

The central role of CD30L/CD30 interactions in allergic rhinitis pathogenesis in mice

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CD30 ligand (CD30L) plays an important role in the amplification and/or activation of effector CD4⁺ T cells, irrespective of Th cell subset. To examine the role of CD30L in allergic rhinitis, we evaluated an OVA model of allergic rhinitis in CD30L knock out (KO) mice on a BALB/c background sensitized with OVA. Symptoms of allergic rhinitis such as eosinophil infiltration into the nasal mucosa were drastically diminished in OVA-sensitized CD30L KO mice following intranasal challenge with OVA. The levels of OVA-specific IgE in the sera and the Th2 response in nasopharynx-associated lymphoid tissues and cervical LNs of CD30L KO mice were significantly lower than those of WT mice following intranasal challenge with OVA. Intranasal administration of CD30-Ig during the effector phase with OVA significantly prevented the development of allergic rhinitis in WT mice. These results suggest that CD30L plays an important role in allergic rhinitis and that the inhibition of CD30L/CD30 signaling might be useful as a novel biological therapy for allergic rhinitis.

Key words: Allergic rhinitis animal model • CD30L • Soluble CD30-Ig • Th2 cells

Introduction

CD30 ligand (CD30L, CD153), one of the TNF superfamily members, is a 40-kDa type II membrane-associated glycoprotein [1] and is expressed on effector CD4⁺ T cells [2–4]. The receptor for CD30L is CD30, a member of the TNF receptor (TNFR) superfamily, and is preferentially expressed on effector or memory Th cells [5–7]. A number of lines of evidence suggest that a CD30L/CD30-mediated signal is involved in the development of diseases associated with both Th1 and Th2 cell responses,

such as diabetes in young non-obese diabetic mice [8], mycobacterial infection [9], and allergic inflammation [10]. Recent work has proved that the CD30L/CD30 signal is important in naturally occurring Treg-cell responses in CD4⁺ T-cell-mediated GVHD [11]. Moreover, recently, we have found that the CD30L/CD30 signal executed by the T-cell–T-cell interaction plays a critical role in Th1 and Th17 cell differentiation in vitro and in vivo [12–15]. Thus, CD30L/CD30 signaling might not be directly correlated with a physiological step for the specific Th cell differentiation subset but might be important for amplification and/or activation of effector and/or memory Th cells.

Predominance of Th2 response, synthesis of antigen-specific IgE, and eosinophil infiltration in the nasal mucosa are characteristics of allergic rhinitis [16]. There are mainly two phases

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during the allergic response, the induction phase and effector phase [17–19]. In the induction phase, Th2 cells and IgE specific for the allergen are generated in secondary lymphoid tissues. In the effector phase, allergic inflammation occurs in nasal mucosa and is further divided into two reactions: early-phase reaction and late-phase reaction. The early-phase reaction is characterized by sneezing, pruritus, nasal obstruction, and rhinorrhea, which are mediated by mast cells bound with IgE. The late-phase reaction is characterized with nasal congestion, inflammatory cells including basophils, eosinophils, mast cells, mononuclear cells, and neutrophils infiltration mediated by Th2 responses in mucosa and nasopharynx-associated lymphoid tissues (NALT) [20–24].

In the present study, to investigate the effect of CD30/CD30L signal on the development of allergy rhinitis, the allergic rhinitis mouse model was investigated using CD30L KO mice. We showed that CD30L positively modulates the allergic symptoms and CD4⁺ Th2 cell responses during the effector phase. Based on this result, the feasibility of CD30-Ig as an application in the treatment of allergic rhinitis has been confirmed. Implication of these findings for therapeutic application of CD30L/CD30 modulation for allergic rhinitis are discussed.

Results

CD30L KO mice showed impaired allergic inflammation in allergic rhinitis

We assessed the role of CD30L in a mice model of allergic rhinitis [25–27]. WT mice and CD30L KO mice were sensitized with OVA (25 µg) or PBS/aluminum hydroxide hydrate gel (alum) three times at 1-wk intervals, and then, from day 21 to day 35 after the first sensitization with OVA; mice were i.n. challenged with 20 µL of PBS or OVA (20 µg/µL) in the bilateral nostrils (Fig. 1A). Over a 5-min period after the last nasal drop of OVA or PBS, we counted the frequencies of sneeze and nasal rubbing of each mouse. The frequencies of nasal rubbing and sneezes after OVA challenge were significantly reduced in CD30L KO mice than those in WT mice (Fig. 1B). No sneezing or nasal rubbing occurred in non-sensitized group after either PBS or OVA challenge (Fig. 1B and data not shown).

