

Direct evidence for a critical role of CD30 in the development of allergic asthma

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Background: CD30 is a costimulatory molecule belonging to the TNF receptor superfamily that is expressed on activated T and B cells. Several studies have demonstrated a positive correlation between expression of CD30 or increased levels of soluble CD30 and the development and severity of allergic diseases. However, thus far, the evidence for a role of CD30 in allergic diseases, such as asthma, is only indirect.

Objective: The aim of the study was to directly investigate the role of CD30 in a murine asthma model.

Methods: CD30-deficient (B6.129P2-Tnfrsf8^{tm1Mak/J}) and wild-type (WT) mice were immunized to ovalbumin (OVA) to induce an asthma-like phenotype and compared in our murine asthma model. Moreover, CD30/CD30 ligand signaling was blocked in OVA-immunized WT animals by using mAbs against CD30 receptor and its ligand, CD153.

Results: The absence of CD30 in OVA-immunized CD30-deficient mice resulted in significantly reduced airway inflammation, serum IgE levels, and T_H2 cytokine levels. The same effect was observed when CD30/CD153 signaling was blocked in OVA-immunized WT animals with mAbs against CD30 or CD30 ligand.

Conclusion: Our results directly demonstrate that CD30/CD153 interaction plays an important role in the induction of T_H2 cell-mediated allergic asthma.

Clinical implications: These findings provide evidence for a role of the costimulatory molecule CD30 in allergic asthma.

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Key words: Asthma, costimulation, T_H2 cells, CD30, immunotherapy

Activation and differentiation of T cells plays a critical role in the pathogenesis of allergies and asthma. Activation of T cells is initiated by the formation of conjugates between T cells and antigen-presenting cells (APCs) in the context of peptide antigen. The T-cell

Abbreviations used

AHR: Airway hyperreactivity
BAL: Bronchoalveolar lavage
CD30L: CD30 ligand
CTLA: Cytotoxic T lymphocyte-associated antigen
H&E: Hematoxylin and eosin
OVA: Ovalbumin
sCD30: Soluble CD30
TNFR: TNF receptor
Treg: T regulatory
WT: Wild-type

receptor alone provides insufficient signals for optimal T-cell function. Instead, a second set of signals provided by costimulatory molecules is needed to amplify and fine-tune the response.¹

One of these costimulatory molecules is CD30 (Ki-1), a member of the TNF receptor (TNFR) family that was originally identified on Reed-Sternberg cells of Hodgkin's disease² and is expressed on activated T and B cells.³ CD30 is a type I membrane protein that can be cleaved by metalloproteases, producing a soluble form (sCD30).³ The ligand of CD30 (CD30L; CD153) is expressed on activated T cells, primarily CD4⁺ T cells of both T_H1 and T_H2 phenotypes, as well as on B cells and a subset of accessory cells.⁴ Several data support the concept that CD30 might play a role in the T_H2 cell-mediated disease allergic asthma⁵: Increased levels of sCD30 have been shown in allergic diseases like atopic dermatitis and allergic asthma,^{6,7} therapy of asthmatic patients decreased CD30 expression on PBMCs derived from these patients,⁸ and stimulation of T cells through CD30 resulted in the generation of T_H2 cytokines that are associated with allergic diseases. However, these data are all indirect, and moreover, contradictory data exist that do not support a role of CD30 in allergic diseases.⁹

The aim of this study was to examine directly the role of the CD30/CD153 pathway in a murine model of allergic asthma. For this purpose, we examined mice deficient in CD30 and compared them with wild-type (WT) animals. Moreover, we blocked the CD30/CD30L interaction in ovalbumin (OVA)-immunized mice by using mAbs directed against the CD30 receptor¹⁰ or against the ligand of CD30 (anti-CD153 mAb).¹¹ All 3 approaches clearly demonstrated a role for CD30/CD30L interaction in the development of allergic asthma.

