

Immunopharmacology and inflammation

IgE and IgA produced by OX40–OX40L or CD40–CD40L interaction in B cells–mast cells re-activate FcεRI or FcαRI on mast cells in mouse allergic asthma

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ABSTRACT

Mast cells are major effector cells of allergic diseases related to IgE. This study was undertaken to determine whether IgE or IgA, produced by CD40–CD40L or OX40–OX40L interactions between B cells and mast cells, re-activate FcεRI or FcαRI on mast cell surface. C57BL mice were sensitized and subjected to OVA challenge to induce asthma. Bone marrow-derived mast cells (BMMCs) and primary B cells were co-cultured. Mast cell recruitment into airways was stained by May–Grünwald Giemsa, the expression of markers or signaling molecules was determined by immunohistochemistry or Western blotting, and co-localization of B cells and mast cells by immunofluorescence. Anti-CD40 plus anti-OX40L Abs synergistically reduced IgE and IgA production, and mediators (histamine, LTs and cytokines) released in mast cells, and additively reduced other responses, such as, numbers of mast cells, the expression of markers (tryptase, mMCP5, B220 and CD19), surface molecules (CD40, CD40L, OX40 and OX40L), FcεRI or FcαRI and the co-localization of BMMCs and B cells, and IgE- or IgA-producing cells, as compared with individual blocking Ab treatment which reduced responses in BAL cells or lung tissues of OVA-challenged mice or in co-culture of B and mast cells. The data suggest that IgE and IgA, produced by OX40–OX40L or CD40–CD40L interaction between B cells and mast cells, may re-activate receptors of FcεRI and FcαRI on mast cell surfaces, followed by more mediator release, and furthermore, that treatment with anti-CD40 plus anti-OX40L Abs offers a potential treatment for allergic asthma.

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1. Introduction

Mast cells are well-known major effector cells of allergic asthma (Elias et al., 2003) and are activated by the cross-linking of antigen-specific IgE bound to high-affinity receptor (FcεRI) on their membranes, followed by secretion of mediators [histamine, leukotrienes (LTs), and cytokines], which mediate a series of events, such as, the development of airway inflammation and B cell Ig isotype class switching (Galli et al., 2005; Hong et al., 2013a). However, FcαRI receptor is not expressed in mouse mast cells, although human eosinophils and mast cells express FcαRI receptor (Gloudemans et al., 2013; Monteiro and van Del Winkel, 2003). Mast cells also express co-stimulatory molecules like CD40L, which is a natural ligand of CD40 and a member of the TNF-α family (Kim et al., 2010). CD40, a type I transmembrane protein belonging to the TNFR superfamily, is expressed on various cell types, such as, B cells and

dendritic cells (Kim et al., 2010). CD40L-expressing mast cells can trigger IgE synthesis in vitro (Hong et al., 2013a) or IgA synthesis (Avery et al., 2008; Merluzzi et al., 2010) through B cell Ig class switching via CD40–CD40L interaction and cytokines (IL-4, IL-13 or IL-6) (Bishop, 2012).

A fully human anti-CD40 monoclonal Ab (ASKP1240) showed modest clinical activity in multiple myeloma (Bensinger et al., 2012), predicted organ allograft survival in animal models (Watanabe et al., 2013), and is being tested as a treatment for malignancies (Hassan et al., 2014). Although the effects of anti-CD40 Ab on allergic asthma have not been characterized in vivo, they have been shown in vitro (Hong et al., 2013a). CD40–CD40L interaction induces a variety of signaling molecules such as TNF receptor-associated factors 2 and 6 (TRAF2/6), MEK kinase 1 (MEKK1) and TGFβ-activated kinase 1 (TAK1) in various cell types (Häcker et al., 2011).

OX40, a transmembrane protein found on activated CD4⁺ T cell surfaces, controls memory Th2 cells that induce lung inflammation (Salek-Andakani et al., 2003), and OX40L is expressed on various cells including B and mast cells (Hong et al., 2013b). The OX40–OX40L interaction regulates T cell-mediated inflammatory diseases (Ishii et al., 2010; Wang et al., 2013), inflammation to sepsis (Karulf et al.,

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2010), the pathogenesis of idiopathic inflammatory myopathies (Papadopoulos et al., 2013), allergic inflammation (Gauvreau et al., 2014; Kaur and Brightling, 2012; Li et al., 2011; Siddiqui et al., 2010), and mast cell activation via Treg cells (Park et al., 2013).

As demonstrated by the above, mast cells are associated with the productions of IgE and IgA by B cell Ig class switching via the CD40–CD40L, but not via the OX40–OX40L. Furthermore, anti-CD40 and anti-OX40L Abs therapies did not completely reduced production of IgE/IgA and mediator release in allergic asthma. Therefore, we hypothesized that IgE/A are produced via OX40–OX40L interaction as well as via CD40–CD40L between B and mast cells in allergic asthma, and the produced IgE or IgA may re-activate FcεRI- and FcαRI-mediated mast cells. We observed that IgE and IgA, produced via the OX40–OX40L and CD40–CD40L interactions in B cells and mast cells in the presence of cytokines, re-activate each receptor on mast cell surface.

2. Materials and methods

2.1. Materials

Ovalbumin (Grade V), aluminum hydroxide gel adjuvant (2% Alhydrogel), DNP-HSA, DNP-KLH and ethidium bromide (EtBr) were purchased from Sigma-Aldrich (St. Louis, MO); Diff-Quik from System Corporation (Kobe, Japan); anti-CD40 Ab, anti-OX40L Ab, IgG Ab and OX40 agonist from BioLegend (San Diego, CA); CD40L agonist from Enzo Life Science Inc. (Farmingdale, NY); May-Grünwald–Giemsa solution from Merck (Darmstadt, Germany); mounting solution from Fisher Chemicals (Fair Lawn, NJ); Abs against CD19, B220, CD40, OX40, TAK and MEKK1 from Abcam (Cambridge, MA); Abs against HRP-conjugated goat anti-mouse, mMCP5, IgE, IgA FITC or TEXAS-Red-conjugated anti-IgG from Zymed Laboratories Inc. (San Francisco, CA); diaminobenzidine (DAB) from Dako (Glostrup, Denmark); aqueous mounting medium from Thermo Shandon (Pittsburgh, MA); aprotinin and leupeptin from Roche (Basel, Switzerland); nitrocellulose membranes, [γ -³²P]ATP (specific activity, 3000 Ci/mmol) and G-25 spin column from Amersham Biosciences (Buckinghamshire, UK); Trizol reagent from Invitrogen (Carlsbad, CA); amfiRivert one-step RT-PCR kit from GenDEPOT (Barker, TX) and all primers from Bionics, Korea; ELISA kit for total and specific IgE, HRP-conjugated goat anti-mouse or HRP-conjugated rabbit anti-goat IgG or HRP-conjugated goat anti-rabbit IgG from Bethyl Laboratories (Montgomery, TX); ELISA kit for total and specific IgA from Antibodies-online.com (Atlanta, GA); ELISA kit for each cytokines from BD Bioscience (San Jose, CA); ELISA kit for histamine or LTs from Neogen Corp. (Lexington, KY) and Cayman Chemical (Ann Arbor, MI), respectively; NF-κB oligonucleotide from Promega (Madison, WI); MagCelect mouse B cell isolation kit from R&D Systems, Inc. (Minneapolis, MN); anti-mouse IgM Ab from Jackson ImmunoResearch Laboratories (West Grove, PA); Abs against mMCP5, OX40L, TRAF2/6, MAP kinases from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); Abs against p-TAK, p-MEKK1, p-MAP kinases from Cell signaling (Beverly, MA).

2.2. Animals

Female C57BL/6 mice weighing 20 g (8 weeks old) were obtained from ORIENT BIO (Seongnam, South Korea). All animals were bred and maintained in specific pathogen-free conditions at the Laboratory Animal Research Center at Sungkyunkwan University (Suwon, South Korea). Animals were treated in accordance with the guidelines issued by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC), and all animal experiments were approved by the Institutional Review Board in the Laboratory Animal Research Center of Sungkyunkwan University.

2.3. Sensitization, challenge, and experimental protocol

C57BL/6 mice were divided into six groups (eight mice/group): NC (negative control) or OVA groups (mice were sensitized and nebulized with PBS or OVA, respectively); αCD40, αOX40L, αCD40 plus αOX40L or IgG Ab [OVA mice pretreated with anti-CD40 blocking Ab (Clone HM40-3), anti-OX40L blocking Ab (Clone RM134L) or both Abs once a day 30 min before OVA challenge for 3 days]. The mice were sensitized with 20 μg/200 μl OVA adsorbed in 1 mg/50 μl aluminum hydroxide gel adjuvant delivered by i.p. injection on days 1 and 15. Mice were then challenged with 5% OVA in PBS delivered nasally for 30 min once daily for 3 days (from day 22 to day 24) using a nebulizer (Mega Medical, Seoul, Korea). The NC mice were sensitized and nebulized with PBS. All mice were killed on day 25 (Hong et al., 2013a).

To block CD40–CD40L or OX40–OX40L interactions, anti-CD40 (Clone HM40-3), anti-OX40L Ab (Clone RM134L) or IgG Ab (300 μg) were dissolved in PBS and administered i.p. daily 30 min before OVA challenge for 3 days (from days 22 to days 24) (Hong et al., 2013a). IgG Ab used as a positive control Ab was pretreated in the same manner as the other two blocking Abs.

2.4. Bronchoalveolar lavage (BAL) fluid and May-Grünwald–Giemsa staining

BAL fluid was collected, and inflammatory cells infiltrated into BAL fluid were stained with Diff-Quick, and mast cells in BAL cells and lung tissues were stained with May-Grünwald–Giemsa and quantified by light microscopy. Numbers of mast cells were quantified in 5 sites of 200 × 200 μm² (5 areas/each slide × 8 mice/each group = 40 areas) (Hong et al., 2013a) (see Supplementary data).

2.5. Immunohistochemistry (IHC) and immunofluorescence

Deparaffinized and blocked lung sections (3 μm) were prepared as described previously (Hong et al., 2013a) (see Supplementary data).

2.6. Western blot analysis

Immunoblotting was performed on the protein extracts of BAL cells, BMMCs, B cells (1 × 10⁶ cells), or lung tissues (50 mg/50 μl) using a previously described method (Hong et al., 2013a) (see Supplementary data).

2.7. Measurement of total and OVA-specific serum IgE and IgA

Total and OVA-specific IgE or total and OVA-specific IgA were determined in sera obtained from blood samples, BAL fluid (in vivo) and supernatants (200 μl) isolated from the media of B cells co-cultured with BMMCs or from the media of B cells stimulated with CD40L agonist (soluble mouse recombinant CD40L) or OX40 agonist (OX40 purified anti-CD134 Ab; LEATTM) using ELISA (in vitro). The lowest detection limits for total and OVA-specific IgE or IgA were better than 2.1 ng/ml and 1.5 ng/ml, 3.5 ng/ml and 5 ng/ml, respectively (Hong et al., 2013a) (see Supplementary data).

2.8. Immunoassay for cytokines, histamine and LTs

In BAL fluid, the supernatants from lung tissue homogenates (20,000 × g, 10 min) or from media of B cells stimulated with CD40L or OX40 agonist, amounts of cytokines were determined using an ELISA kit. The lowest detection limit for IL-4 was better than 31.3 pg/ml, for IL-6 was 7.8 pg/ml, and for IL-13 and TGF-β was better than 15.6 pg/ml.

The amounts of histamine and LTs in BAL fluid or each supernatant were determined using ELISA kit. The lowest detection limits for histamine or LTs were 2.5 ng/ml and 7.8 pg/ml, respectively (Hong et al., 2013a) (see Supplementary data).

2.9. Culture and activation of bone marrow-derived mast cells (BMMCs)

Bone marrow cells, which were flushed from mouse femurs and tibias, were cultured for 5 weeks in RPMI-1640 containing IL-3. The purity for BMMCs as determined by May-Grünwald-Giemsa staining was more than 95% of total cells (Hong et al., 2013a, 2013b) (see Supplementary data).

BMMCs (1×10^6 cells) were sensitized with 0.1 μ g/ml anti-DNP IgE Ab for Fc ϵ RI-mediated responses or 10 μ g/ml anti-DNP IgA for Fc α RI-mediated responses overnight at 37 °C. Cells were then washed twice and challenged with 10 ng/ml DNP-HSA and 10 μ g/ml DNP-KLH, respectively (act-BMMCs) for 5 h or the times indicated at 37 °C (Decot et al., 2005; Hong et al., 2013a).

To examine the activation of BMMCs by CD40L (soluble mouse recombinant CD40L) and OX40 (OX40 purified anti-CD134 Ab; LEATTM) as agonists, BMMCs (1×10^6 cells) were activated with 400 ng/ml of CD40L agonist or OX40 agonist (referred to as CD40L-sti-BMMCs or OX40-sti-BMMCs, respectively). Cells were incubated for 5 h or for the times indicated at 37 °C, and after activation, cells were examined for the expression of signaling molecules (Richman and Vonderheide, 2014).

2.10. Isolation and activation of B cells or activation of B cells co-cultured with mast cells

B cells were isolated from the spleens of C57BL/6 mice using the MagSelect mouse B cell isolation kit, and confirmed the CD19 and B220 in B cell surface (Meher et al., 2014; Kanda et al., 2010) using a LSM5 EXCTTER confocal microscope (Carl Zeiss, Oberkochen, Germany). B cell purities were more than 94% of total cells.

B cells (1×10^6 cells) were incubated with 0.5 μ g/ml anti-mouse IgM Ab for the times indicated (5 h for expression of surface molecules; 48 h for IgE or IgA production) (referred to as act-B cells) (Hong et al., 2013a). act-B cells (1×10^6 cells) were added on non-act-BMMCs (BMMCs alone) or act-BMMCs (1×10^6 cells) that were activated with Ag/Ab for 5 h, and then co-cultured for 72 h.