Histopathologically, CD30L KO mice showed a mild inflammatory cell infiltration in the mucosa as compared with WT mice 12 h after the final OVA i.n. challenge (Fig. 2A). Moreover, after the last OVA i.n. challenge, we also measured the eosinophils in nasal mucosa by using a microscope. We found that the accumulation of eosinophils in the nasal mucosa was drastically lower in CD30L KO mice than that in WT mice (Fig. 2B and C). Only a few eosinophils were detected in non-sensitized group of mice challenged with PBS or OVA (Fig. 2 and data not shown).

We next examined the cell accumulation in tissues such as nasal mucosa, NALT, cervical LNs, and spleen after the final i.n. challenge with PBS or OVA. The total numbers of cells were significantly lower in the nasal mucosa, NALT, and cervical LNs of

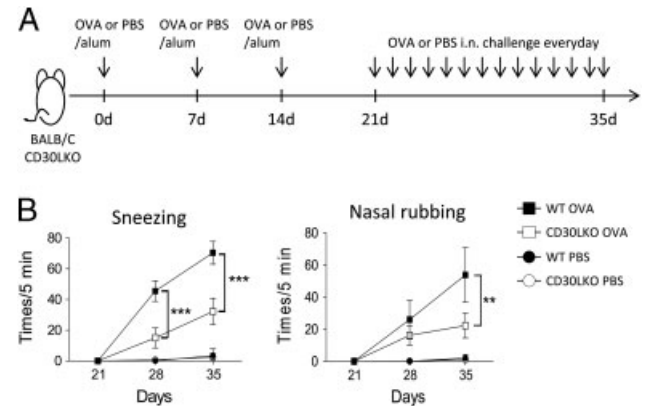


Figure 1. Allergic symptoms following nasal challenge with OVA in OVA-sensitized CD30L KO and WT mice. (A) Experimental protocol, as described in the Materials and Methods. (B) The numbers of sneezes and nasal rubbing. The number of sneezes (left) and incidences of nasal rubbing (right) were counted 5 min after the final OVA i.n. challenge. Data are geometric means $\pm$ SD and are representative of three independent experiments. Five to eight mice were used in each experiment (** $p < 0.01$, *** $p < 0.001$, Student's t-test).

CD30L KO mice than in WT mice (Fig. 3). The numbers of CD4⁺ T cells were significantly lower in these tissues of CD30L KO mice and the number of B cells was significantly lower in NALT and cervical LNs of CD30L KO mice than WT mice (Fig. 3). The numbers of eosinophils (CD11b⁺ Gr-1^{dull}) and mast cells (c-kit⁺ FcεRI⁺ IgE⁺) were significantly lower in the nasal mucosa of CD30L KO mice than WT mice (Fig. 3). Thus, CD30L KO mice showed impaired symptoms and allergic inflammation in allergic rhinitis.

CD30L KO mice showed impaired Th2 responses with IgE production in allergic rhinitis

We measured OVA-specific antibodies in the serum of OVA-sensitized WT mice and CD30L KO mice before/after intranasal challenge with OVA. On day 21 after the first OVA sensitization, a significant level of OVA-specific IgE was detected in the serum, but a similar level of the OVA-specific IgE was detected both in OVA-sensitized CD30L KO mice and WT mice (Fig. 4A). The OVA-specific IgE and IgG2a levels increased after intranasal challenge with OVA but not with PBS. The level of OVA-specific IgE was significantly lower in CD30L KO than that in WT mice on days 28 and 35 after the OVA challenge (Fig. 4A). No difference was found in the OVA-specific IgG2a level between OVA-sensitized CD30L KO and WT mice after OVA challenge (Fig. 4A).

We next assessed the cytokine secretion by CD4⁺ T cells. Cells were obtained from several tissues such as spleen, cervical LNs, and NALT after the last i.n. challenge with OVA and then were cultured 96 h in the presence or absence of OVA. The CD30L^{-/-} CD4⁺ T cells obtained from NALT and cervical LNs produced lower levels of IL-4, IL-5, and IL-13 in response to OVA as compared with CD4⁺ T cells from WT mice (Fig. 4B). We did not find any difference in IFN-γ and IL-17A production by CD4⁺