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METHODS

Mice

C57BL/6 mice, CD30^{-/-} (Tnfrsf8^{tm1Mak}) mice, BALB/c mice, and DO11.10 mice (6–8 weeks of age) were obtained from the Jackson Laboratory (Bar Harbor, Me) and bred and maintained in our animal facility at the BioCenter in Halle/Saale, Germany. All animal experiments involved groups of 3 to 6 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.

Immunization and antibody treatments

Mice were immunized with OVA (20 µg; Sigma-Aldrich, Steinheim, Germany) adsorbed to 2 mg of an aqueous solution of aluminum hydroxide and magnesium hydroxide (Imject Alum; Perbio Science, Bonn, Germany) intraperitoneally on days 1 and 14, followed by 20 µg of OVA in 40 µL of normal saline administered intranasally on days 14 to 16 and 21 to 23. Control mice received intraperitoneal injections of alum alone and normal saline intranasally. Anti-CD153 mAb (RM153, rat IgG_{2b}, anti-mouse CD153 mAb, 150 µg), anti-CD30 mAb (mCD30.1, Armenian hamster IgG anti-mouse CD30 mAb, 150 µg), or control mAb (rat IgG_{2b}, Armenian hamster IgG, 150 µg) was applied intraperitoneally on days 0 and 13 to block CD30/CD30L interaction. Airway hyperreactivity (AHR) was measured on day 24, and mice were killed on day 25. In a reversal protocol mice were immunized with OVA on day 1, followed by OVA in normal saline intranasally on days 8 to 10. Control mice received alum intraperitoneally and normal saline intranasally. AHR was measured the following day to confirm the induction of AHR by means of OVA sensitization. Anti-CD30/anti-CD153 mAb or control mAb was injected intraperitoneally 12 hours after AHR measurement on day 11. Intranasal OVA challenge was repeated on days 15 to 17, and AHR was measured on day 18. Mice were killed the following day.

Collection of bronchoalveolar lavage fluid

Animals were killed by means of CO₂ asphyxiation. The trachea was cannulated, and the right lung was lavaged 3 times with 400 µL of 0.9% NaCl. Cells in the lavage fluid were counted with a hemocytometer, and bronchoalveolar lavage (BAL) cell differentials were determined on cytospin slide preparations stained with May-Grünwald-Giemsa (Merck, Darmstadt, Germany) on blinded samples by an independent investigator. At least 200 cells were differentiated by means of light microscopy based on conventional morphologic criteria.

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

The left lung was fixed in 10% formalin and stained with hematoxylin and eosin (H&E, Merck) or with periodic acid–Schiff (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; 5 shots per lung) and analyzed with HistoClick Software (provided by Ingo Hohmann and Wiebke Scherf, Halle, Germany) based on morphometric image analysis developed in our laboratory.

Measurement of airway responsiveness

Airway responsiveness was assessed on the basis of methacholine-induced airflow obstruction from conscious mice placed in a whole-body plethysmograph (EMKA Technologies, Paris, France), as described previously.¹² Mice were exposed for 2 minutes to

aerosolized 0.9% NaCl produced by a sonicator (model LS 290-990N), followed by increasing amounts of methacholine. Enhanced pause measurements were made for 4 minutes. Data are expressed as the percentage of the average values of baseline enhanced pause values after 0.9% NaCl exposure.

OVA-specific IgE and IgG₁ assay

OVA-specific IgE and IgG₁ serum levels were measured by means of sandwich ELISA, according to a standard protocol described previously.¹³

In vitro proliferation and cytokine production

One day after airway function testing, splenocytes or lung cells (5×10^6 cells per well) were restimulated *in vitro* with OVA (200 µg/mL) in culture medium (RPMI medium supplemented with 10% FCS, 100 U/mL penicillin, and 100 µg/mL streptomycin). For proliferation measurements, spleen cells were restimulated with 0.2 to 200 µg/mL OVA in 96-well tissue-culture plates in 200 µL of culture medium or not stimulated for control specimens. After 48 hours, the cultures were pulsed with 0.25 µCi of tritiated thymidine for 18 hours, and the incorporated activity was measured in a Betaplate scintillation counter (MicroBeta Trilux; Wallac, Turku, Finland). Lung cells were pulsed with tritiated thymidine immediately after preparation for 24 hours. Cytokines were measured in supernatants from restimulated splenocytes or lung cells after 3 days by using DuoSet ELISA kits (R&D Systems, Minneapolis, Minn), according to the manufacturer's instructions.