To block CD40–CD40L and OX40–OX40L interactions, anti-CD40 Ab or anti-OX40L Ab (300 ng/ml) were pretreated 1 h before B cell activation. Total IgE or IgA levels secreted to culture media were determined by ELISA. The optimal incubation time (72 h) and ratio (1:1) for co-culture were as we previously reported (Hong et al., 2013a). Optimal times and doses required for the expression of surface molecules in B cells and BMMCs or for blocking Abs were determined by preliminary experiments.

2.11. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from BMMCs, B cells, BAL cells (1×10^6 cells), and lung tissues (50 mg/500 μ l), and EMSA was performed as described previously (Hong et al., 2013a) (see Supplementary data).

2.12. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated from BAL cells (1×10^6 cells) or lung tissues (50 mg/500 μ l) using Trizol reagent. RT-PCR was performed in a final volume of 50 μ l using a amfiRivert one-step RT-PCR kit (GenDEPOT, Barker, TX) in an automated thermal cycler (BIOER Technology, Hangzhou, China). PCR assays were performed over 35 cycles, and products were analyzed using 1% agarose gel containing ethidium bromide (EtBr) (Hong et al., 2013a) (see Supplementary data).

2.13. Statistical analysis

Experimental data are presented as mean $\pm$ S.E.M. ($n=8$). Multi-group comparisons were performed using one-way ANOVA followed by Scheffe's *posthoc* test using SPSS (SPSS Inc., Chicago). *P* values of <0.05 were regarded significant. Densitometry analyses of western blots were performed using Quantity One version 4.6.3 (Bio-Rad, Hercules). Histograms of densitometry results are presented as the mean $\pm$ S.E.M. ($n=4$) of four independent experiments.

3. Results

3.1. Effects of anti-CD40 Ab and anti-OX40 Ab on surface molecules in BAL cells and lung tissues of OVA-challenged mice

In order to investigate Ig class switching via the OX40–OX40L interaction, we used CD40–CD40L interaction as a positive control and their blocking Abs. We first observed that surface molecules, such as CD40, CD40L, OX40 and OX40L, were enhanced in the BAL cells (Fig. 1A and B) and lung tissues (Supplementary Fig. 1A and B) of OVA-challenged mice. Anti-CD40 Ab or anti-OX40L Ab reduced the expression of CD40–CD40L and OX40–OX40L, respectively, in the BAL cells and lung tissues of OVA mice. Combined pretreatment with both Abs specifically inhibited the expression of surface molecules in a similar manner to that observed for separately blocking Ab-pretreated mice.

IgG Ab as positive control instead of blocking Abs did not affect expression of surface molecules in BAL cells and lung tissues of OVA-challenged mice. Therefore, hereafter, we did not describe any more about the data for all IgG due to no responses in IgG treatment.

3.2. Effects of anti-CD40 Ab and anti-OX40 Ab on IgE and IgA production in the sera or BAL fluid of OVA-challenged mice

We found that total and OVA-specific IgE and IgA levels were elevated in the sera of OVA-challenged mice (Fig. 1C and D). Anti-CD40 Ab reduced total and OVA-specific IgE (total, 194 ± 12.8 ng/ml; specific, 142 ± 8.4 ng/ml) and IgA serum levels (total, 305 ± 18.4 ng/ml; specific, 59 ± 12.5 ng/ml) versus OVA mice, which showed increased IgE and IgA levels (total, 342 ± 16.5 ng/ml; specific, 246 ± 10.5 ng/ml for IgE; total, 526 ± 23.2 ng/ml; specific, 98 ± 10.5 ng/ml for IgA) versus NC mice (total, 21 ± 1.1 ng/ml; specific, 1 ± 0.5 ng/ml for IgE; total, 34 ± 6.5 ng/ml; specific, 1 ± 0.5 ng/ml for IgA).

Anti-OX40L Ab also reduced total and OVA-specific IgE (total, 277 ± 13.2 ng/ml; specific, 197 ± 7.5 ng/ml) and IgA levels (total, 421 ± 20.5 ng/ml; specific, 79 ± 11.3 ng/ml) in serum versus OVA mice. When both Abs were pretreated, they synergistically inhibited IgE levels (total, 96 ± 10.2 ng/ml; specific, 74 ± 9.2 ng/ml) and IgA levels (total, 151 ± 12.5 ng/ml; specific, 29 ± 10.4 ng/ml) versus each Ab-pretreated mice (Fig. 1C and D). These observations suggest that IgE and IgA are produced through B cell Ig class switching via the OX40–OX40L as well as CD40–CD40L interaction. However, OVA mice showed much higher total IgA levels than total IgE levels, but showed much lower OVA-specific IgA levels than OVA-specific IgE levels.

Total and OVA-specific IgE and IgA levels were also elevated in BAL fluid, but the produced Abs showed much lower levels than those in sera (Supplementary Fig. 1C and D). Anti-CD40 or anti-OX40L Ab reduced total and OVA-specific IgE and IgA levels in the similar pattern to those in sera.

We also examined levels of cytokines (IL-4 and IL-13), which are required to B cell Ig class switching for IgE, and of cytokines (IL-6 and TGF- β) for IgA in BAL fluid. OVA mice increased levels of these cytokines (from 20 ± 10.4 to 325 ± 45.2 pg/ml for IL-4; from 62 ± 10.2 to 724 ± 50.4 pg/ml for IL-13; from 50 ± 11.5 pg/ml to 542 ± 32.5 pg/ml for IL-6; from 46 ± 10.8 to 625 ± 50.4 pg/ml for

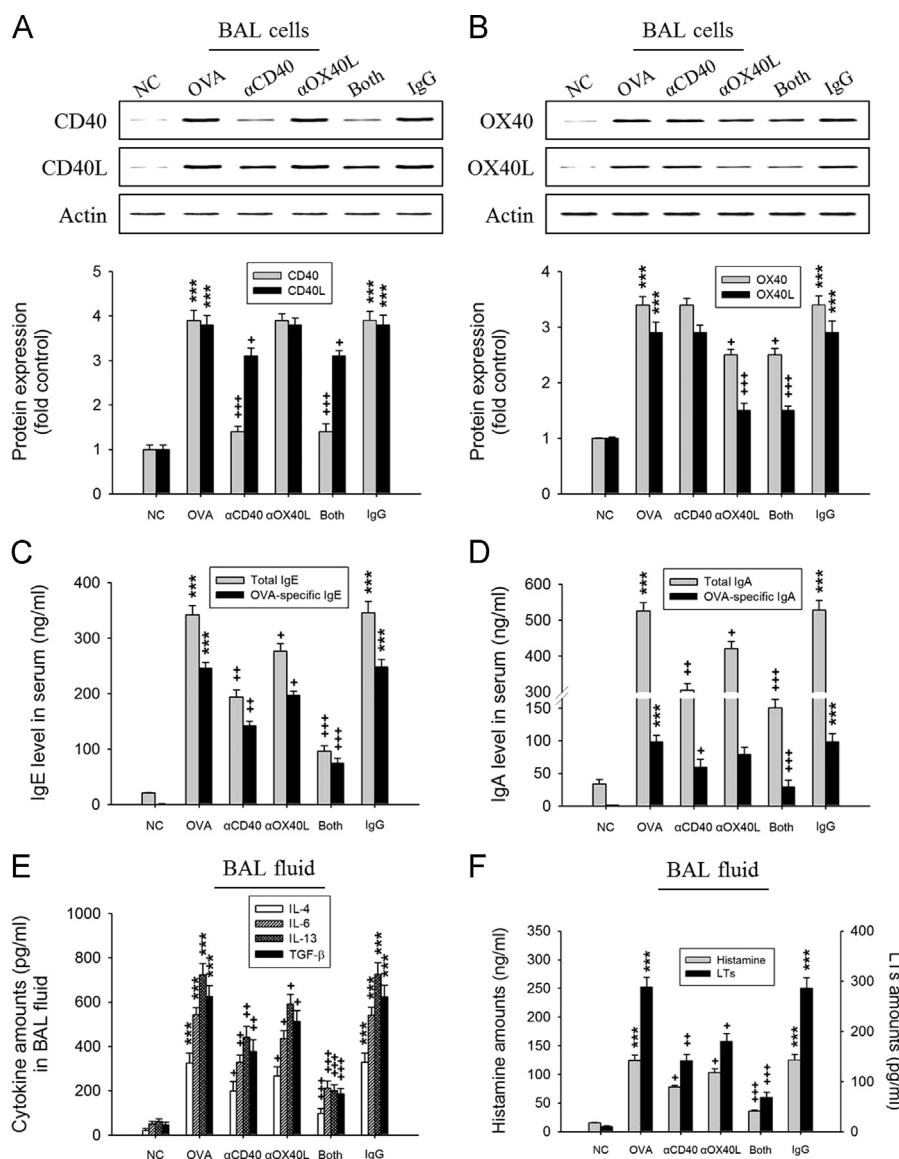


Fig. 1. Effects of anti-CD40 and anti-OX40L Abs on the expression of surface molecules, serum total and OVA-specific IgE and IgA levels, and on levels of mediators in BAL cells of OVA-challenged mice. C57BL/6 mice were divided into six groups (eight mice/group), as follows: NC (negative control) or OVA groups (mice were sensitized and nebulized with PBS or OVA, respectively), α CD40, α OX40L, α CD40 plus α OX40L or IgG Ab (used as a positive control) groups [OVA mice were pretreated with anti-CD40 blocking Ab (clone HM40-3), anti-OX40L blocking Ab (clone RM134L), anti-CD40 plus anti-OX40L Abs or IgG once daily 30 min before OVA challenge for 3 days]. Mice were sensitized with 20 μ g/200 μ l OVA adsorbed in 1 mg/50 μ l of aluminum hydroxide gel adjuvant delivered by i.p. injection on days 1 and 15, and then challenged nasally with 5% OVA in PBS for 30 min once daily for 3 days (from day 22 to day 24) using a nebulizer. NCs were sensitized and nebulized with PBS. All mice were killed on day 25. Expression of surface molecules was determined in protein extracts isolated from BAL cells by Western blotting. Levels of total and OVA-specific IgE and IgA in sera, and amounts of cytokines, histamines and LTs in BAL fluids were determined by ELISA. (A) and (B) Expression of CD40 and CD40L, and OX40 and OX40L, respectively. The histogram of densitometry results shows mean $\pm$ S.E.M. ($n=4$) of the ratios of the band densities of surface molecules versus the control and actin of four independent experiments. (C) and (D) Levels of total and OVA-specific IgE and IgA in sera, respectively. (E) Amounts of cytokines related to Ig class switching in BAL fluid. (F) Amounts of histamine and LTs in BAL fluid. *** $P < 0.001$ versus NC mice. + $P < 0.05$; ++ $P < 0.01$; +++ $P < 0.001$ versus OVA mice.

TGF- β) (Fig. 1E). Mice treated with both Abs synergistically reduced amounts of IL-4 (to 97 ± 23.8 pg/ml), IL-13 (to 201 ± 23.2 pg/ml), IL-6 (to 212 ± 31.5 pg/ml), and of TGF- β (to 185 ± 24.2 pg/ml) in BAL fluid versus mice treated with anti-CD40 or anti-OX40L Abs (to 198 ± 43.2 pg/ml or to 267 ± 40.2 pg/ml for IL-4; to 442 ± 48.2 or to 592 ± 42.3 pg/ml for IL-13; to 328 ± 32.8 pg/ml or to 436 ± 36.4 pg/ml for IL-6; to 378 ± 52.4 pg/ml or to 512 ± 50.2 pg/ml for TGF- β , respectively). That is, anti-CD40 or anti-OX40L Ab reduced the levels of all cytokines versus OVA mice.

In lung tissue homogenates, mice pretreated with anti-CD40 plus anti-OX40L Abs showed lower amounts of IL-4 (from 889 ± 32.4 pg/ml to 235 ± 12.3 pg/ml), IL-13 (from 1832 ± 42.8 pg/ml to 784 ± 18.5 pg/ml), and of TGF- β (from 1684 ± 70.4 pg/ml to $503 \pm$

30.1 pg/ml) than mice treated with anti-CD40 or anti-OX40L Ab (Supplementary Fig. 1E).

Anti-CD40 (77 ± 3.2 ng/ml for histamine; 141 ± 12.8 pg/ml for LTs) or anti-OX40L Abs (104 ± 7.1 for histamine; 180 ± 15.3 pg/ml for LTs) reduced levels of histamine and LTs in BAL fluid versus OVA mice (128 ± 10.8 ng/ml for histamine; 288 ± 19.8 pg/ml for LTs), which showed higher levels of both versus NC mice (13 ± 0.7 ng/ml for histamine; 10 ± 1.4 pg/ml for LTs) (Fig. 1F). Mice pretreated with anti-CD40 plus anti-OX40L Abs showed synergistic inhibition of histamine (38 ± 2.6 ng/ml) and LTs (68 ± 10.8 pg/ml). In the lung tissues of OVA-challenged mice, anti-CD40 plus anti-OX40L Abs remarkably inhibited histamine and LT levels versus anti-CD40 or anti-OX40L Abs pretreated mice (Supplementary Fig. 1F and G).

3.3. Effects of anti-CD40 and anti-OX40L Abs on the recruitment and surface marker expression of mast cells or B cells into BAL fluid or lung tissues of OVA-challenged mice

We examined the effects of blocking Abs on mast cell infiltration in the BAL cells or lung tissues of OVA-challenged mice. Anti-CD40 Ab (18 ± 2.6 cells for BAL cells; 17 ± 1.3 cells for lung tissues) or anti-OX40L Ab (24 ± 2.7 cells for BAL cells; 28 ± 2.4 cells for lung tissues) pretreated mice reduced the increased mast cell infiltration (32 ± 2.9 cells for BAL cells; 37 ± 1.9 cells for lung tissues) versus NC mice (1 ± 0.2 cells for BAL cells; 2 ± 0.3 cells for

lung tissues) (Fig. 2A and B). Anti-CD40 plus anti-OX40L Abs pretreated mice (11 ± 1.0 cells for BAL cells; 17 ± 1.3 cells for lung tissues) showed additive reductions in the infiltration of mast cells into BAL fluid or lung tissues versus each blocking Ab.