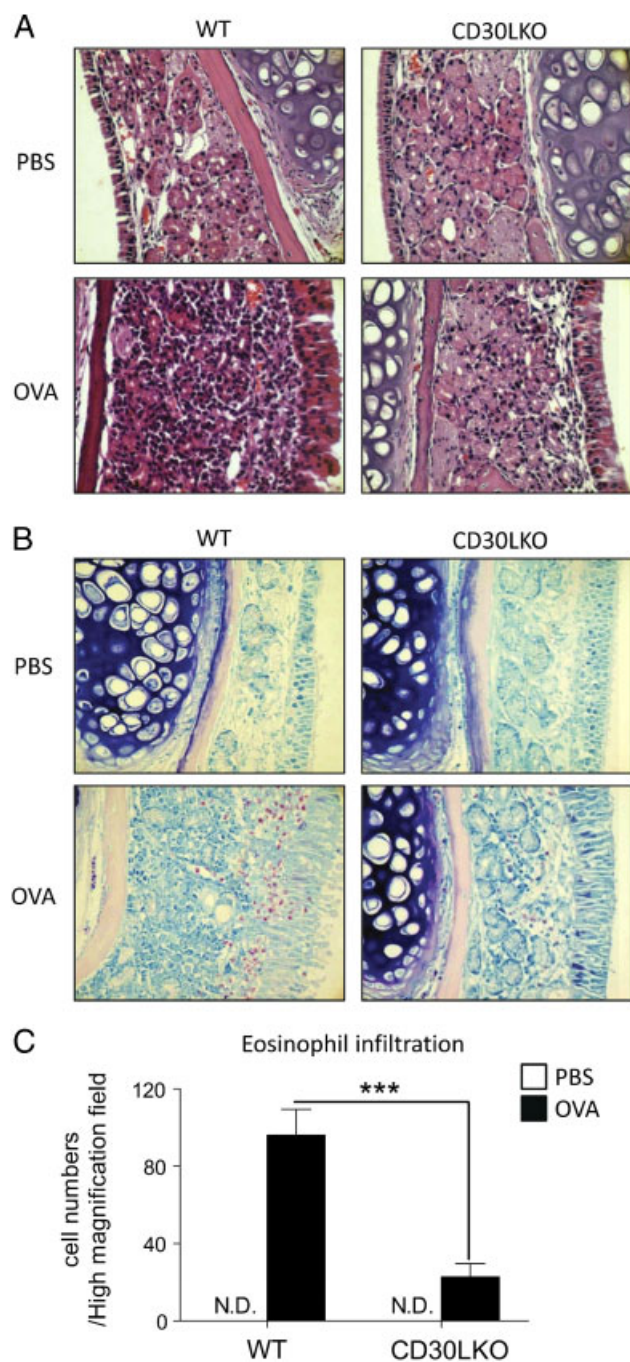


Figure 2. Pathohistology of allergic rhinitis in CD30L KO and WT mice. Coronal nasal sections were stained with (A) hematoxylin and eosin or (B) Hansel staining. Results are shown at 100 $\times$ magnification and are representative of three independent experiments. (C) The absolute number of eosinophils in the nasal mucosa was counted microscopically by Hansel staining. Data are geometric means $\pm$ SD and are representative of three independent experiments. Five to eight mice were used in each experiment (*** p <0.001, Student's *t*-test).

T cells from cervical LNs and NALT in response to OVA between CD30L KO and WT mice (Fig. 4B). Only low, if any, levels of IL-4, IL-5, IL-13, IFN- γ , and IL-17A were detected in the culture supernatants of CD4 $^{+}$ T cells from cervical LNs and NALT in the absence of OVA (Fig. 4B). In non-sensitized mice, we did not

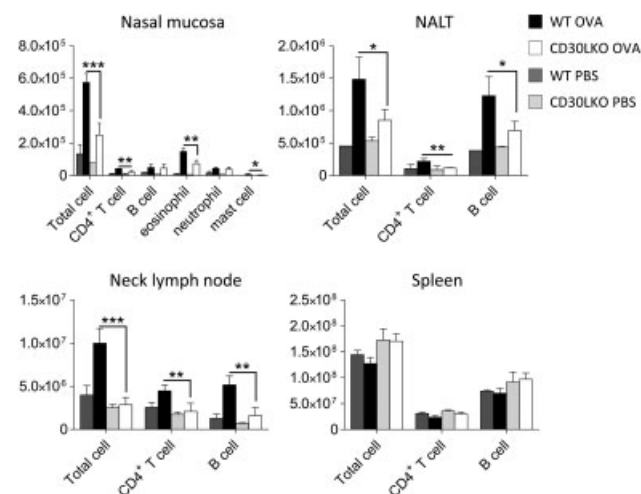


Figure 3. The numbers of cells in the nasal mucosa, NALT, cervical LNs, and spleens of OVA-sensitized CD30L KO and WT mice after i.n. challenge with OVA. The mononuclear cells were harvested from nasal mucosa, NALT, cervical LNs, and spleens of mice 12 h after the final OVA i.n. challenge and stained with various combinations of mAbs (B cell: B220 $^{+}$ CD3 $^{-}$; eosinophil: CD11b $^{+}$ Gr-1 ^{dull} ; neutrophil: CD11b $^{+}$ Gr-1 ^{high} ; mast cell: c-kit $^{+}$ Fc ϵ RI α $^{+}$ IgE $^{+}$). The stained cells were analyzed by flow cytometry. The absolute numbers of each of the subsets were counted by multiplying the percentage of the corresponding cells by the absolute number of cells. Each group consisted of five mice. Data are presented as mean $\pm$ SD. Statistically significant differences between WT and CD30L KO mice are indicated with asterisks (* p <0.05, ** p <0.01, *** p <0.001, Student's *t*-test).