Flow cytometric analysis

Flow cytometric analysis was performed with fluorescein isothiocyanate-, phycoerythrin-, or peridinin-chlorophyll-protein complex–Cy5.5–conjugated mAbs to mouse CD3, CD4, CD8α, CD19, and CD25 (BD Biosciences Pharmingen, Heidelberg, Germany). Cells (1×10^6) were preincubated with 2.4G2 mAb (10 µg/mL) to block FcγR and then incubated with the relevant mAbs for 45 minutes at 4°C. Cells were washed twice with 1% BSA in PBS. For cytotoxic T lymphocyte-associated antigen (CTLA) 4 staining, cells were permeabilized with Cytotfix/Cytoperm-buffer (BD Bioscience) and incubated with phycoerythrin-labeled anti-CTLA4 mAb. Cells were analyzed with FACScan (BD Biosciences Pharmingen).

Statistical analysis

The Student *t* test was used to determine the statistical significance of differences between groups. *P* values of less than .05 were considered significant.

RESULTS

Absence of CD30 leads to reduced airway inflammation in OVA-immunized mice

The role of CD30 in allergic asthma was investigated by means of comparative analysis of CD30^{-/-} versus WT mice in our well-established murine asthma model.^{12,14,15} One of the major features of allergic asthma is the eosinophilic airway inflammation. Analysis of the cell number in the BAL fluid revealed significantly increased total cell numbers in OVA-immunized mice compared with those in nonimmunized control animals. Impressively, the increase in inflammatory cells in the BAL fluid of OVA-immunized CD30^{-/-} mice was only 20% of that of OVA-immunized WT animals (50.52 ± 6.36 vs 9.64 ± 3.3 , *P* < .001; Fig 1, A). Histologic analysis of