Anti-CD40 plus anti-OX40L Abs pretreatment additively inhibited other inflammatory cells (macrophages, lymphocytes, neutrophils and eosinophils) in BAL (Supplementary Fig. 1H) and in lung tissues (data not shown) versus anti-CD40 or anti-OX40L Ab pretreated mice.

Furthermore, we observed that anti-CD40 plus anti-OX40L Abs pretreated mice (brown color) showed remarkable reduced

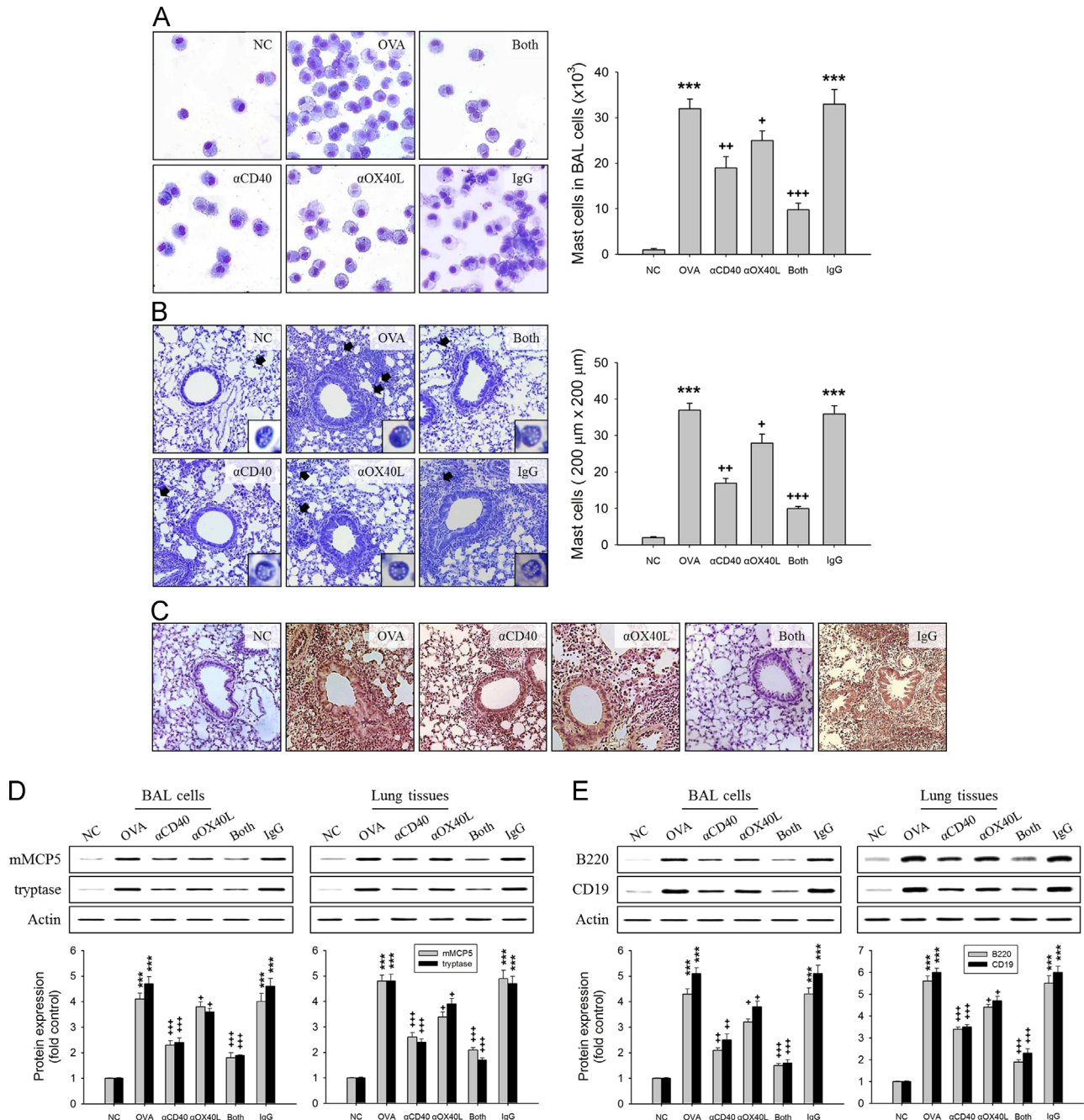


Fig. 2. Effects of anti-CD40 and anti-OX40L Abs on the population and markers of mast cells or B cells in BAL cells and lung tissues of OVA-challenged mice. Experimental details for animal models of allergic asthma, treatments with blocking Abs, and definitions for all symbols used are provided in the legend of Fig. 1. Numbers of mast cells stained with May-Grünwald-Giemsa were quantified at 5 sites in areas of 200 μ m $\times$ 200 μ m (5 sites/slide $\times$ 8 mice/group = 40 areas) under a microscope. Population of B cells was determined in lung tissue sections (3 μ m) by immunohistochemistry. Expression of surface markers was determined in protein extracts isolated from BAL cells or lung tissues by Western blotting. (A) and (B) Numbers of mast cells in BAL cells and lung tissues, respectively. (C) Population of B cells (brown color) using CD19 Ab. (D) and (E) Expression of markers in mast cells (mMCP5 and tryptase) and B cells (B220 and CD19). The histogram of densitometry results was obtained as described in Fig. 1 legend ($n=4$). *** $P < 0.001$ versus NC mice. + $P < 0.05$; ++ $P < 0.01$; +++ $P < 0.001$ versus OVA mice.

recruitment of B cells (CD19) versus anti-CD40 or anti-OX40L Ab pretreated mice, which inhibited the increased B cell recruitment in lung tissues of OVA mice (Fig. 2C).

We observed expression of mMCP5 and tryptase as mast cell surface markers and B220 and CD19 as B cell surface markers infiltrated into BAL cells and lung tissues (Fig. 2D and E). Anti-CD40 plus anti-OX40L Abs pretreated mice additively reduced increased expression in both cell markers in BAL cells and the lung tissues of OVA mice versus each Ab pretreated mice, which showed inhibition of these increased entities in BAL cells or lung tissues.

3.4. Effects of anti-CD40 and anti-OX40L Abs on the co-localization on mast cells and B cells or IgE/IgA-producing cells in the lung tissues of OVA-challenged mice

Mast cells (mMCP5) and B cells (CD19), IgE-producing cells or IgA-producing cells were co-localized in the lung tissues of OVA-challenged mice (Fig. 3). Furthermore, anti-CD40 plus anti-OX40L Abs (5 ± 0.6 cells) additively inhibited the co-localization of mast cells and B cells as compared with anti-CD40 or anti-OX40L Ab pretreated mice (9 ± 0.8 cells for anti-CD40 Ab; 12 ± 1.4 cells for anti-OX40L Ab), which inhibited the increased co-localization of c-kit and B cells versus OVA mice (23 ± 1.4 cells) (Fig. 3A). Co-localization of mast cells and IgE-producing cells (3 ± 0.4 cells) was reduced in the lung tissues of anti-CD40 plus anti-OX40L Abs pretreated mice versus that observed anti-CD40 or anti-OX40L Abs pretreated mice (7 ± 0.9 cells for anti-CD40 Ab; 10 ± 1.2 cells for anti-OX40L Ab). That is, anti-CD40 or anti-OX40L Abs pretreated mice inhibited the co-localization of mMCP5 and IgE-producing cells versus that observed in OVA mice (19 ± 1.8 cells) (Fig. 3B).

To confirm the co-localization of mast cells and B cells, we used IgG TEXAS-Red as an isotype control for B cells and IgG FITC as an isotype control for mast cells. Intensity of fluorescence for CD19 in B cell surface or for mMCP5 in mast cell surface was not shown by isotype Ab in lung tissues of OVA-challenged mice (Supplementary Fig. 2A and B). Thus, intensity of fluorescence in Fig. 3A was not autofluorescence, but showed image by the co-localization of mast cells and B cells. And, the co-localization of mast cells and B cells was shown using higher magnification ($\times 1000$) in small box in the upper corner (Supplementary Fig. 2C). Thus, both cells showed the interaction.

The co-localization of mast cells and IgA-producing cells (4 ± 1.2 cells) was also reduced in the lung tissues of anti-CD40 plus anti-OX40L Abs pretreated mice as compared with that observed in anti-CD40 or anti-OX40L Abs pretreated mice (8 ± 1.9 cells for anti-CD40 Ab; 12 ± 1.5 cells for anti-OX40L Ab), compared to that observed in OVA mice (22 ± 2.4 cells) (Fig. 3C). These observations indicate that mast cells and B cells, mast cells and IgE-producing cells, and mast cells and IgA-producing cells are co-localized in lung tissues of OVA-challenged mice.

3.5. Effects of anti-CD40 and anti-OX40L Abs on the expression of surface molecules in the BMMCs or B cells separated from co-culture

To investigate whether expression of surface molecules and IgE/IgA production observed in vivo, which has a variety of cell types, occur in vitro, we examined IgE and IgA production using co-culture of B cells and BMMCs (72 h) at a ratio of 1:1, as reported previously (Hong et al., 2013a). The optimal time (5 h) required for expression of surface molecules in BMMCs activated with Ag/Ab reaction (act-BMMCs) or B cells activated with anti-IgM Ab (act-B cells), and the optimal time (60 min) and dose (300 ng/ml) required for inhibition of blocking Ab pretreatment were determined in preliminary experiments (Supplementary Fig. 3A–C). CD40 was originally expressed in non-act-BMMCs (BMMCs alone), and CD40L expression was enhanced in act-BMMCs separated after co-culture (referred to as co-cultured-act-BMMCs). The expression of CD40 was enhanced

in act-B cells separated after co-culture (referred to as co-cultured-act-B cells), but CD40L was not (Fig. 4A). The expression of OX40 and OX40L was also enhanced in co-cultured-act-BMMCs. In co-cultured-act-B cells, the expression of OX40L was enhanced, but OX40 was not expressed (Fig. 4A).

Anti-CD40 Ab or anti-OX40L Ab inhibited expression of CD40 and OX40L, respectively, in co-cultured-act-BMMCs (Fig. 4B left panel). In co-cultured-act-B cells, anti-CD40 Ab or anti-OX40L Ab inhibited the expression of CD40 and OX40L, respectively (Fig. 4B right panel).

3.6. Effects of anti-CD40 and anti-OX40L Abs on IgE and IgA levels in B cells co-cultured with BMMCs

There is no report yet to inhibit IgA levels by anti-OX40L Ab, and to induce IgA production through B cell Ig class switching via OX40–OX40L interaction. Therefore, we found that the production of total IgE and IgA was enhanced in B cells co-cultured with BMMCs (shown as B cells with BMMCs) via OX40–OX40L and CD40–CD40L interaction (Fig. 4C and D).

Anti-CD40 Ab (from 42 ± 1.2 ng/ml to 26 ± 2.8 ng/ml for non-act-B cells with act-BMMCs; from 69 ± 3.8 ng/ml to 42 ± 5.1 ng/ml for act-B cell with act-BMMCs) inhibited IgE levels as compared with non-treated blocking Ab or act-B cells with non-act-BMMCs. Anti-OX40L Ab (to 34 ± 1.5 ng/ml for non-act-B cells with act-BMMCs; to 58 ± 2.1 ng/ml for act-B cell with act-BMMCs) also inhibited IgE levels as compared with non-act-B cells with non-act-BMMCs. Pretreatment with anti-CD40 plus anti-OX40L Abs (to 12 ± 2.4 ng/ml for non-act-B cells with act-BMMCs; 19 ± 2.2 ng/ml for act-B cells with act-BMMCs) synergistically inhibited IgE versus pretreatment with anti-CD40 or anti-OX40L Abs (Fig. 4C).

IgA levels were enhanced in non-act-B cells with non-act-BMMCs (from 0 ± 0 ng/ml to 4 ± 1.2 ng/ml), act-B cells with non-act-BMMCs (to 12 ± 2.5 ng/ml), non-act-B cells with act-BMMCs (to 54 ± 2.4 ng/ml), and in act-B cells with act-BMMCs (to 94 ± 7.4 ng/ml) (Fig. 4D). Anti-CD40 Ab (to 34 ± 4.8 ng/ml for non-act-B cells with act-BMMCs; to 59 ± 5.9 ng/ml for act-B cell with act-BMMCs) inhibited IgA levels as compared with non-treated blocking Ab. Anti-OX40L Ab (to 43 ± 2.1 ng/ml for non-act-B cells with act-BMMCs; to 73 ± 3.2 ng/ml for act-B cells with act-BMMCs) also inhibited IgA levels as compared with non-treated blocking Ab. Anti-CD40 plus anti-OX40L Abs pretreatment (to 16 ± 2.3 ng/ml for non-act-B cells with act-BMMCs; 25 ± 3.2 ng/ml for act-B cells with act-BMMCs) synergistically inhibited IgA versus anti-CD40 or anti-OX40L Ab pretreatment. In vitro results indicated that IgE and IgA may be produced by B cell Ig class switching via OX40–OX40L as well as CD40–CD40L interaction.

3.7. The effects of CD40L agonist or OX40 agonist on signaling molecules and on the productions of IgE and IgA in B cells

CD40–CD40L or OX40–OX40L interactions can support B cell Ig class switching by activating signaling molecules, such as, TRAF2/6, MEKK1, and TAK1. Therefore, we wondered whether CD40L or OX40 agonists activated B cells through the same signaling pathways. B cells stimulated with CD40L agonist (referred to as CD40L-sti-B cells) induced expression or activity of TRAF2–MEKK1 and TRAF6–TAK1 pathway (two signaling pathways). B cells stimulated with OX40 agonist (referred to as OX40-sti-B cells) induced only TRAF6–TAK1 pathway (Supplementary Fig. 4A and B). The optimal dose (400 ng/ml) and time (5 h) for agonists (CD40L and OX40) with respect to the expression of all signaling molecules were determined during preliminary experiments (Supplementary Fig. 4A–D).

