatory pathways in transplantation. *Semin Immunol.* 2011; 23:293–303. [PubMed: 21616680]

5. Ford ML, Adams AB, Pearson TC. Targeting co-stimulatory pathways: transplantation and autoimmunity. *Nat Rev.* 2014; 10:14–24.
6. Greenwald RJ, Freeman GJ, Sharpe AH. The B7 family revisited. *Annu Rev Immunol.* 2005; 23:515–48. [PubMed: 15771580]
7. Schwartz RH. A cell culture model for T lymphocyte clonal anergy. *Science (New York, NY).* 1990; 248:1349–56.
8. Quezada SA, Jarvinen LZ, Lind EF, Noelle RJ. CD40/CD154 interactions at the interface of tolerance and immunity. *Annu Rev Immunol.* 2004; 22:307–28. [PubMed: 15032580]
9. Elgueta R, Benson MJ, de Vries VC, Wasiuk A, Guo Y, Noelle RJ. Molecular mechanism and function of CD40/CD40L engagement in the immune system. *Immunol Rev.* 2009; 229:152–72. [PubMed: 19426221]
10. Larsen CP, Elwood ET, Alexander DZ, et al. Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways. *Nature.* 1996; 381:434–8. [PubMed: 8632801]
11. Rogers PR, Song J, Gramaglia I, Killeen N, Croft M. OX40 promotes Bcl-xL and Bcl-2 expression and is essential for long-term survival of CD4 T cells. *Immunity.* 2001; 15:445–55. [PubMed: 11567634]
12. Stuber E, Strober W. The T cell-B cell interaction via OX40-OX40L is necessary for the T cell-dependent humoral immune response. *J Exp Med.* 1996; 183:979–89. [PubMed: 8642301]
13. Kaur D, Brightling C. OX40/OX40 ligand interactions in T-cell regulation and asthma. *Chest.* 2012; 141:494–9. [PubMed: 22315115]
14. Kinnear G, Wood KJ, Marshall D, Jones ND. Anti-OX40 prevents effector T-cell accumulation and CD8+ T-cell mediated skin allograft rejection. *Transplantation.* 2010; 90:1265–71. [PubMed: 21037534]
15. Demirci G, Amanullah F, Kewalaramani R, et al. Critical role of OX40 in CD28 and CD154-independent rejection. *J Immunol.* 2004; 172:1691–8. [PubMed: 14734751]
16. Yuan X, Salama AD, Dong V, et al. The role of the CD134-CD134 ligand costimulatory pathway in alloimmune responses in vivo. *J Immunol.* 2003; 170:2949–55. [PubMed: 12626546]
17. Dawicki W, Bertram EM, Sharpe AH, Watts TH. 4-1BB and OX40 act independently to facilitate robust CD8 and CD4 recall responses. *J Immunol.* 2004; 173:5944–51. [PubMed: 15528328]
18. Vu MD, Xiao X, Gao W, et al. OX40 costimulation turns off Foxp3+ Tregs. *Blood.* 2007; 110:2501–10. [PubMed: 17575071]
19. Xiao X, Gong W, Demirci G, et al. New insights on OX40 in the control of T cell immunity and immune tolerance in vivo. *J Immunol.* 2012; 188:892–901. [PubMed: 22147766]
20. Jember AG, Zuberi R, Liu FT, Croft M. Development of allergic inflammation in a murine model of asthma is dependent on the costimulatory receptor OX40. *J Exp Med.* 2001; 193:387–92. [PubMed: 11157058]
21. Arestides RS, He H, Westlake RM, et al. Costimulatory molecule OX40L is critical for both Th1 and Th2 responses in allergic inflammation. *Eur J Immunol.* 2002; 32:2874–80. [PubMed: 12355440]
22. Seshasayee D, Lee WP, Zhou M, et al. In vivo blockade of OX40 ligand inhibits thymic stromal lymphopoietin driven atopic inflammation. *J Clin Invest.* 2007; 117:3868–78. [PubMed: 18060034]
23. Gauvreau GM, Boulet LP, Cockcroft DW, et al. OX40L blockade and allergen-induced airway responses in subjects with mild asthma. *Clin Exp Allergy.* 2014; 44:29–37. [PubMed: 24224471]
24. Linhart B, Bigenzahn S, Hartl A, et al. Costimulation blockade inhibits allergic sensitization but does not affect established allergy in a murine model of grass pollen allergy. *J Immunol.* 2007; 178:3924–31. [PubMed: 17339493]
25. Hoffmann A, Jamin A, Foetisch K, et al. Determination of the allergenic activity of birch pollen and apple prick test solutions by measurement of beta-hexosaminidase release from RBL-2H3 cells. Comparison with classical methods in allergen standardization. *Allergy.* 1999; 54:446–54. [PubMed: 10380775]
26. Chitnis T, Najafian N, Abdallah KA, et al. CD28-independent induction of experimental autoimmune encephalomyelitis. *J Clin Invest.* 2001; 107:575–83. [PubMed: 11238558]