detect any levels of IL-4, IL-5, IL-13, IFN- γ , and IL-17 by CD4 $^{+}$ T cells of cervical LNs and NALT in response to OVA (Fig. 4B and data not shown). Thus, CD30L KO mice showed impaired Th2 responses in NALT, especially after i.n. challenge with OVA.

CD30-Ig suppressed allergic rhinitis during the effector phase

We have revealed that the CD30/CD30L signal contributes to the allergic rhinitis development by enhancing Th2 responses. These findings elevate the probability that the regulation of CD30/CD30L signal may be useful to control allergic rhinitis. To provide evidence for this hypothesis, we investigated whether soluble CD30-Ig fusion protein suppresses allergic rhinitis development, which is known to suppress CD30L/CD30 signaling in vivo as well as in vitro [7, 15]. After i.n. challenge of OVA together with CD30-Ig or control murine IgG $_1$ for 14 days, the number of sneezes and nasal rubbing, OVA-specific IgE production, and histological change of nasal mucosa were analyzed. The numbers of sneeze and nasal rubbing decreased by treatment with CD30-Ig during the effector phase (Fig. 5A). In the nasal mucosa, eosinophil infiltration decreased by treatment with CD30-Ig during the effector phase (Fig. 5B). OVA-specific IgE, but not IgG2a, was significantly decreased by the addition of CD30-Ig on day 35 (Fig. 5C).

We further counted the number of cells harvested from nasal mucosa, NALT, cervical LNs, and spleen 12 h after the final OVA