Anti-CD40 Ab inhibited the increased expression and activities of signaling molecules, such as, TRAF2–MEKK1 and TRAF6–TAK1, in CD40L-sti-B cells, but anti-OX40L Ab inhibited only the increased expression of TRAF6 and TAK1 pathway in OX40-sti-B cells (Fig. 5A).

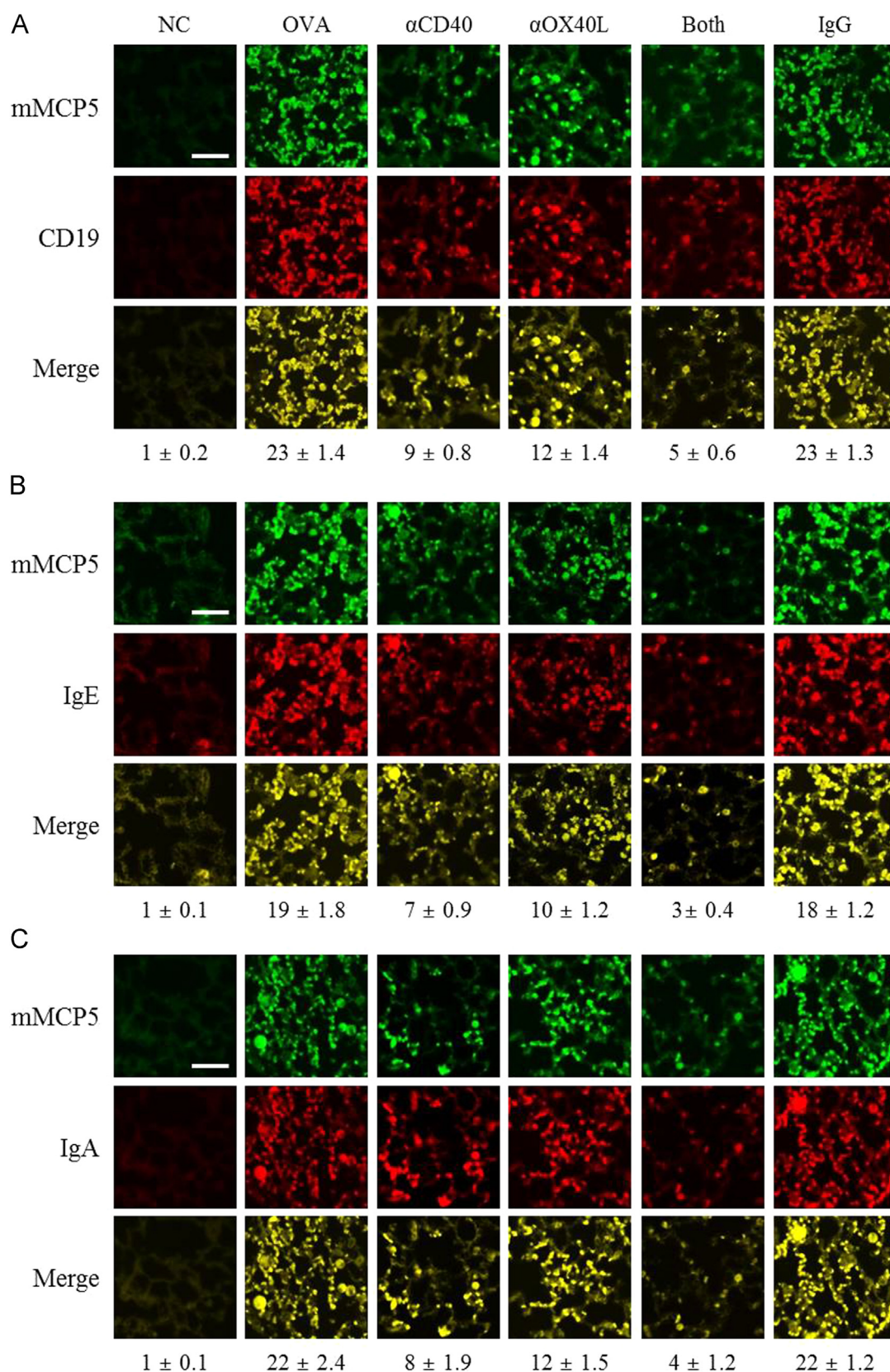


Fig. 3. Effects of anti-CD40 and anti-OX40L Abs on the co-localization of mast cells and B cells, IgE- or IgA-producing cells in the lung tissues of OVA-challenged mice. Experimental details of the animal models of allergic asthma, treatments with blocking Abs, and the definition of all symbols used are provided in Fig. 1 legend. Co-localization of mast cells (mMCP5) and B cells (CD19), IgE-producing cells or IgA-producing cells in lung tissues was determined by immunofluorescence. Degrees of immunofluorescence (yellow) developed by co-localization were quantified by counting numbers of cells at 5 sites of area $200 \mu\text{m} \times 200 \mu\text{m}$ under a microscope (5 areas/slide $\times$ 8 mice/each group = 40 areas). Values (mean $\pm$ S.E.M.) are shown in the merge box below. (A) Co-localization (yellow) of mMCP5 (green) and CD19 (red). (B) Co-localization of mMCP5 and IgE-producing cells (red). (C) Co-localization of mMCP5 and IgA-producing cells (red). IgG Ab was used a positive control instead of blocking Abs. Original magnification in NC (negative control) was $\times 400$ and the scale bars were $100 \mu\text{m}$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

In order to confirm whether these signaling pathways are induced in B cells activated with agonists, we used B cells that CD40L or OX40 is transfected. Expression of signaling molecules was not induced in transfected-CD40-sti-B cells or transfected-OX40L-sti-B cells (Supplementary Fig. 4E; Fig. 5B).

Anti-CD40 or anti-OX40L Abs inhibited activities of MAP kinases and NF- κ B/AP-1 in CD40L- or OX40-sti-B cells versus NC (negative control) (Fig. 5C and D). Anti-CD40 plus anti-OX40L Abs additively or synergistically inhibited their activities versus each blocking Ab. In BAL cells or the lung tissues of OVA-challenged mice, anti-CD40 plus anti-OX40L Abs additively inhibited NF- κ B/AP-1 activities and the mRNA expression of various inflammatory cytokines (IL-4, IL-13, TGF- β , IL-1 β , IL-5, IL-6, IL-8, and TNF- α) (Supplementary Fig. 5A and B).

IgE levels were increased in CD40L-sti-B cells (29 ± 2.1 ng/ml) and in OX40-sti-B cells (19 ± 1.8 ng/ml). Anti-CD40 Ab (8 ± 2.3 ng/ml) or anti-OX40L Ab (6 ± 2.3 ng/ml) inhibited IgE levels produced in CD40L-sti-B cells and OX40-sti-B cells, respectively (Fig. 5E). IgA levels were also increased in CD40L-sti-B cells (41 ± 1.6 ng/ml) or in OX40-sti-B cells (26 ± 1.2 ng/ml). Anti-CD40 (12 ± 1.2 ng/ml) or anti-OX40 Ab (10 ± 1.8 ng/ml) inhibited IgA levels. These findings imply that OX40 or CD40L agonist may induce the productions of IgE and IgA in B cells. After transfection of CD40 or OX40L on B cell surface, production of IgE/IgA was almost not induced in CD40- or OX40L-sti-B cells (Fig. 5E).

3.8. Re-activations of Fc ϵ RI or Fc α RI on mast cell surfaces by IgE and IgA produced via CD40–CD40L or OX40–OX40L interactions in B cells co-cultured with mast cells

Our data can infer that IgE or IgA produced may re-activate Fc ϵ RI or Fc α RI receptor on mast cell surfaces adjacent to B cells. Therefore, we first examined whether mouse mast cell surfaces express Fc α RI. We observed that Fc α RI was originally present on BMMCs surfaces and weakly expressed in BMMCs activated by Fc ϵ RI-mediated reaction (DNP-HSA/anti-DNP IgE Ab) (Fig. 5F). BMMCs activated by Fc α RI-mediated reaction (DNP-KLH/anti-DNP IgA Ab) also enhanced the expression of Fc α RI, Fc ϵ RI, CD40L, OX40, and MAP kinases (Fig. 5F and G; Supplementary Fig. 6A and B), and this was followed by mediator release (Supplementary Fig. 6C and D) via MAP kinases (Supplementary Fig. 6E) and NF- κ B/AP-1 activities (Supplementary Fig. 6F). Furthermore, Fc α RI-mediated BMMCs activation had lower responses in mast cell activation than those in Fc ϵ RI-mediated mast cell responses. The expression of Fc ϵ RI and Fc α RI was enhanced in the mast cells isolated from lung tissues (Fig. 5H), BAL cells or lung tissues (Supplementary Fig. 6G) of OVA-challenged mice. Anti-CD40 plus anti-OX40L Abs pretreated mice showed Fc ϵ RI and Fc α RI inhibition in mast cells isolated from lung tissues or BAL cells of OVA mice.

4. Discussion

We demonstrate that mast cells activated by Ag/Ab reaction show enhanced expression of the co-stimulatory (surface) molecules (CD40L and OX40) and mediator release. Furthermore, we

show that binding of CD40 on B cell surfaces to CD40L on BMMCs surfaces may be activated via the TRAF2–MEKK1 and TRAF6–TAK1 signaling pathways, and that binding of OX40L on B cell surface to OX40 on BMMCs surface may be activated only through TRAF6–TAK1 signaling pathways, followed by IgE and IgA production via B cell Ig class switching in the presence of cytokines. The produced IgE or IgA causes more mediator release due to the re-activation of Fc α RI as well as Fc ϵ RI on mast cell surfaces. In addition, we demonstrate that treatment with anti-CD40 plus anti-OX40L Abs synergistically reduces IgE and IgA levels and mediator release, and additively reduces all allergic responses caused by OVA-challenge in mice.

As mast cells and B cells are known under normal conditions to localize at different sites, it is difficult to imagine how these cell types could cross-talk. However, it has been reported that mast cells co-localize with B cells in inflammatory sites (Merluzzi et al., 2010). Our data also suggest that mast cells may participate in cell-to-cell cross-talk with B cells, as shown in Fig. 3.

Mast cells play key roles in inflammatory conditions like allergic asthma (Amin, 2012), and in B cell survival and proliferation (Merluzzi et al., 2010) by inflammatory mediators, such as, histamine, LTs, and various cytokines released by cross-linking IgE-bound Fc ϵ RI. IgE-Ag-mediated mast cells drive B cell differentiation toward IgA-producing plasma cells via IL-6, IL-5 and TGF- β , and via CD40–CD40L interaction (Bemark et al., 2012; Hong et al., 2014; Merluzzi et al., 2010, 2014). Our data are in agreement with previous reports described above.

The OX40–OX40L interaction participates in the pathogenesis of various diseases, such as idiopathic inflammatory myopathies and systemic lupus erythematosus (Farres et al., 2011; Karulf et al., 2010; Papadopoulos et al., 2013), including allergic asthma (Gauvreau et al., 2014). And, anti-OX40L Ab plays a critical role in the development of pathogenic Th2 in asthma model (Hoshino et al., 2003). However, no report that IgE-Ag mediated mast cell activation induces the production of IgE and IgA through B cell Ig class switching via OX40–OX40L interaction has been issued. Thus, our results suggest that IgE and IgA may be produced by B cell Ig class switching via the OX40/OX40L interaction in the presence of cytokines (IL-4, IL-13, IL-6 and TGF- β) expressed/secreted from Fc ϵ RI-mediated mast cell activation, as both Abs (IgE/A) were produced by B cell Ig class switching via the CD40/CD40L interaction.

Signaling pathways in CD40–CD40L is associated with TRAF2–MEKK1 and TRAF6–TAK1 in the various cell types, but not in B cells (Häcker et al., 2011). Our data can infer that binding of OX40 on mast cells to OX40L on B cell surfaces may be activated only by the TRAF6–TAK1 signaling pathway, and that binding of CD40L on mast cells to CD40 on B cells may be induced by TRAF2–MEKK1 and TRAF6–TAK1 (Fig. 6), as demonstrated by the data shown via transfection of B cell surface molecules.