27. Taylor PA, Noelle RJ, Blazar BR. CD4 (+)CD25(+) immune regulatory cells are required for induction of tolerance to alloantigen via costimulatory blockade. *J Exp Med*. 2001; 193:1311–8. [PubMed: 11390438]
28. Thornton AM, Shevach EM. Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen non-specific. *J Immunol*. 2000; 164:183–90. [PubMed: 10605010]
29. Wang YH, Liu YJ. OX40-OX40L interactions: a promising therapeutic target for allergic diseases? *J Clin Invest*. 2007; 117:3655–7. [PubMed: 18074001]
30. Salek-Ardakani S, Song J, Halteman BS, et al. OX40 (CD134) controls memory T helper 2 cells that drive lung inflammation. *J Exp Med*. 2003; 198:315–24. [PubMed: 12860930]
31. Hoshino A, Tanaka Y, Akiba H, et al. Critical role for OX40 ligand in the development of pathogenic Th2 cells in a murine model of asthma. *Eur J Immunol*. 2003; 33:861–9. [PubMed: 12672051]
32. Ford ML, Koehn BH, Wagener ME, et al. Antigen-specific precursor frequency impacts T cell proliferation, differentiation, and requirement for costimulation. *J Exp Med*. 2007; 204:299–309. [PubMed: 17261633]
33. Graca L, Honey K, Adams E, Cobbold SP, Waldmann H. Cutting edge: anti-CD154 therapeutic antibodies induce infectious transplantation tolerance. *J Immunol*. 2000; 165:4783–6. [PubMed: 11045999]
34. Ferrer IR, Wagener ME, Song M, Kirk AD, Larsen CP, Ford ML. Antigen-specific induced Foxp3+ regulatory T cells are generated following CD40/CD154 blockade. *Proc Natl Acad Sci USA*. 2011; 108:20701–6. [PubMed: 22143783]

Fig. 1.

Blockade of OX40L has no relevant effect on the allergen-specific response in an IgE-mediated allergy model. Allergen-specific antibody levels were analysed by ELISA (IgE, IgG1, IgG2a, IgG3, IgA and IgM) in sera of mice before treatment (pre-immune, d 0), 3 weeks after the first immunization (d 21) and 3 weeks after the second immunization (d 42). Treatment protocols are described in detail in Table 1. (a–f) Allergen-specific isotype levels are shown for immunized mice (group A, designated as control group), immunized mice with early anti-OX40L treatment (days 0, 2, 4, 8, group B) and immunized mice with late

anti-OX40L-treated mice (days 21, 23, 25 and 29, group C) ($n = 6/\text{group}$). Antibody levels are displayed as OD values in box-and-whisker plots. (g) Effector function was measured by allergen-specific β -hexosaminidase release of serum-loaded RBL cells in response to Phl p 5. Serum samples of day 0, 21 and 42 were tested and results are represented as box-and-whisker plot ($n = 6$). (h) Phl p 5-specific T cell reactivity was tested in a proliferation assay. Bars represent Phl p 5-specific stimulation indices (SI) of splenocytes as means of 6 mice per group with SEM (week 7). ns indicates not significant.

Fig. 2.

OX40L blockade together with anti-CD40L/CTLA4Ig treatment has no relevant additional effect. Allergen-specific antibody levels were analysed by ELISA (IgE, IgG1, IgG2a, IgG3, IgA and IgM) in serum samples collected in a span of 3 weeks (a–f). Allergen-specific isotype levels are shown for the control group (group A), immunized mice treated early with anti-CD40L/CTLA4Ig (group D, two independent experiments) or with anti-CD40L/CTLA4Ig/anti-OX40L (group E, three independent experiments) ($n = 6/\text{group}$). Results of group D and E are representative of two and three independent experiments, respectively.

Effector function of IgE was analysed by allergen-specific β -hexosaminidase release of serum loaded RBL cells in response to Phl p 5. Samples were collected to the depicted time points, and results are shown as box-and-whisker plots ($n = 6/\text{group}$) (g) T cell reactivity was tested in a proliferation assay. Bars represent Phl p 5-specific stimulation indices (SI) of splenocytes as means of 6 mice per group with SEM (week 15) (h) ns indicates not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 3.

Blockade of OX40L/CD40L/CD28 little effect on ongoing allergen-specific response.