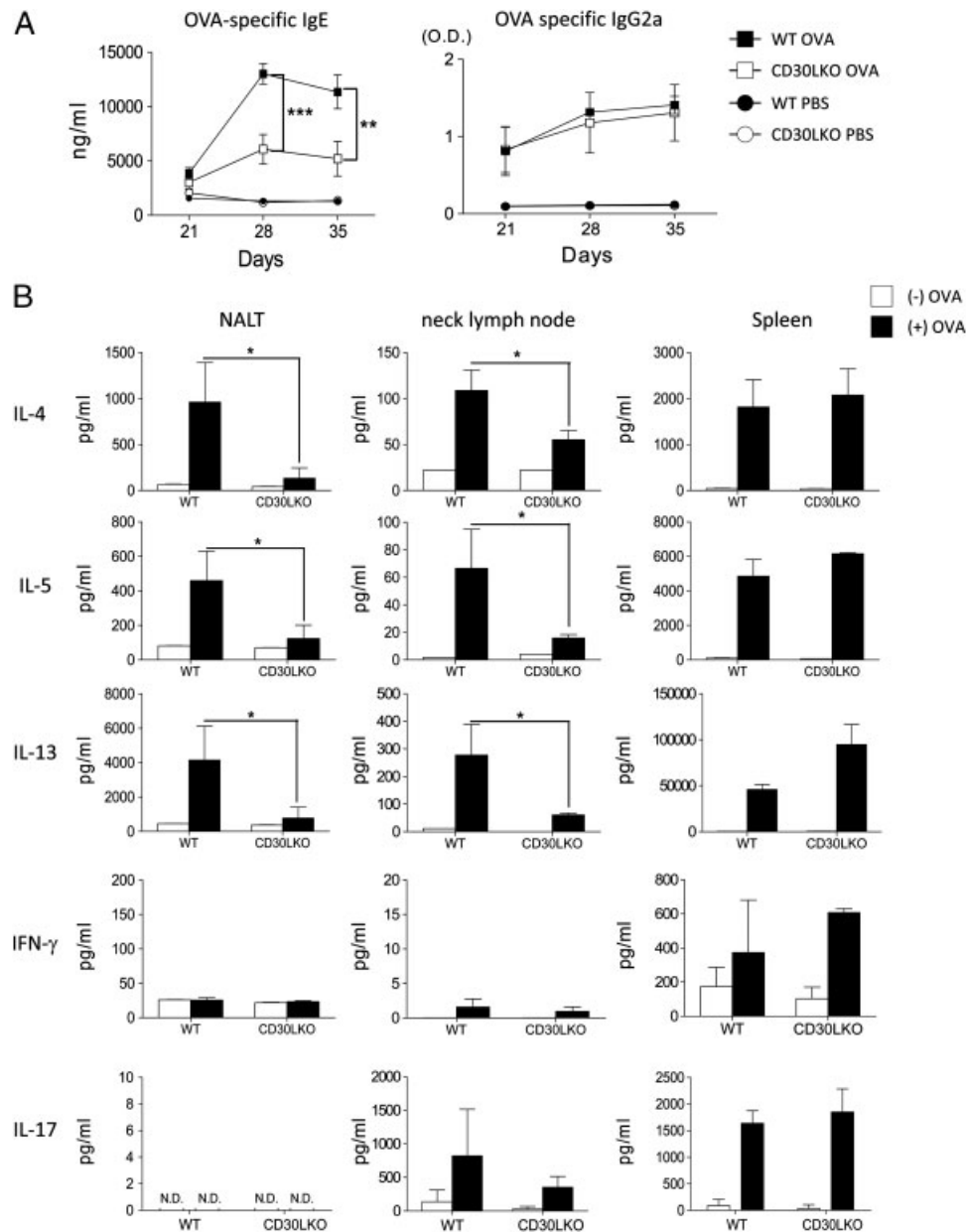


Figure 4. OVA-specific Th2 responses with OVA-specific Abs in CD30L KO and WT mice. (A) OVA-specific IgE (left) and IgG2a (right) in the sera of OVA-sensitized CD30L KO and WT mice before and after intranasal challenge with OVA. Mice were killed 12 h after the final OVA i.n. challenge. Data are geometric means $\pm$ SD and are representative of three independent experiments. Five to eight mice were used in each experiment (** $p < 0.01$; *** $p < 0.001$, Student's *t*-test). (B) Cytokine production by CD4⁺ T cells in cervical LNs, NALT, and spleen of CD30L KO or WT mice. Purified CD4⁺ T cells (2×10^5) were cultured with or without the stimulation of OVA (200 μ g/mL) together with irradiated spleen cells (1×10^6) for 96 h at 37°C. The data are means of triplicate determinations $\pm$ SD and are representative of three independent experiments using pooled cells from three to four mice. (* $p < 0.05$, Student's *t*-test).

challenge together with CD30-Ig or control mAb. The total numbers of cells were significantly lower in the nasal mucosa, NALT, and cervical LNs of CD30-Ig-treated mice than in control mice (Fig. 6). The numbers of CD4⁺ T cells were significantly lower in these tissues of CD30-Ig-treated mice, and the numbers of B cells were significantly lower in NALT and cervical LNs of CD30-Ig-treated mice than control mice (Fig. 6). The numbers of eosinophils (CD11b⁺ Gr-1^{dim}) and mast cells (c-kit⁺ FcεR1⁺ IgE⁺) were significantly lower in the nasal mucosa of CD30-Ig-treated

mice than control mice (Fig. 6). Thus, it is possible for the administration of CD30-Ig during the effector phase to suppress the development of allergic rhinitis.

Discussion

In this study, we investigated the role of CD30L in the development of allergic rhinitis with the use of CD30L KO mice.