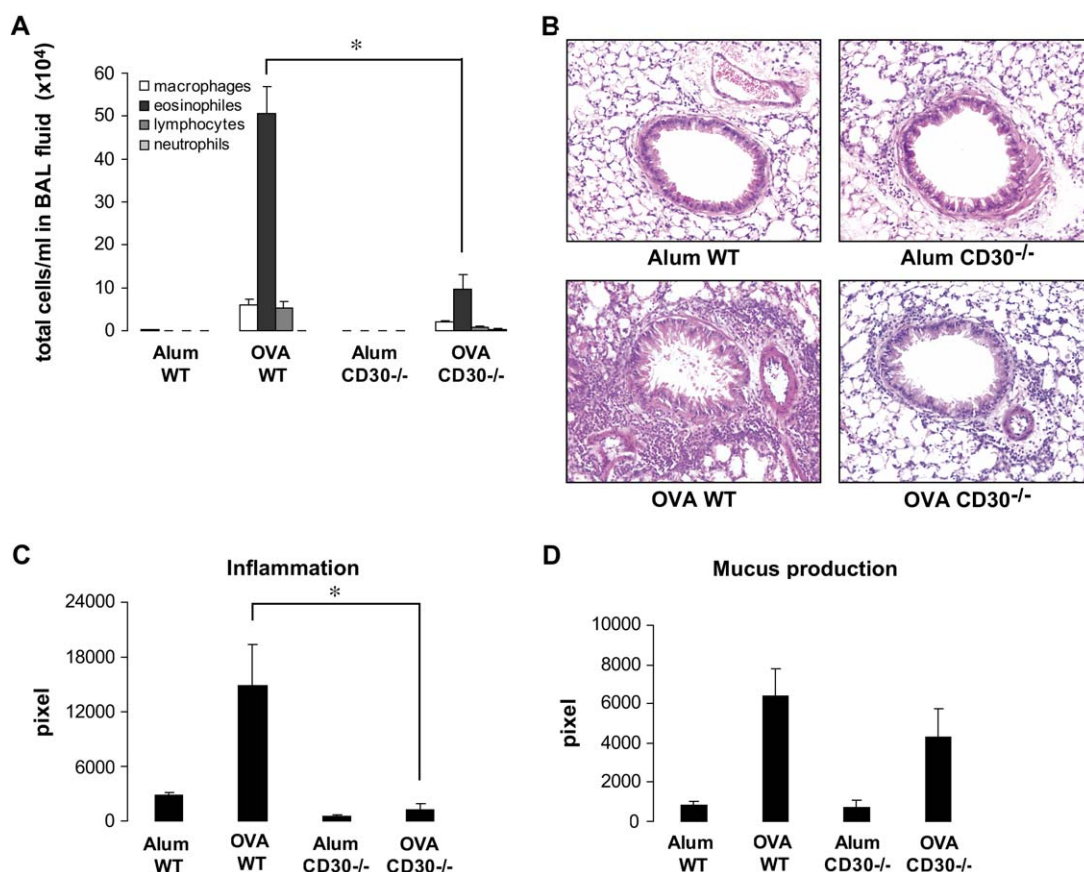


FIG 1. Absence of CD30 leads to reduced airway inflammation in OVA-immunized mice. Total cell numbers in BAL fluid (**A**) and inflammation of lung tissues examined by means of lung histology (**B**; H&E, magnification $\times 100$) and quantified by using a computer-based image-analyzing program (**C**) were significantly reduced in OVA-immunized CD30^{-/-} mice, whereas mucus production (**D**) was not significantly altered. Data are expressed as means $\pm$ SEM ($n \geq 9$ animals per group). * $P < .05$.

H&E-stained lung sections isolated from OVA-immunized mice on day 25 showed substantially less inflammatory infiltrates in the airways of CD30^{-/-} mice compared with those seen in WT animals (Fig 1, B). An objective, investigator-independent computer program based on morphometric image analysis of lung sections from at least 9 animals per group confirmed these results and demonstrated significant differences in airway inflammation in CD30^{-/-} versus WT animals (Fig 1, C). In contrast, mucus production, examined in periodic acid-Schiff-stained lung sections, was only slightly reduced in CD30^{-/-} mice compared with that seen in WT control animals (Fig 1, D).

Absence of CD30 leads to significantly reduced IgE serum levels, significantly reduced T_H2 cytokine production, and significantly less proliferation of splenocytes in OVA-immunized mice

Another hallmark of allergic asthma is the production of allergen-specific IgE. To determine whether the absence of CD30/CD153 interaction leads to differences in the production of OVA-specific IgE, IgG₁, and IgG_{2a}, we

obtained serum on day 25, and Ig levels were measured by means of ELISA. OVA immunization of WT mice resulted in markedly increased serum levels of OVA-specific IgE and IgG₁. This increase was significantly lower in CD30^{-/-} mice for IgE and clearly reduced for IgG₁, although there the level of significance was not reached (Fig 2, A). In contrast, there was no difference in IgG_{2a} levels between CD30^{-/-} and WT mice (data not shown).

Spleen and lung cells were isolated and restimulated *in vitro* with OVA for 3 days to investigate cytokine production in the different animals. The increase of IL-13 and IL-5 production by OVA immunization was significantly greater in WT mice relative to CD30^{-/-} mice. This was true for supernatants derived from lung cell cultures, as for supernatants derived from spleen cell cultures. In contrast, there were no significant differences in IFN- γ production (Fig 2, B). Furthermore, levels of IL-10, as well as TGF- β , both cytokines that are produced by specific populations of T regulatory (Treg) cells, were measured. Although IL-10 levels were decreased, TGF- β levels were not altered in OVA-immunized CD30^{-/-} mice.