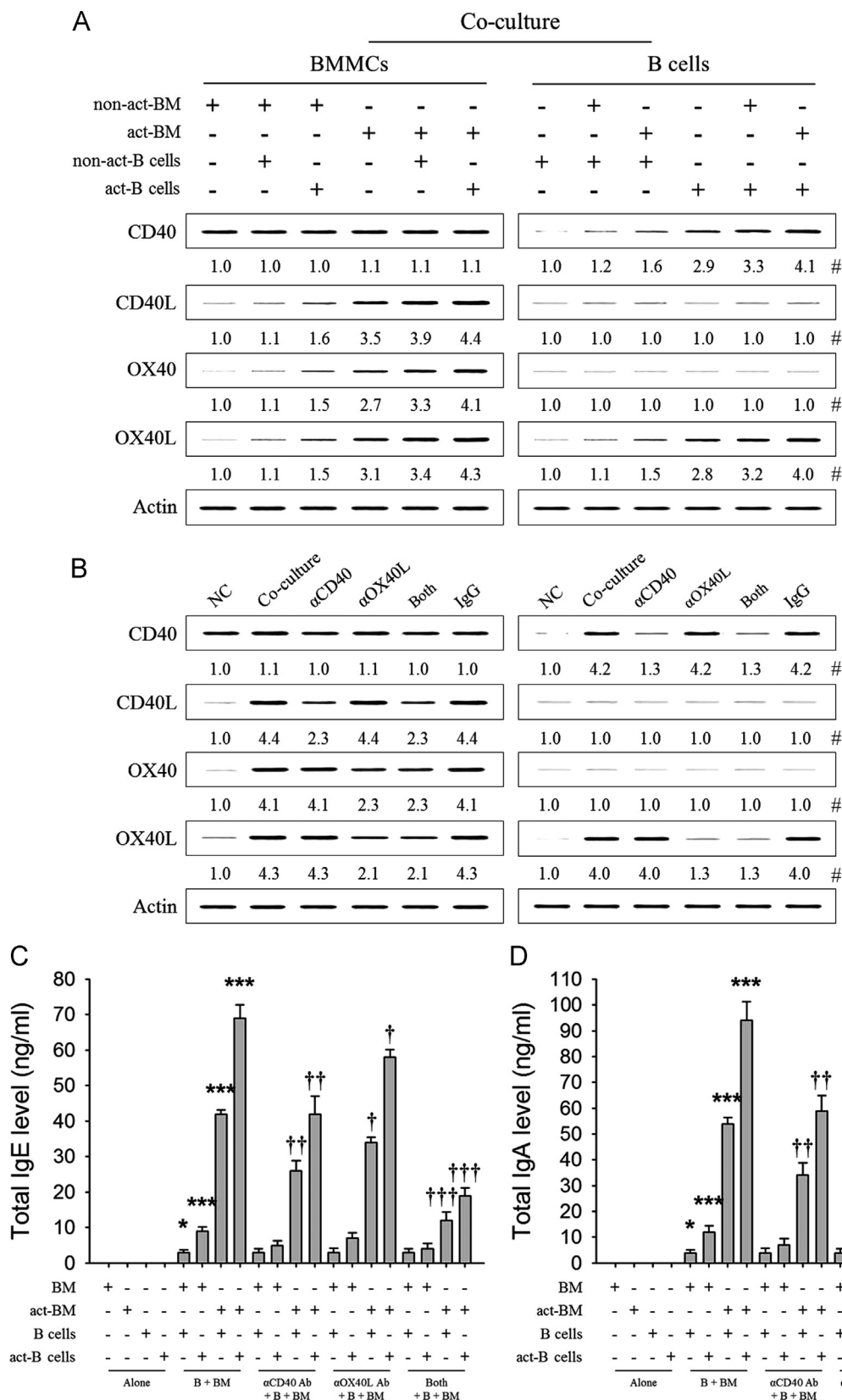
The IgA receptor (Fc α RI or CD89) is expressed on human mast cells, eosinophils and neutrophils (Dahl et al., 2004), but not on mouse mast cells (Gludemans et al., 2013; Monteiro and van Del Winkel, 2003). However, in this study, we observed for the first

Fig. 4. Effects of anti-CD40 and anti-OX40L Abs on the expression of surface molecules and on total IgE and IgA levels in B cells co-cultured with BMMCs. BMMCs (1×10^6 cells) were activated with 10 ng/ml DNP-HSA and 0.1 μ g/ml anti-DNP IgE Ab for 5 h (referred to as act-BMMCs). B cells (1×10^6 cells) were activated with 0.5 μ g/ml of anti-mouse IgM Ab (referred to as act-B cells) for 5 h (for the expression of surface molecules) or for 48 h (for the production of IgE or IgA). act-B cells (1×10^6 cells) were added to non-act-BMMCs (BMMCs alone) or act-BMMCs (1×10^6 cells), and then co-cultured for 72 h. To block cell-to-cell interactions, anti-CD40 Ab, anti-OX40L Ab (300 ng/ml), anti-CD40 plus anti-OX40L Abs or IgG Ab (300 ng/ml) were pretreated at 1 h before B cell activation. Expression of CD40, CD40L, OX40 and OX40L was determined in protein extracts isolated from BMMCs or B cells separated after co-culture by Western blotting. Total IgE and IgA levels in media were determined by ELISA. (A) The expression of CD40, CD40L, OX40 and OX40L in BMMCs or B cells separated after co-culture. (B) Expression of CD40, CD40L, OX40 and OX40L in BMMCs and B cells separated after pretreatment with blocking Abs and co-culture. IgG was used as a positive control instead of blocking Abs. (C) Total IgE levels produced in B cells co-cultured with BMMCs. (D) Total IgA levels produced in B cells co-cultured with BMMCs. BM, BMMCs; B, B cells; α CD40 or α OX40L, pretreatment with anti-CD40 and anti-OX40L Abs, respectively; both, pretreatment with anti-CD40 plus anti-OX40L Abs. Numbers below bands are mean values ($n=4$) of ratios of the band image densities of surface molecules versus the control and actin as determined by four independent experiments. – or +, without or with B cell and BMMCs. * $P < 0.05$; *** $P < 0.001$ versus non-act-BMMCs, act-BMMCs, non-act-B cells, or act-B cells. $^{\dagger}P < 0.05$; $^{\dagger\dagger}P < 0.01$; $^{\dagger\dagger\dagger}P < 0.001$ versus corresponding B cells co-cultured with BMMCs.

time that Fc α RI is originally expressed and enhanced in BMMCs activated with IgE-Ag-mediated BMMCs or IgA-Ag-mediated BMMCs, followed by mediator release (histamine, LTs and cytokines) via MAP kinases and NF- κ B-AP-1. Thus, our data suggest that IgA, produced in B cells via CD40-CD40L or OX40-OX40L

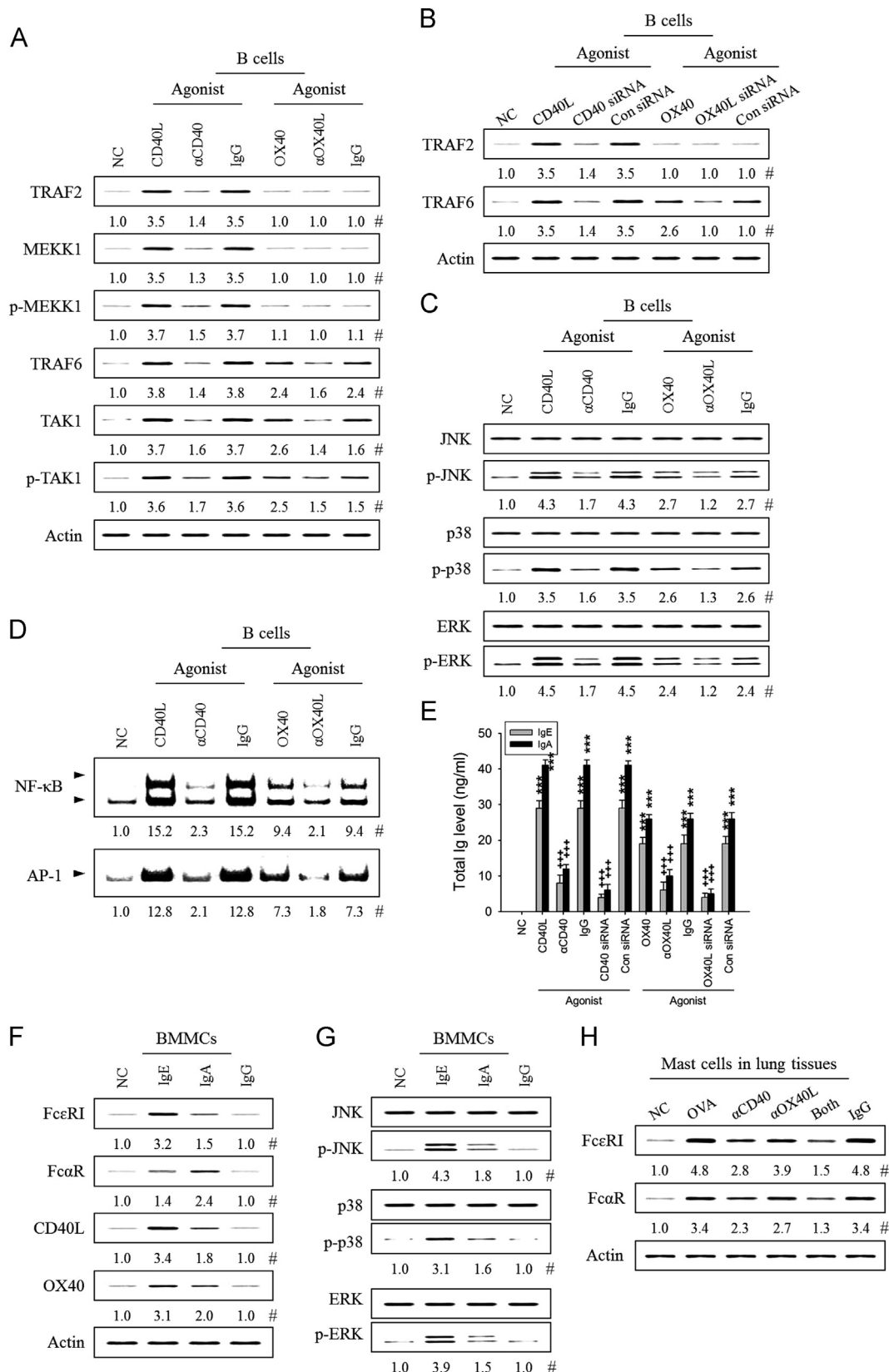
interactions, may re-activate Fc α RI-mediated mast cells in the same signaling pathways as those caused in Fc ϵ RI-mediated responses

Furthermore, combination therapy by anti-CD40 plus anti-OX40L Abs additively inhibited some responses and synergistically



inhibited others, particularly the production of IgE and IgA and mediator release. This data suggest that synergistic reduction in IgE and IgA production could be due to the direct blocking of B cell

surface molecules which are related to Ig class switching, and due to blocking the re-activations of Fc ϵ RI and Fc α RI caused by the Igs produced. Synergistic effects on mediator release by combination



therapy can also be inferred from the reduced re-activation of mast cells via IgE or IgA produced in B cells, and from the down-regulations of the expression of FcεRI and FcαRI due to reduction of IgE or IgA production. However, further study is needed to investigate the mast cell re-activation caused by the IgE and IgA produced.

Of the cytokines released by mast cell activation, IL-5 is necessary for the differentiation, proliferation and activation of eosinophils, IL-8 plays an important role in immediate and late phase allergic reactions as a potent chemotactic cytokine, and TNF-α plays pivotal role in chronic inflammation and innate immunity (Hong et al., 2013a). Thus, our data suggest that anti-CD40 plus anti-OX40L Abs therapy (combination therapy) might additively inhibit the infiltration of eosinophils and other inflammatory immune cells into lung tissues by down-regulating the production of various cytokines/chemokines.

In general, IgA have a protective role in allergic diseases like asthma (Gloude-mans et al., 2013; Hajek et al., 2008). However, eosinophils and neutrophils, which are recruited into airway in allergic asthma, express receptors for IgA that can activate the cells upon binding of IgA immune complexes, and then these cells can release important mediators in severe asthma (Monteiro and van Del Winkel, 2003). IgA complexes can also cause severe inflammation and pathology like immune complex glomerulonephritis (Tomino, 2012). Serum total and OVA-specific IgE and total IgA are elevated in OVA-challenged mice (Takeda et al., 2013). Thus, our data suggest that IgA produced in mast cells may aggravate rather than promoting tolerance in allergic asthma, as demonstrated by the data shown in FcαRI-mediated mast cell activation. However, relationship between IgE and IgA produced from interaction of B cells and mast cells of allergic asthma model further needs to be investigated.

In conclusion, the present study demonstrates that mast cells activated by Ag/Ab reaction enhance the expression of surface molecules (CD40L and OX40) and the releases of various mediators (histamine, LTs and cytokines), and that these surface molecules may bind to CD40 and OX40L, respectively, on the surfaces of B cells located alongside mast cells. The B cells activated by the CD40–CD40L interaction in the presence of cytokines (secreted by activated mast cells) may support Ig class switching to facilitate the production of IgE and IgA via the TRAF2–MEKK1 and TRAF6–TAK1 pathways. The B cells activated by the OX40–OX40L interaction and cytokines may also support the production of IgE and IgA via one signaling pathway like TRAF6–TAK1, and then that the IgE or IgA produced might re-activate mast cells via the binding of FcεRI and FcαRI, respectively, on mast cell surfaces, followed by more mediator release. Thus, our findings suggest that anti-CD40 Ab or anti-OX40L Ab may reduce the production of IgE and IgA Abs through blocking each signaling pathway induced by CD40–CD40L and OX40–OX40L interaction (Fig. 6).

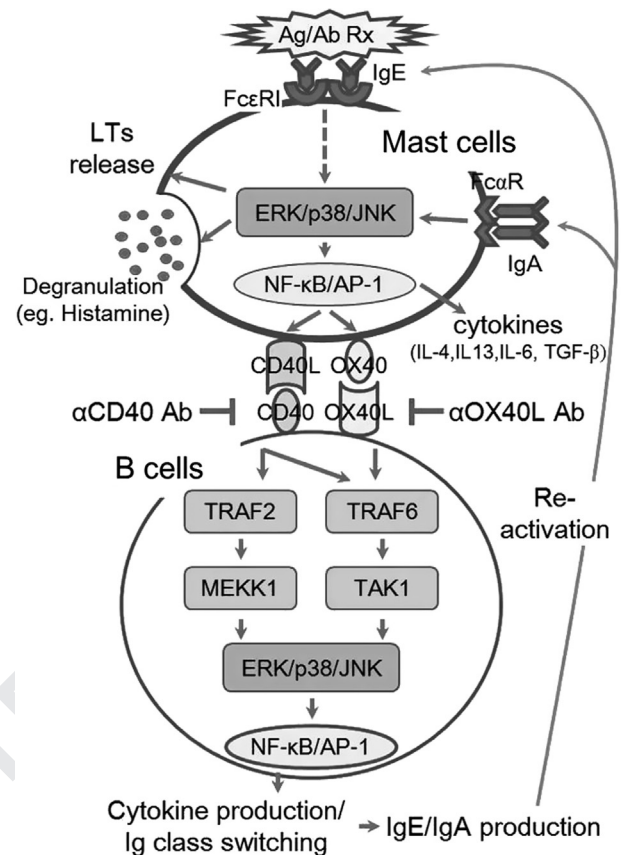


Fig. 6. Schematic diagram for signaling pathways associated with interaction between B cells and mast cells. Mast cells activated by the Ag/Ab reaction enhance the expression of surface molecules, such as CD40L and OX40, and release various mediators (histamine, LTs and cytokines). These surface molecules may bind to CD40 and OX40L on B cells located alongside mast cells. B cells activated by the CD40–CD40L interaction may support production of IgE and IgA through B cell Ig class switching via two signaling pathways, such as, TRAF2–MEKK1 and TRAF6–TAK1, in the presence of cytokines (IL-4, IL-13, IL-6 and TGF-β) secreted by mast cells activated with Ag/Ab reaction. Furthermore, B cells activated by the OX40–OX40L interaction may also support production of IgE and IgA through Ig class switching via one signaling pathway like TRAF6–TAK1, in the presence of cytokines secreted from activated mast cells. Thus, our data suggest that the IgE produced may re-activate mast cells by binding to FcεRI on mast cell surfaces to induce more mediator release, and that the IgA produced may re-activate mast cells by binding to FcαRI, which is enhanced in IgA–Ag-mediated mast cells. Our findings also suggest that anti-CD40 Ab may reduce the productions of IgE and IgA by blocking the signaling pathways, such as TRAF2–MEKK1 and TRAF6–TAK1, via the CD40–CD40L interaction, and that anti-OX40L Ab may reduce Abs' production by blocking TRAF6/TAK1 signals via OX40–OX40L interaction. The dotted arrow indicates that no response was observed in the present study, but has been reported previously in mast cells, and indicates inhibition by blocking Ab pretreatment.