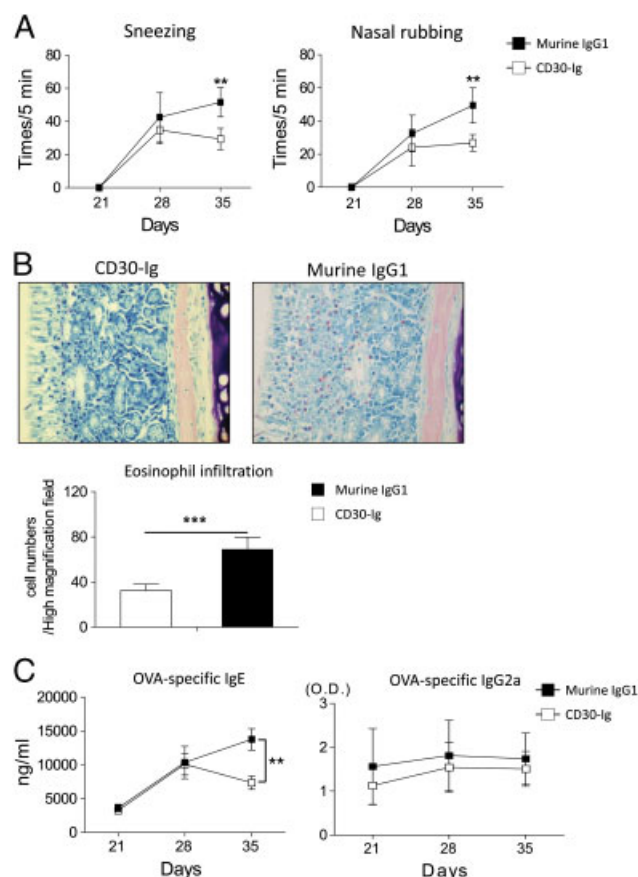


Figure 5. Effects of soluble CD30-Ig fusion protein on allergic rhinitis. (A) The numbers of sneezes and nasal rubbing. OVA-sensitized WT mice were challenged i.n. with OVA together with CD30-Ig fusion protein or murine IgG1 on days 21–35. The numbers of sneezes (left) and incidences of nasal rubbing (right) were counted 5 min after the last i.n. challenge with CD30-Ig or murine IgG1. (B) The absolute number of eosinophils in the nasal mucosa. Coronal nasal sections were stained with Hansel solution. Results are shown at 100 $\times$ magnification and are representative of three independent experiments. (Bottom) The absolute number of eosinophils infiltrated in the nasal mucosa was counted microscopically 12 h after final OVA i.n. challenge together with CD30-Ig fusion protein or murine IgG1. (C) OVA-specific Abs in CD30-Ig treated mice. OVA-specific IgE (left) and IgG2a (right) were measured in the sera of OVA-sensitized WT mice treated with murine IgG1 or CD30-Ig. Data are geometric means $\pm$ SD and are representative of three independent experiments. Five to eight mice were used in each experiment (** p <0.01, *** p <0.001, Student's *t*-test).

After OVA sensitization, the normal level of Ag-specific IgE production could be detected in CD30L KO mice. However, allergic inflammation was attenuated in OVA-sensitized CD30L KO mice after i.n. challenge with OVA as determined by the allergic symptoms, eosinophil accumulation, and Th2 cell response in NALT. The memory OVA-specific CD4⁺ Th2 cell response with IgE production was amplified during the effector step of allergic rhinitis in the presence of CD30L but not in the absence of CD30L. Thus, these results demonstrate that CD30L plays an important role in controlling allergic rhinitis development through the amplification of the Th2 cell response during the effector phase. Notably, *in vivo* treatment with CD30-Ig

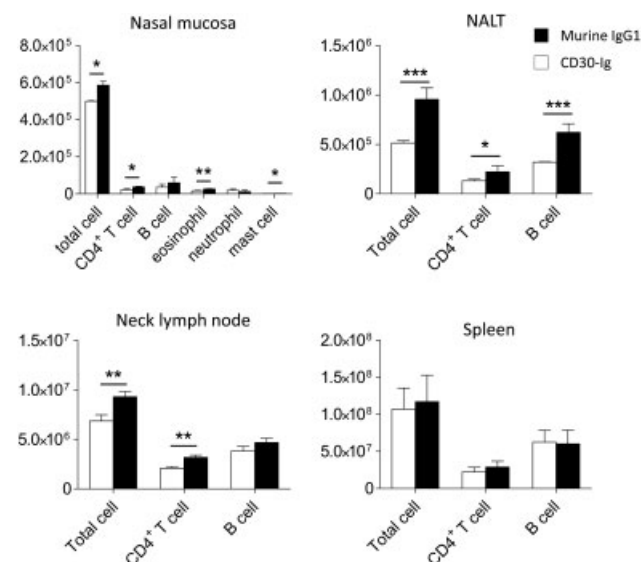


Figure 6. Cell numbers in nasal mucosa, NALT, cervical LNs and spleens of CD30-Ig-treated mice. Cells in nasal mucosa, NALT, cervical LNs, or spleens of OVA-sensitized WT mice were harvested after the final OVA i.n. challenge together with CD30-Ig or murine IgG1 (B cell: B220⁺CD3⁺; eosinophil: CD11b⁺Gr-1^{dim}; neutrophil: CD11b⁺Gr-1^{high}; mast cell: c-kit⁺FcεRIα⁺IgE⁺). The stained cells were analyzed by flow cytometry. The absolute numbers of each of the subsets were determined by multiplying the percentage of the corresponding cells by the absolute number of cells. Each group consisted of five mice. Data are geometric means $\pm$ SD and are representative of three independent experiments. Five to eight mice were used in each experiment (* p <0.05, ** p <0.01, *** p <0.001, Student's *t*-test).

markedly suppressed the development of allergic rhinitis, suggesting that soluble CD30 can be useful to suppress the development of allergic inflammation.