Proliferation of splenocytes isolated from OVA-immunized WT and CD30^{-/-} mice was significantly decreased

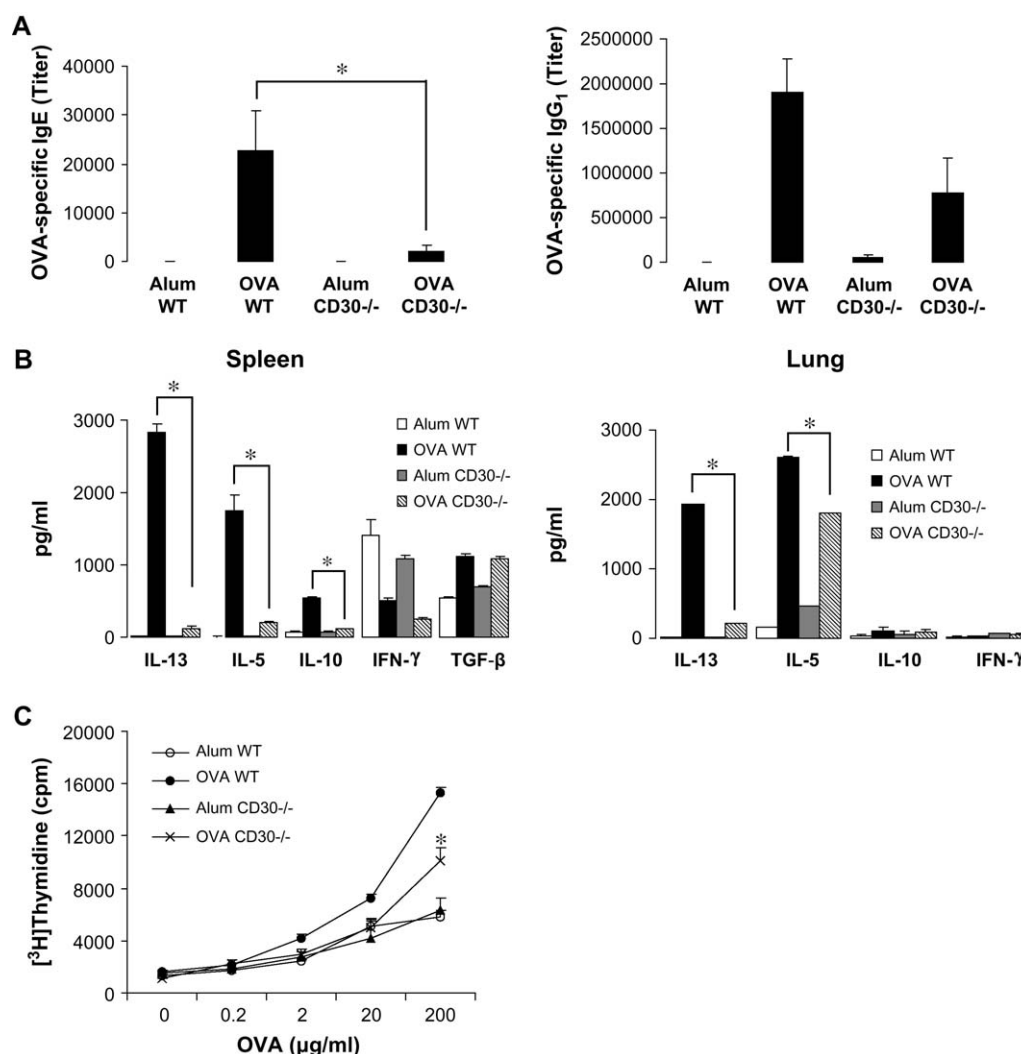


FIG 2. Absence of CD30 leads to reduced T_H2 cytokine production, IgE levels, and cell proliferation in OVA-immunized mice. OVA-specific IgE serum levels (**A**), IL-13 and IL-5 production in spleen and lung cell cultures (**B**), and proliferation of splenocytes after restimulation (**C**) were significantly reduced in OVA-immunized CD30^{-/-} mice compared with that seen in OVA-immunized WT mice. Data are expressed as means $\pm$ SEM ($n \geq 9$ animals per group). * $P < .05$.

in CD30^{-/-} compared with WT mice after restimulation with OVA (Fig 2, C).