Fig. 5. Effects of CD40L or OX40 agonist on the expression of various signaling molecules or IgE and IgA production from B cells, or expression of receptors and surface molecules in FcεRI- or FcαRI-mediated BMMCs. B cells (1×10^6 cells) were stimulated with 400 ng/ml of CD40L (soluble mouse recombinant CD40L) or OX40 (OX40 purified anti-134 Ab) agonist (referred to as CD40L-sti-B cells or OX40-sti-B cells) for 5 h. BMMCs (1×10^6 cells) were also sensitized/activated with DNP-HAS/anti-DNP IgE Ab (10 ng/0.1 μg/ml) for FcεRI-mediated responses or DNP-KLH/anti-DNP IgA (10 μg/ml/10 μg/ml) for FcαRI-mediated responses for the time periods indicated or 5 h. Experimental details of animal models of allergic asthma, treatment with blocking Abs, and the definitions of the symbols used are provided in Fig. 1 legend. The expression of signaling or receptor molecules was determined in protein extracts isolated from B cells or BMMCs by Western blotting. Transfection for CD40L or OX40 on B cell surface was carried out according to the manufacturer's method. The activities of transcription factors were determined by EMSA. The levels of IgE and IgA secreted into supernatants by sti-B cells were determined by ELISA. (A) The expression of TRAFs, MEKK1, and TAK1, and the phosphorylations of MEKK1 and TAK1 in CD40L-sti- or OX40-sti-B cells after blocking Ab pretreatment. (B) Expression of TRAF2/6 after transfection of CD40 and OX40L on B cell surface. (C) and (D) Activities of MAP kinases and NF-κB/AP-1 in CD40L- or OX40-sti-B cells after blocking Ab pretreatment, respectively. (E) Amounts of IgE or IgA secreted into media of CD40L- or OX40-sti-B cells after blocking Ab pretreatment or after transfection of B cell surface molecules. (F) Expression of receptors and surface molecules in BMMCs activated with Ag/Ab reaction (IgE, DNP-HSA/anti-DNP IgE Ab; IgA, DNP-KLH/anti-DNP IgA Ab). (G) Phosphorylation of MAP kinases in BMMCs activated with Ag/Ab reaction. (H) Expression of receptors in mast cells isolated from lung tissues after pretreatment with blocking Abs. IgG was used as positive control. Numbers below bands are the mean values ($n=4$) obtained, as described in the legend of Fig. 4. CD40L or OX40, B cells stimulated with CD40L or OX40 agonist, respectively; αCD40 or αOX40L, pretreatment with anti-CD40 or anti-OX40L Abs, respectively; IgE, DNP-HSA/anti-DNP IgE Ab reaction; IgA, DNP-KLH/anti-DNP IgA Ab reaction. *** $P < 0.001$ versus NC (negative control). + $P < 0.05$; + + $P < 0.01$ versus CD40L or OX40 agonist.

Acknowledgments

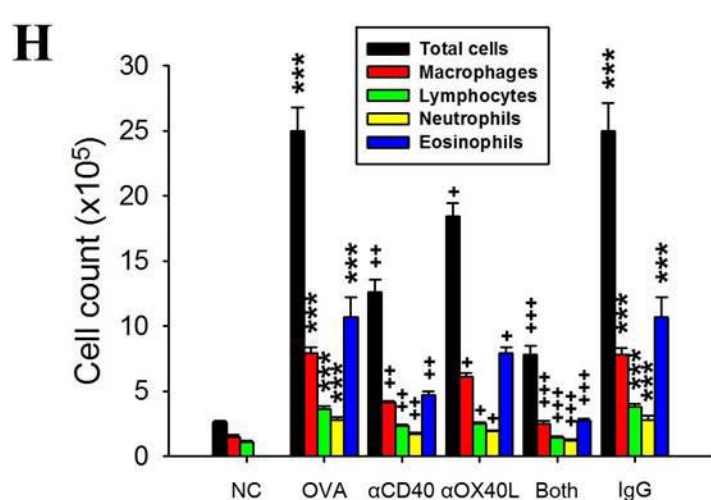
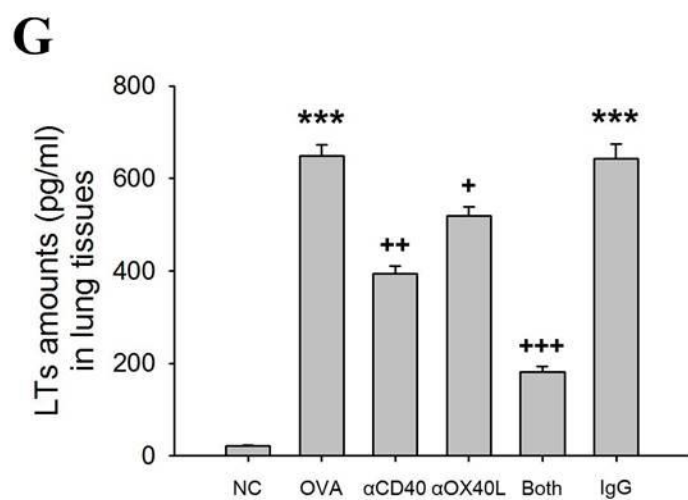
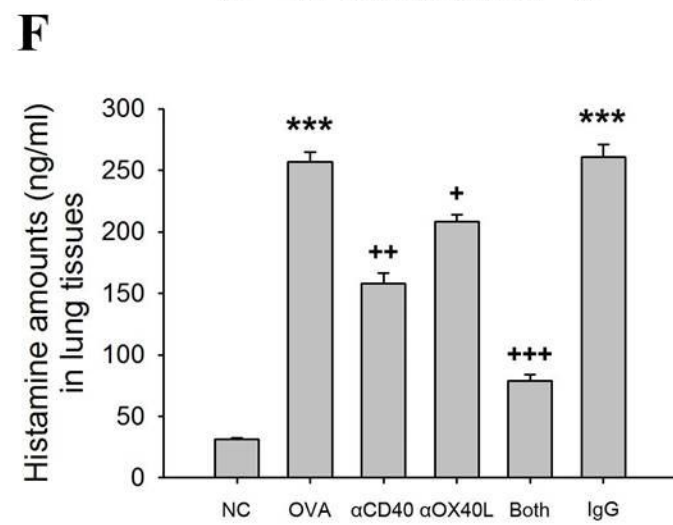
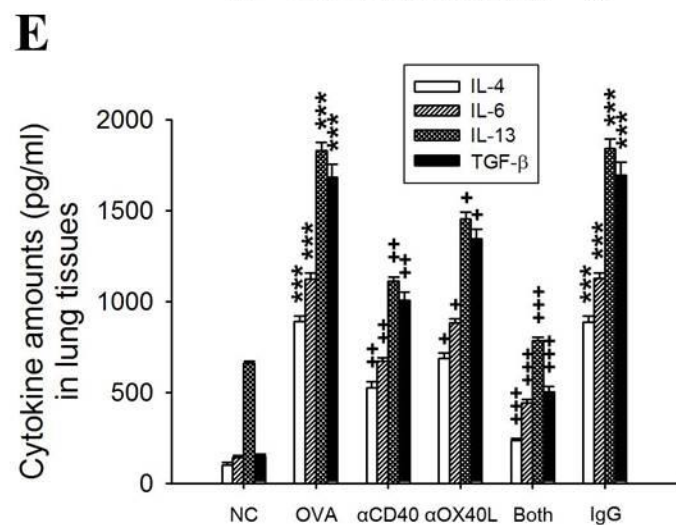
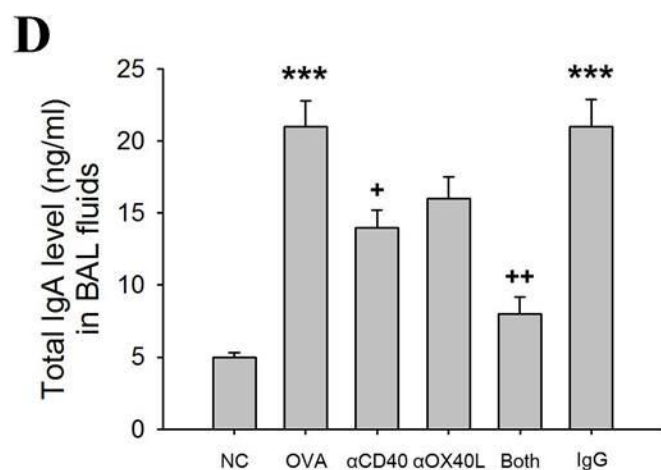
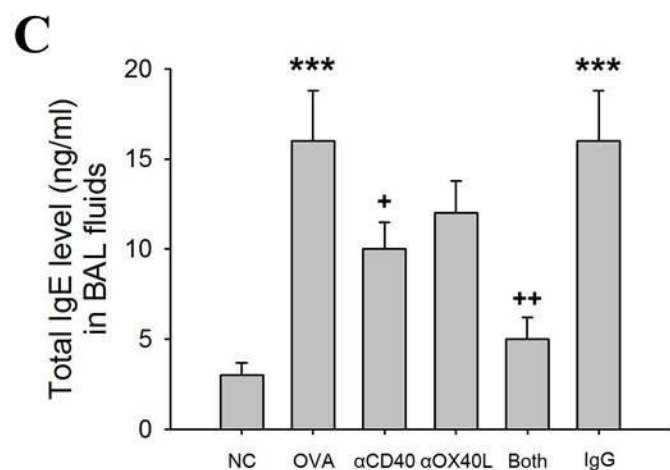
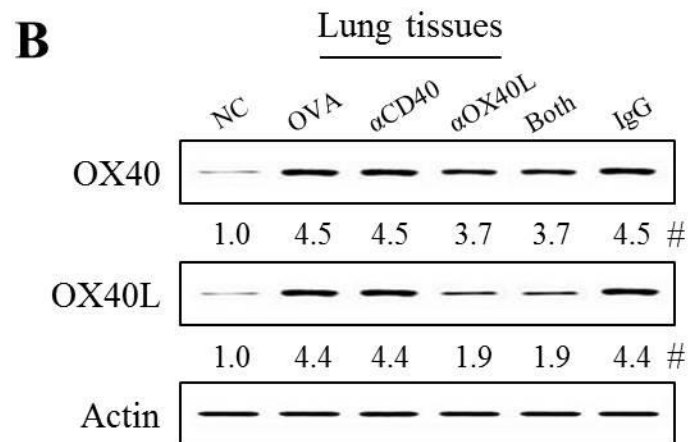
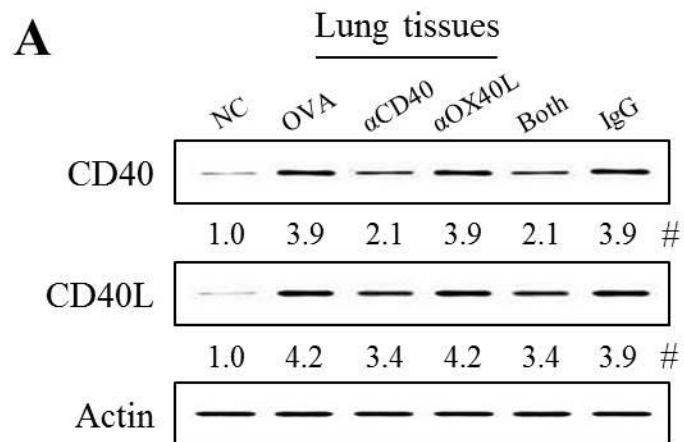
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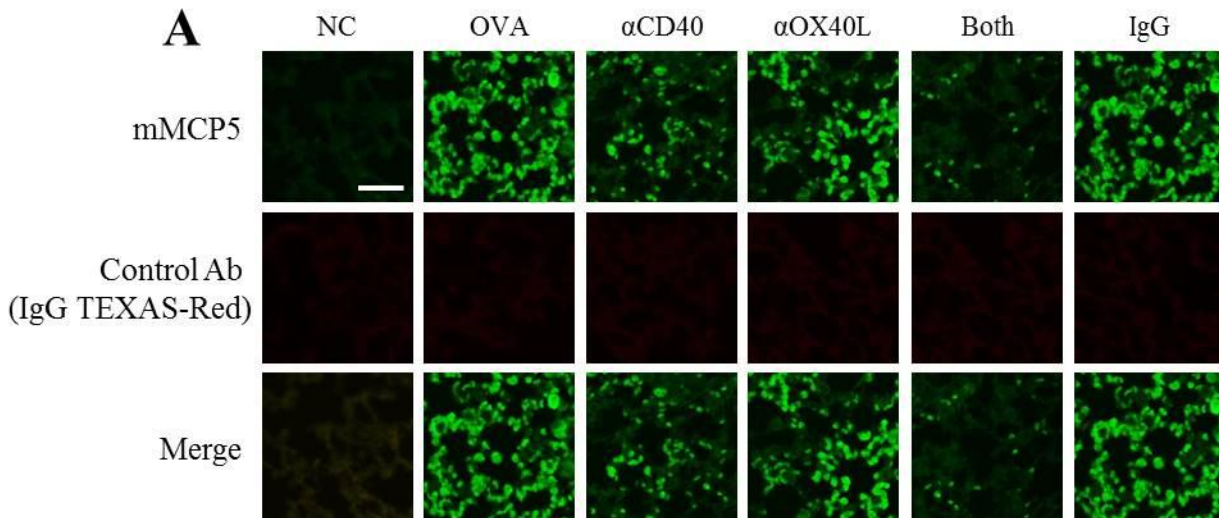
Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ejphar.2015.02.023>.