It is reported that the expression of CD30 at the surface of different immune cells or in a soluble form in the serum could be linked to Th2 polarization and allergic diseases including asthma [10]. In the initial studies, the concentration of the soluble form of CD30 is higher in patients with asthma or atopic dermatitis than in healthy controls and correlates directly with the severity of the disease [28]. It was demonstrated that the level of CD30 expression at the surface of CD4⁺ T cells was correlated with the production of IL-4 after restimulation of CD4⁺ T cells isolated from allergic patients [29, 30]. Polte et al. have recently reported, using CD30KO mice and anti-CD30 or CD30L mAb-treated mice, that CD30L/CD30 signaling plays a critical role in the development of acute airway inflammation in a murine asthma model, and speculated that CD30 seems to mainly modulate the initial phase of antigen/allergen contact when T-cell priming occurs [10, 31]. This finding suggested that CD30 might contribute to the generation of a large amount of Ag-specific T cells at the peak of primary response through proliferating and surviving signals. Although the proliferation of spleen cells of CD30KO mice was reduced as compared with WT mice, they were not completely anergic but still responded to OVA. In the present study, we found that normal production of OVA-specific IgE was measured

in CD30L KO mice during the induction phase after OVA sensitization, but severely impaired IgE production after i.n. challenge with OVA during the effector phase. The effector phase in allergic rhinitis has characteristics of rhinorrhea, sneezing, pruritus, nasal congestion, and infiltration of the inflammatory cells such as eosinophils, neutrophils, basophils, mast cells, and mononuclear cells, which are induced mainly by memory CD4⁺ T cells [17, 18]. It was also shown that Th2 cell responses mediated by OVA-specific CD4⁺ T cells in NALT and neck LNs are impaired during the effector phase. There are several lines of evidence that preactivated T cells could be stimulated by CD30L, instead of resting naïve T cells [7, 32]. CD30 activates NF- κ B activation [33] resulting in increased T-cell survival [34]. Thus, CD30L/CD30 signaling might promote preactivated Th2 cell survival after first OVA sensitization and accelerate the Th2 response after intranasal OVA challenge. CD30L/CD30 signaling may play an important role in the proliferation of differentiated effector or memory Th2 cells.

It was demonstrated that CD30L/CD30 signaling is involved in Th1 cell response-associated diseases [8, 9]. We previously reported that Th1-type colitis of trinitrobenzene sulfonic acid (TNBS)-induced acute colitis was suppressed in CD30L KO mice. Furthermore, Th1-type cytokine IFN- γ production by T cells of the lamina propria decreased significantly in TNBS-treated CD30L KO mice as compared with WT mice [12]. We also found that CD30L KO mice showed reduced Th1 responses and increased susceptibility to infection with *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) [13]. These results demonstrated that CD30L is indeed associated with Th1-cell response-associated diseases. Furthermore, it was reported that naturally occurring Treg cells suppress allograft rejection mediated by memory CD4⁺ T cells in a CD30-dependent manner [11]. We have recently found that CD30L/CD30 signaling plays a critical role in Th17 cell differentiation in vitro and in vivo [13]. Thus, CD30L/CD30 signaling might be essential for the proliferation and/or activation of effector or memory Th cells, irrespective of Th subtype, but not be directly associated with the physiological step for the specific Th-cell differentiation.

Our results obtained by using CD30L KO mice elucidated that allergic rhinitis was impaired or never fully developed during the effector phase in the absence of CD30L. These findings suggest that blocking of CD30L might be useful for the therapy of allergic rhinitis. CD30-Ig might be useful for treating allergic rhinitis. Although cross-linking with CD30-Ig can induce reverse signaling from CD30L [35], a soluble form of CD30-Ig significantly inhibited Th1 and Th17 responses in vivo and in vitro [15]. We have recently found that the administration of CD30-Ig in vivo prevented dextran sulphate sodium-induced colitis, which is associated with both Th1 and Th17 responses in intestinal mucosa [15]. In fact, in this study, it was shown that CD30-Ig treatment inhibited the development of allergic rhinitis mediated by allergen in mice. Polte et al. recently showed that the application of inhibitory mAbs directed against the CD30 receptor (anti-CD30mAb) or ligand (anti-CD153mAb) to OVA-sensitized BALB/c mice that already had an asthma-like phenotype did not reverse this established phenotype

[10]. At present, we cannot explain the discrepancy between their asthma model and our rhinitis model. However, it is possible that direct administration of CD30-Ig at a local site in allergic responses is more effective than systemic administration of inhibitory mAb to CD30 or CD30L. Our findings suggest that CD30L/CD30 signaling is more important in the secondary response at the allergic inflammatory site rather than during the initial phase of antigen priming in the LNs and spleen. The suppression of CD30L/CD30 signaling by soluble CD30-Ig can suppress or regulate rhinitis development and might be useful for therapy for allergic rhinitis.