Blocking of CD30/CD153 signaling inhibits the development of airway inflammation and AHR

To further investigate the significance of our findings in CD30^{-/-} mice, we chose a different approach and blocked signalling between CD30 and CD153 in OVA-immunized WT animals by using mAbs against CD30 receptor (anti-CD30 mAb) or CD153 (anti-CD153 mAb), the ligand of CD30. Anti-CD30 mAb and anti-CD153 mAb were applied 1 day before OVA immunization was started and 13 days afterward in a 23-day immunization protocol. Both mAbs were able to inhibit the development

of eosinophilic airway inflammation in OVA-immunized BALB/c mice as demonstrated by significantly reduced total cell numbers in the BAL fluid of the treated animals compared with those seen in control animals that received control antibody (total cell numbers: OVA, $38.95 \pm 5.3 \times 10^4$ cells/mL; OVA + anti-CD30 mAb, $20.90 \pm 3.1 \times 10^4$ cells/mL [$P < .05$]; OVA + anti-CD153 mAb, $11.68 \pm 2.9 \times 10^4$ cells/mL [$P < .01$]; Fig 3, A). Accordingly, histologic examination and computer-based analysis of H&E-stained lung sections isolated from OVA-immunized mice that had received anti-CD30 mAb or anti-CD153 mAb before immunization revealed significantly decreased airway inflammation in the anti-CD30- and anti-CD153-treated groups compared with in the control animals (Fig 3, B). As in CD30^{-/-} mice, mucus production was only slightly inhibited by