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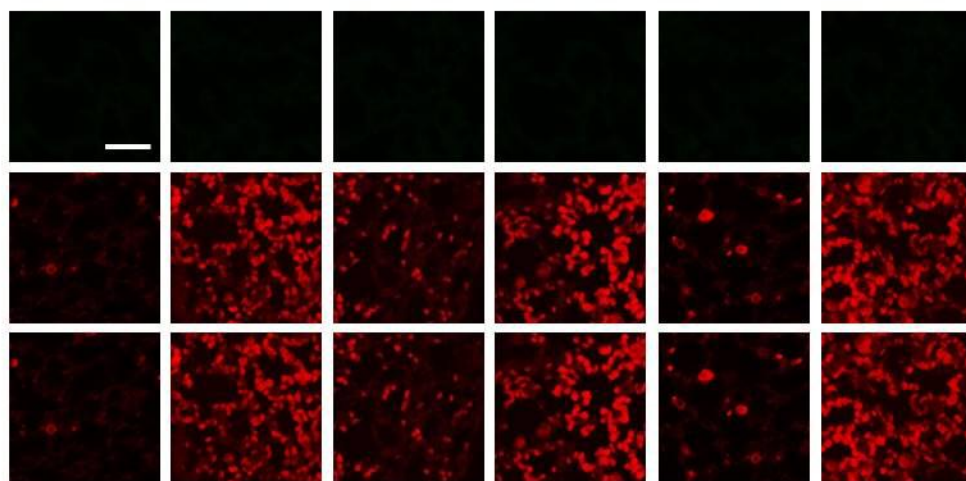
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A**B**Control Ab
(IgG FITC)