Allergen-specific antibody levels were analysed by ELISA (IgE, IgG1, IgG2a, IgG3, IgA and IgM) in serum samples collected at baseline (pre-immune, d 0), 3 weeks after the first immunization (d 21 and 3 weeks after the second immunization (d 42). (a–f) Allergen-specific isotype levels are shown for immunized mice (group A, designated as control group), immunized mice with late anti-CD40L/CTLA4Ig/anti-OX40L treatment according to group F in Table 1 ($n = 6/\text{group}$). Antibody levels are displayed as OD values in box-and-

whisker plots. T cell reactivity was tested in a proliferation assay. Bars represent means of 6 mice per group with SEM (week 7) (g). ns indicates not significant. * $P < 0.05$, ** $P < 0.01$.

Fig. 4.

Potency of remaining anti-CD40L/CTLA4Ig/anti-OX40L 3 weeks before immunization. (a) Remaining function of circulating anti-CD40L, CTLA4Ig and anti-OX40L was tested by administration 21 days prior to the first immunization. Three different groups were followed, the control group (group A), mice treated with anti-CD40L/CTLA4Ig/anti-OX40L early (i.e. d 0, 2 and 4; group E) and mice receiving the same treatment at day -21 (group G). Serum samples were collected as depicted in the diagram. Shown is a box-and-whisker plot with OD values ($n = 6$). (b) T cell response of mice receiving antibodies 3 weeks before the first immunization was analysed at week 12 and compared to the other groups in a T cell proliferation assay. Bars represent Phl p 5-specific stimulation indices (SI) of splenocytes, mean values of 3 mice with SEM are shown. ** indicates $P < 0.01$.

Fig. 5.

Depletion of CD25 positive cells restores allergic T cell response. (a) Influence of CD25⁺ cell depletion in allergen-specific IgE production was tested. Groups of mice received anti-CD25 early (group I) or late (group J) and IgE levels were compared to the control group. Mean OD values are shown as box-and-whisker plot. Depletion of CD25⁺ was verified in flow cytometric analysis (b). (c) Effect of CD25⁺ cells on antibody-treated mice was assessed by depletion at day 16, 5 days before the second immunization. Shown are control group (group A), anti-CD40L/CTLA4Ig/anti-OX40L (early)-treated mice (group E) and

same regime with CD25⁺ cell depletion (group H). Serum samples were collected every 3 weeks. Allergen-specific IgE levels are depicted in OD values ($n = 6$) and presented as box-and-whisker plot. One of two independent experiments is shown. (d) T cell proliferation was assessed in response to Phl p 5 at week 15. Bars represent Phl p 5-specific stimulation indices (SI) of splenocytes as mean SI values of 6 mice per group are shown in a column bar graph with SEM. To analyse Treg function mice were immunized with additional Bet v 1 starting at day 21 (group K, L and M, $n = 6$). Serum samples of double immunized mice were collected (group K) and the control group was represented in this graph. Further groups shown are mice treated with anti-CD40L/CTLA4Ig/anti-OX40L (group L) and the last group with CD25 depletion on day 16 (group M). OD values of Bet v 1-specific IgE are shown as box-and-whisker plots, groups are described in the figure (e). (f) shows the β -hexosaminidase release of serum-coated RBL cells in response to Bet v 1 at week 12. (g) T cell proliferation was assessed at week 12 ($n = 3$). Bars represent Bet v 1-specific stimulation indices (SI) of splenocytes as SEM (one of two independent experiments). ns indicates not significant, $**P < 0.01$.

Table 1
Immunization and treatment protocol

Group	Immunization		Treatment			
	Phl p 5	Bet v 1	Anti-OX40L	CTLA4Ig	Anti-CD154	Anti-CD25
A	✓					
B	✓		Early			
C	✓		Late			
D	✓			Early	Early	
E	✓		Early	Early	Early	
F	✓		Late	Late	Late	
G	✓		d -21, -19, -17, -13	d -21, -19, -17	d -21, -19, -17	
H	✓		Early	Early	Early	16
I	✓					0, 4, 8
J	✓					21, 25, 29
K	✓	Start d 21				
L	✓	Start d 21	Early	Early	Early	
M	✓	Start d 21	Early	Early	Early	16

Groups of BALB/c mice (6/group) were immunized with the allergen rPhl p 5 and/or rBet v 1 adsorbed to aluminium hydroxide every 3 weeks (days 0, 21, 42,...). Groups were treated additionally with combinations of anti-OX40L (*early*: days 0, 2, 4, 8 or *late*: days 21, 23, 25 and 29), CTLA4Ig (*early*: days 0, 2, 4 or *late*: days 21, 23 and 24), anti-CD40L (*early*: days 0, 2 and 4) or (*late*: days 21, 23 and 25) and/or anti-CD25 (*early*: days 0, 4 and 8; *late*: 21, 25, 29 or day 16).