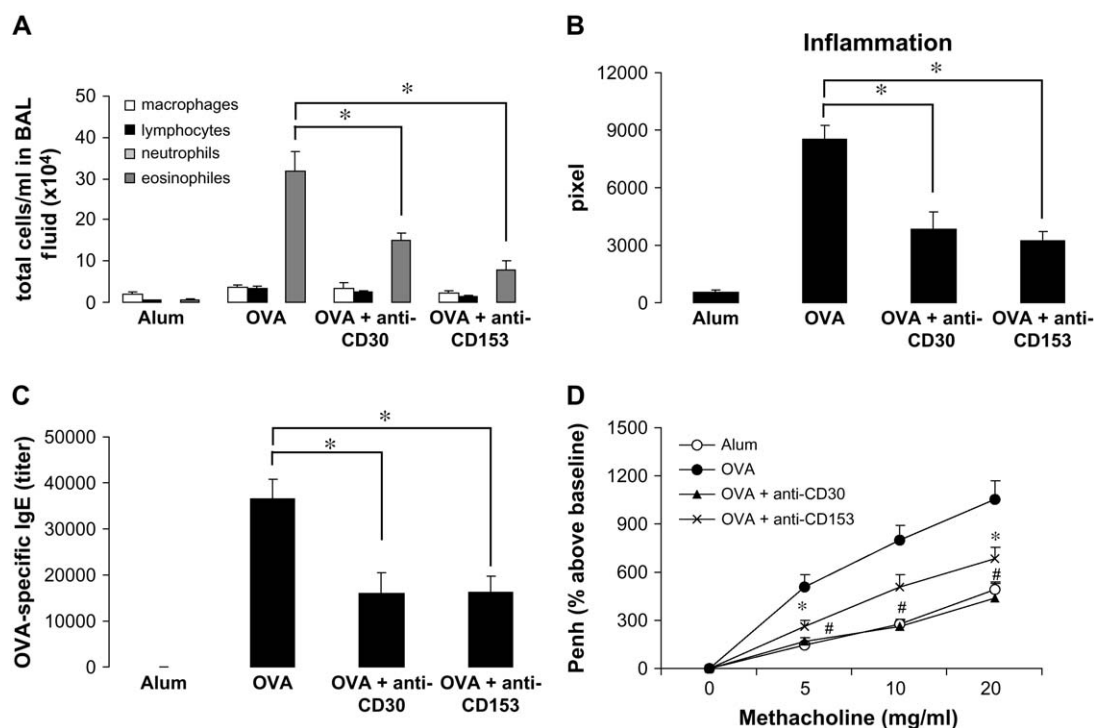


FIG 3. Blocking of CD30/CD153 interaction by anti-CD30 and anti-CD153 mAbs inhibits the development of airway inflammation, IgE production, and AHR. Anti-CD30 mAb and anti-CD153 mAb significantly reduced the number of eosinophils (**A**), lung inflammation (**B**), OVA-specific IgE serum levels (**C**), and AHR (**D**). Data are expressed as means $\pm$ SEM ($n \geq 9$ animals per group). * $P < .05$, OVA vs OVA plus anti-CD153; # $P < .05$, OVA vs OVA plus anti-CD30.

application of anti-CD30 and anti-CD153 mAb (data not shown). In addition, and similar to the results seen with CD30^{-/-} mice, serum levels of OVA-specific IgE were significantly reduced by anti-CD30 mAb or anti-CD153 mAb (Fig 3, C).

AHR was measured in response to methacholine in a whole-body-plethysmograph 1 day after the last intranasal challenge with OVA on day 24 of the immunization protocol to test whether blocking of CD30/CD153 signaling can modify AHR in OVA-immunized BALB/c mice. Although OVA-immunized mice that had received isotype control mAb had increased AHR in response to methacholine challenge, application of anti-CD30 mAb or anti-CD153 mAb significantly reduced the degree of AHR (Fig 3, D).

To investigate the therapeutic potential of blocking the CD30/CD153 costimulation by antagonistic antibodies, we administered anti-CD30 mAb or anti-CD153 mAb after an asthma-like phenotype was already established. Both anti-CD30 mAb and anti-CD153 mAb were unable to inhibit an already established asthma phenotype in our model (data not shown).

DISCUSSION

Several studies in the past have shown indirect evidence for a role of CD30 in allergic diseases by

correlating CD30 expression and sCD30 levels to severity and outcome of allergic asthma and atopic dermatitis.^{7,16,17} However, thus far, no study could demonstrate direct evidence for a role of CD30/CD153 signaling in these diseases. Moreover, contradictory data questioned the evidence for a role of CD30 in allergies.⁹

By using CD30^{-/-} mice and mAbs directed against CD30 receptor or ligand, we could clearly demonstrate in our well-established murine model of asthma that absence of CD30 signaling or blocking of CD30 signaling significantly reduces eosinophilic pulmonary inflammation. Moreover, CD30 signaling was critical for T_H2 cytokine production, as well as IgE production, in the murine asthma model because absence of CD30 expression or blocking of CD30 signaling significantly inhibited these parameters in OVA-immunized mice.

The finding that blocking of CD30 costimulation inhibits T_H2 cytokine production has been shown before in an *in vitro* model using human T cells.¹⁶ However, although this study has demonstrated increased levels of the T_H1 cytokine IFN- γ , indicating that CD30 blockade induces a shift from a T_H2 to a T_H1 response, we could not detect increased levels of IFN- γ after CD30 blockade.¹⁸ In some instances IFN- γ has been shown to inhibit the asthma phenotype. However, IFN- γ is unlikely to be a mediator of the suppressed phenotype in our model because we could not detect increased levels of IFN- γ in the lungs or IFN- γ -dependent antibodies in the serum.

Therefore in our model the inhibitory effects of anti-CD30 and anti-CD153 mAb cannot be based on a shift from T_H2 to T_H1 cell immunity.

An alternative to control immune response is the suppression of T_H2 effector cells by $CD4^+$ Treg cells. Several forms of antigen-specific Treg cells have been described in the last years that are induced after exposure to specific antigen.¹⁹ These include $CD4^+CD25^+$ T cells, T cells producing IL-10 or TGF- β , or T cells expressing CTLA4 or foxp3.²⁰ However, in our study we did not find evidence for the induction of Treg cells by blocking CD30/CD153 signaling or when CD30/CD153 signaling was completely absent in $CD30^{-/-}$ mice. Neither the number of $CD4^+CD25^+$ T cells nor the production of IL-10 and TGF- β were increased. Moreover, we could not find increased CTLA4 expression or increased foxp3 expression measured by means of FACS analysis and real-time PCR after CD30/CD153 blockade (unpublished data).