Materials and methods

Mice

Age- and sex-matched BALB/c mice were purchased from Japan KBT (Shizuoka, Japan). The generation and preliminary characterization of CD30L KO mice were previously described [11, 36] and have been backcrossed onto the BALB/c strain more than eight times. All mice were maintained under specific pathogen-free conditions, were offered food and water ad libitum, and were used at the age of 6–8 wk. This study was approved by the Committee on Ethics on Animal Experiments, Kyushu University Faculty of Medicine.

Abs and reagents

FITC-conjugated anti-mouse IgE, FITC-conjugated CD3, FITC-conjugated anti-mouse CD11b, PE-conjugated Gr1, PE-conjugated c-kit, PE-conjugated B220 and allophycocyanin-conjugated CD4 mAbs were purchased from BD PharMingen (San Diego, CA, USA). Biotin-conjugated anti-mouse/human CD45R (RA3-6B2) and anti-mouse NK1.1 (PK136), purified anti-mouse CD11b (M1/70), and PE-conjugated anti-CD117 (ckit, 2B8) were obtained from eBioscience (San Diego, CA, USA). Biotin-conjugated anti-mouse CD11c (HL3) and anti-mouse I-A^b (25-9-3) and PE-conjugated anti-Fc ϵ RI α (MAR-1) were obtained from Becton Dickinson (Franklin Lakes, NJ, USA). MicroBeads conjugated to monoclonal rat anti-mouse CD4 (L3T4) antibodies (GK1.5), MicroBeads conjugated to goat anti-rat IgG, and MicroBeads conjugated to streptavidin were purchased from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany). OVA (grade V) was purchased from Sigma (St. Louis, MO, USA). Alum was obtained from LSL (Tokyo, Japan).

Sensitization and challenge

Mice were sensitized by i.p. injection with 25 μ g OVA or PBS mixed with 1 mg alum on days 0, 7, and 14. Each day from day 21 to day 35 after the first sensitization, mice were i.n. challenged with 20 μ L of OVA dissolved in PBS (20 μ g/mL) or PBS only into the bilateral nostril.

Nasal signs

As per the previous description [25], mice were placed into an observation cage (one animal/cage) for about 10 min and then were i.n. challenged with OVA or PBS. After the i.n. challenge with OVA, the frequencies of sneezes in 5 min were counted using the method of Sugimoto et al. [37].

Measurement of OVA-specific Abs

OVA-specific antibodies productions on days 21, 28 and 35 were determined, as described previously [25].

Measure OVA-specific cytokine production by CD4⁺ T cells in NALT, cervical LNs, and spleen

Mononuclear cells from NALT were isolated as described [23, 38]. In brief, the mononuclear cells were isolated by gentle teasing through stainless steel screens, and FACS analysis was performed. NALT and spleen cells were harvested 12 h after the final OVA i.n. challenge, and then CD4⁺ T cells from NALT, cervical LNs, or spleen were purified by autoMACS (Miltenyi Biotec GmbH). 2×10^5 of purified CD4⁺ T cells were cultured in round-bottom microtiter plates with irradiated spleen cells (1×10^6) and OVA (200 µg/mL) in complete RPMI 1640 medium containing 10% FBS, 5 µM 2-ME, 10 U/mL penicillin, and 100 µg/mL streptomycin for 96 h at 37°C in 5% CO₂. The levels of cytokines in the supernatants were determined by ELISA using a Cytokine Development Kit (GT, Minneapolis, MN, USA).

Histological examination

Mice were killed 12 h after the final OVA i.n. challenge, and then histologic examination was implemented. Cell numbers of eosinophils were counted on each side of the posterior edge of the nasal septum microscopically in a high-power field, as described previously [25].

In vivo treatment with soluble CD30-Ig

A soluble murine CD30 and Ig fusion protein (CD30-Ig) was obtained using a method previously described [7, 15]. The CD30-Ig fusion cDNA was expressed in the vector pBMGNeo by NIH 3T3 cells. The secreted CD30-Ig protein by growing NIH 3T3 cells with serum-free medium (SFM101; Nissui Pharmaceutical, Tokyo, Japan) was purified by HiTrap Protein G HP (Amersham Biosciences) and analyzed by SDS-PAGE for purity. CD30-Ig, diluted to 0.5 mg/mL in PBS, was stored at –80°C until use. For in vivo neutralization, 400 µL of CD30-Ig or isotype control murine IgG1 (Biolegend, San Diego, CA, USA) was i.n. injected into OVA-sensitized WT mice on days 21–35. Counting the

frequencies of sneezes and histologic examination of nasal mucosa were implemented as described above.

Statistical analysis

The statistical significance of the data was determined by Student's *t*-test. A value of $p < 0.05$ was considered significant.

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Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: Alum: aluminum hydroxide hydrate gel · CD30L: CD30 ligand · NALT: nasopharynx-associated lymphoid tissues

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