To further analyze the mechanism that might be responsible for the observed effects, we looked at proliferation of splenocytes derived from OVA-immunized $CD30^{-/-}$ or WT mice. Absence of CD30 resulted in significantly reduced proliferation after restimulation with OVA. This finding goes along with the hypothesis that CD30 might provide proliferation and surviving signals to allow the generation of high numbers of antigen-specific T cells at the peak of primary response.²¹⁻²³ However, although splenocytes derived from $CD30^{-/-}$ mice did proliferate less than splenocytes derived from WT mice, they were not completely anergic but still responded to OVA. This finding might support a combination of different mechanisms for the inhibitory effect of CD30/CD153 signaling blockade.

To determine whether administration of anti-CD30 mAb could induce deletion of OVA-specific T cells, we transferred $CD4^+$ T cells from DO11.10 mice that are transgenic for the OVA receptor into BALB/c mice. Afterward, anti-CD30 mAb was applied intraperitoneally, and OVA was administered the following day. Spleen cells were isolated 2 days later, and apoptotic/necrotic $CD4^+KJ1-26^+$ T cells were identified by means of FACS analysis. Treatment with anti-CD30 mAb led to an increase of necrotic and postapoptotic cells of OVA-specific splenocytes compared with those seen in OVA control mice (OVA, 19.2%; OVA + anti-CD30, 24.4%), whereas the percentage of the Annexin V⁺ apoptotic cells was nearly unchanged (unpublished data). These data support the concept that the effects of CD30 can be based on, in part, deletion of antigen-specific $CD4^+$ T cells. However, the mechanism by which CD30 enhances T_H2 -dependent diseases, such as asthma, remains to be further explored.

Our data with $CD30^{-/-}$ mice, as well as our data with blocking antibodies to CD30 receptor (anti-CD30 mAb) and its ligand (anti-CD153 mAb), directly showed that CD30/CD153 interaction is critically involved in the development of asthma in a murine asthma model. These results prompted us to further look at the therapeutic potential of CD30 blockade in allergic asthma.

Application of inhibitory mAbs directed against CD30 receptor (anti-CD30 mAb) or ligand (anti-CD153 mAb) to OVA-immunized BALB/c mice that had already had an asthma-like phenotype did not reverse this established phenotype (data not shown). These findings suggest that CD30 signaling in our model is required early, in the initial phase of antigen priming, to inhibit or modulate the development of asthma but does not seem to be a sufficient target for asthma therapy. However, because of species differences, it is possible that in human subjects CD30 plays a different role in asthma compared with that seen in mice and might even be a possible target for asthma therapy.

In contrast to this observation with anti-CD30 mAb, our previous study with CD137, another costimulatory molecule of the TNFR family, showed impressive therapeutic potential of CD137 mAb. CD137 mAb very effectively prevented and also reversed an already established allergic asthma phenotype.¹³ Although both costimulatory molecules of the TNFR family that we tested, CD30 and CD137, are clearly involved in asthma pathogenesis, CD30 seems to mainly modulate the initial phase of antigen/allergen contact when T-cell priming occurs, whereas CD137 seems to be involved as well in the initial phase of T-cell priming, as in the effector phase.

Earlier studies have shown that other costimulatory pathways, such as CD28/B7/inducible costimulator (ICOS) or OX40, are involved in allergic asthma.²⁴⁻²⁷ However, as demonstrated in basic studies of T-cell responses, activation, expansion, and effector function are controlled by a complex interplay of multiple costimulatory receptors^{1,28} that is by far not fully understood today. Further studies that analyze the different roles of the different costimulatory molecules and their interplay in the pathogenesis and therapy of asthma are needed.

In conclusion, the findings reported here show, for the first time, directly that CD30 signaling plays a central role in the development of T_H2 cell-mediated allergic immune responses but do not support the concept that CD30 is a sufficient target for asthma therapy.

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