CD19

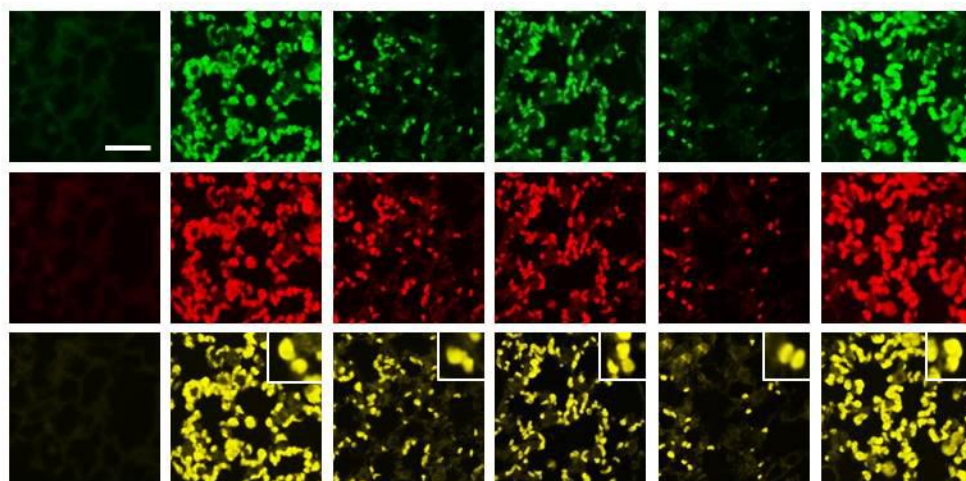
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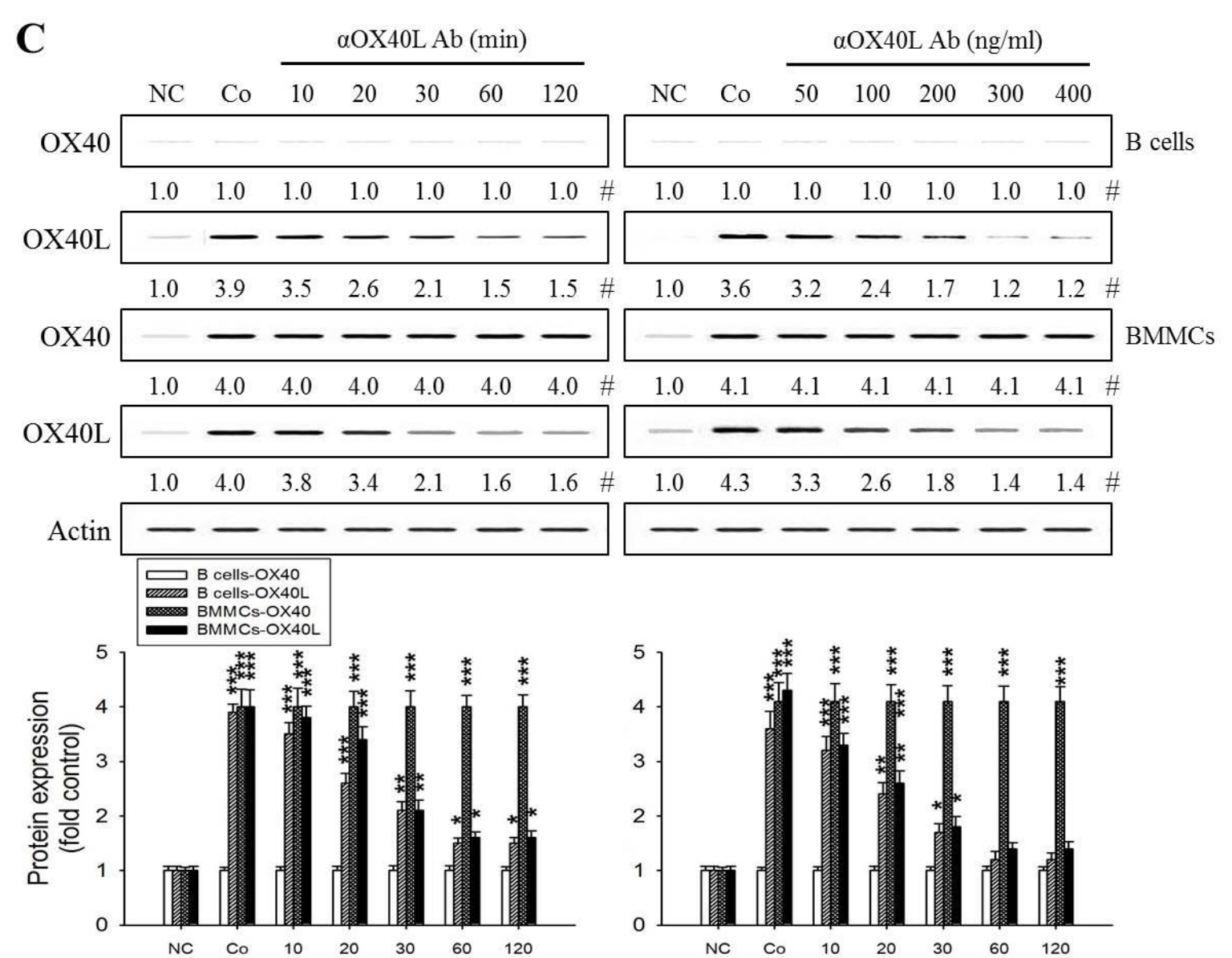
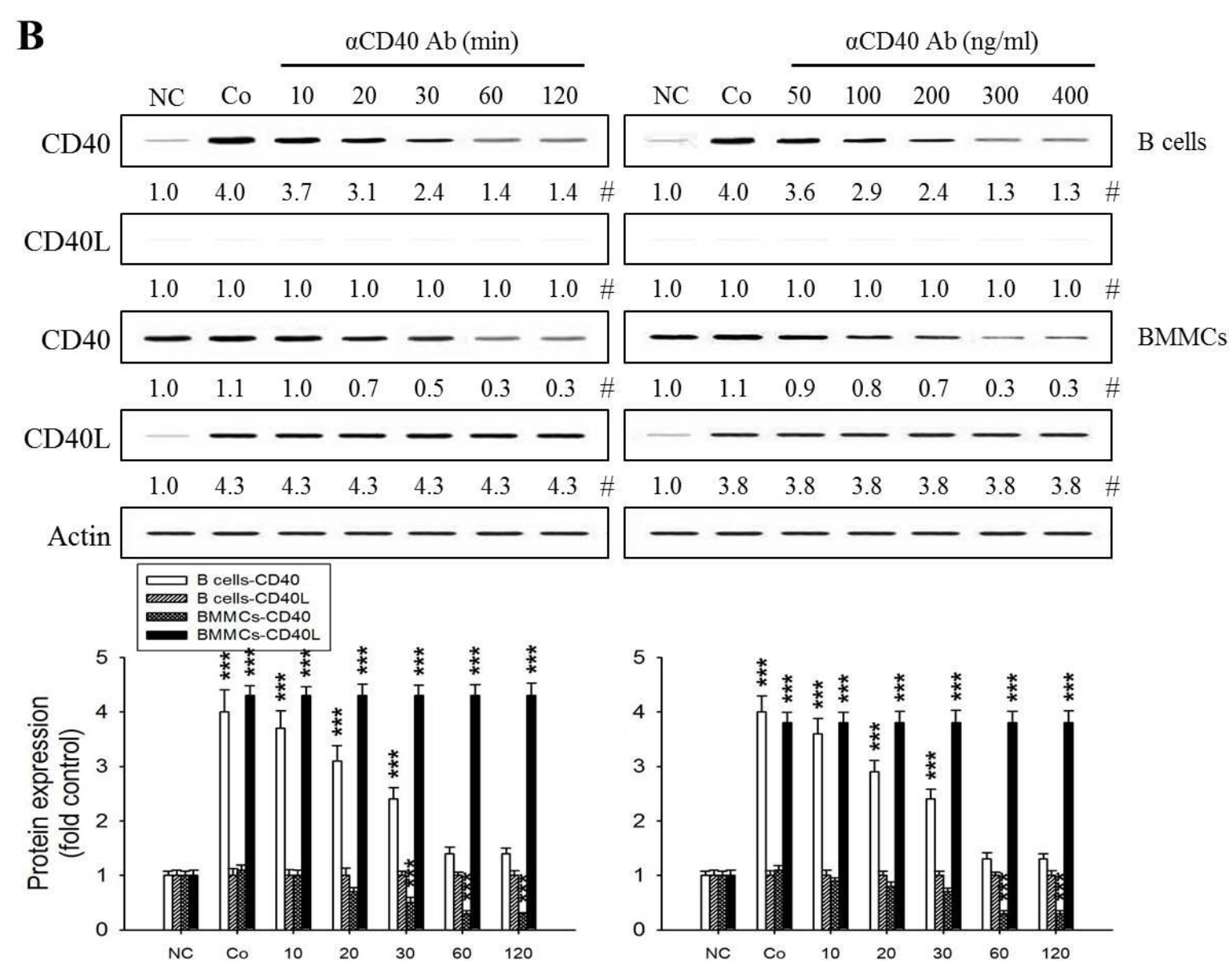
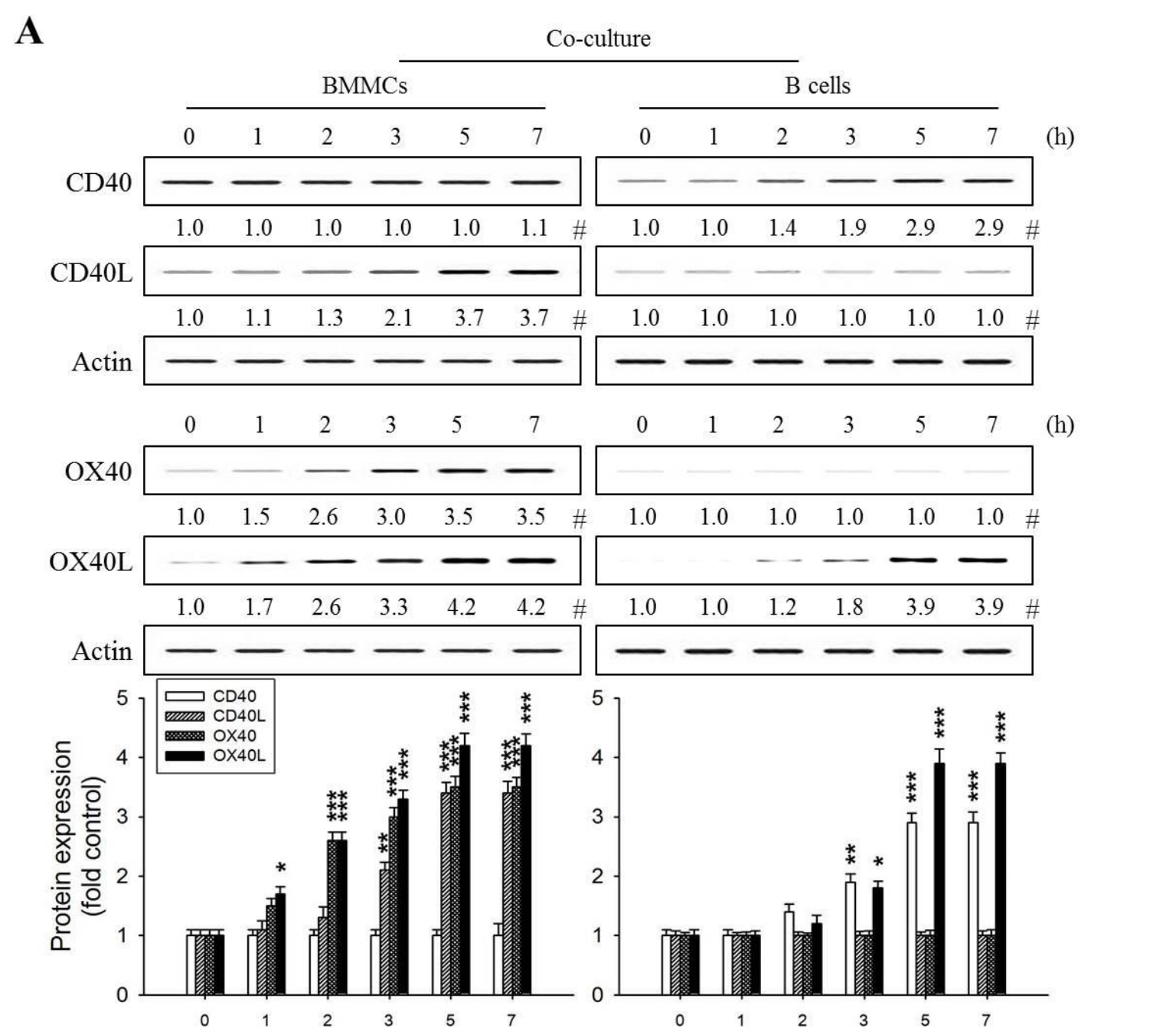
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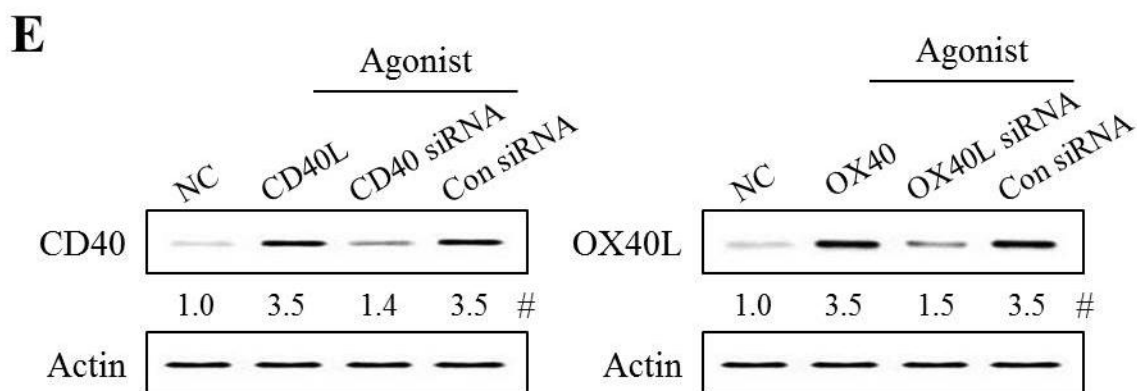
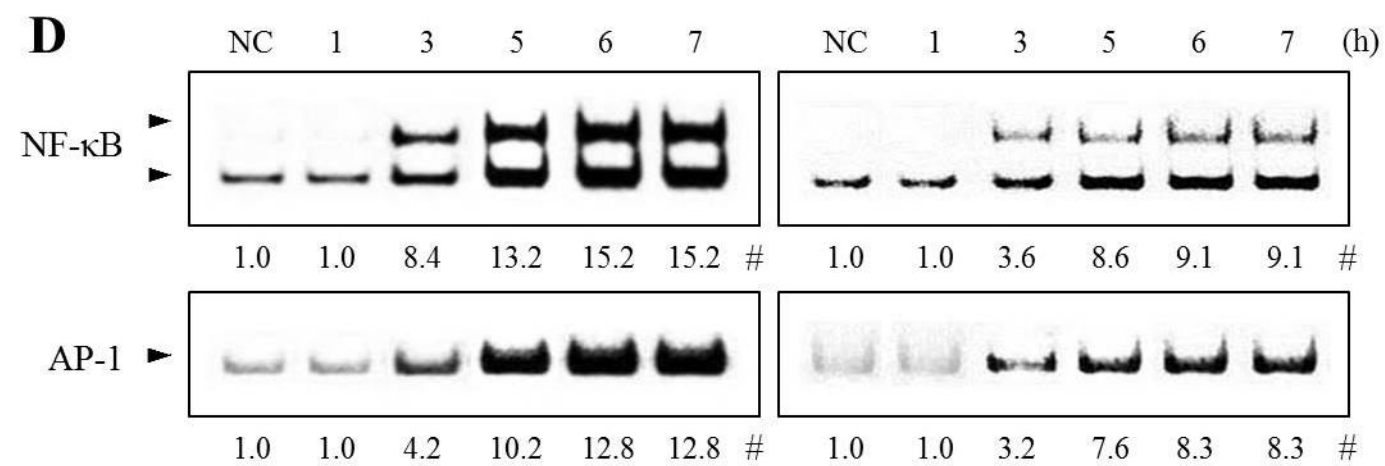
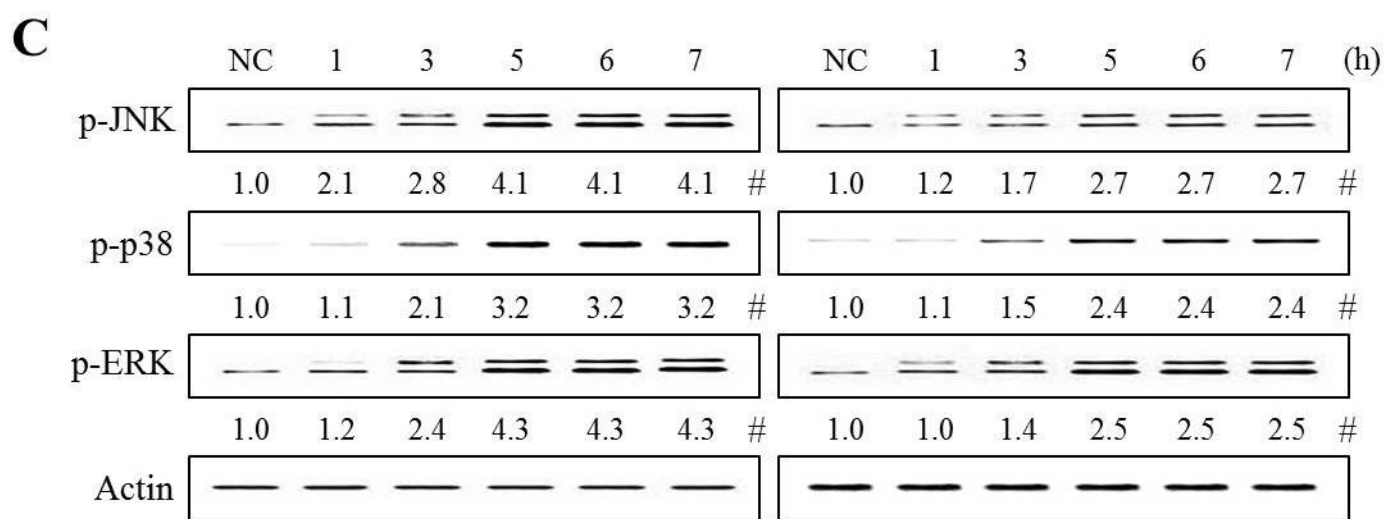
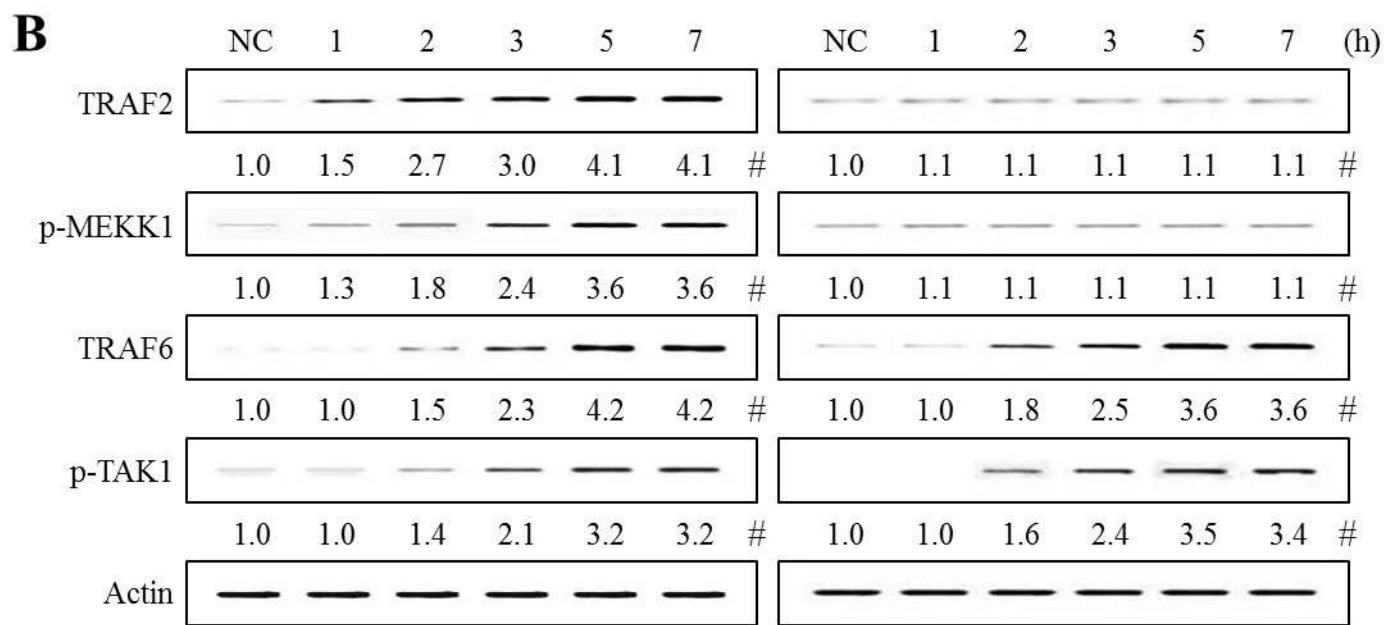
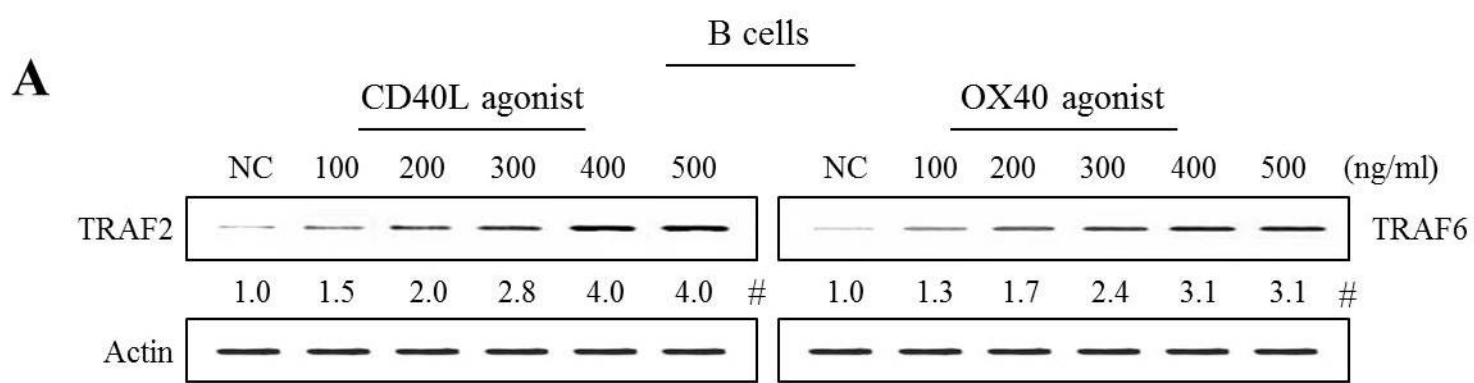
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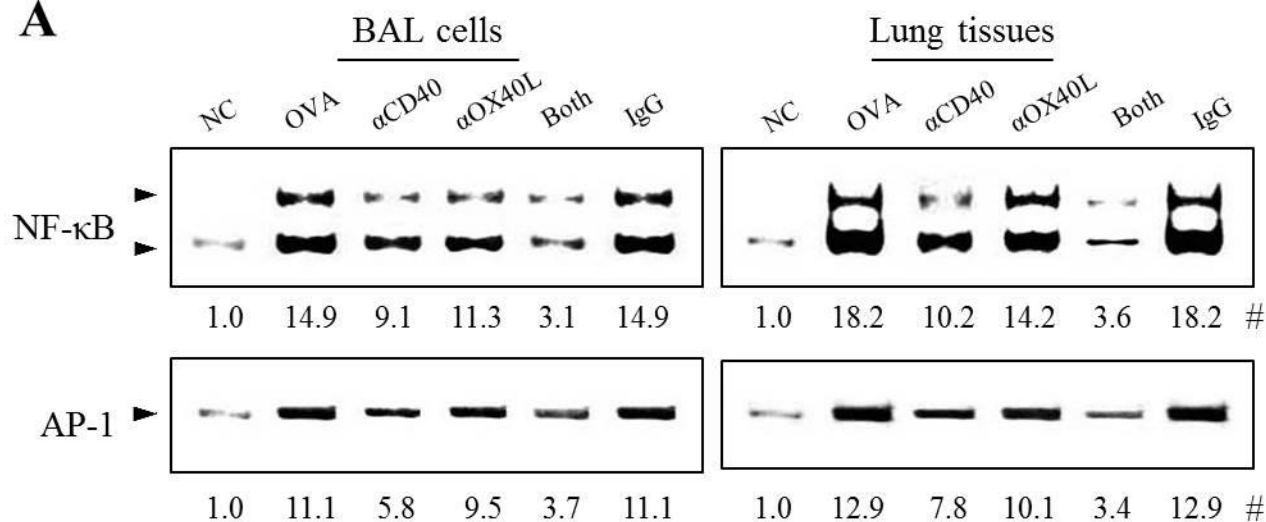
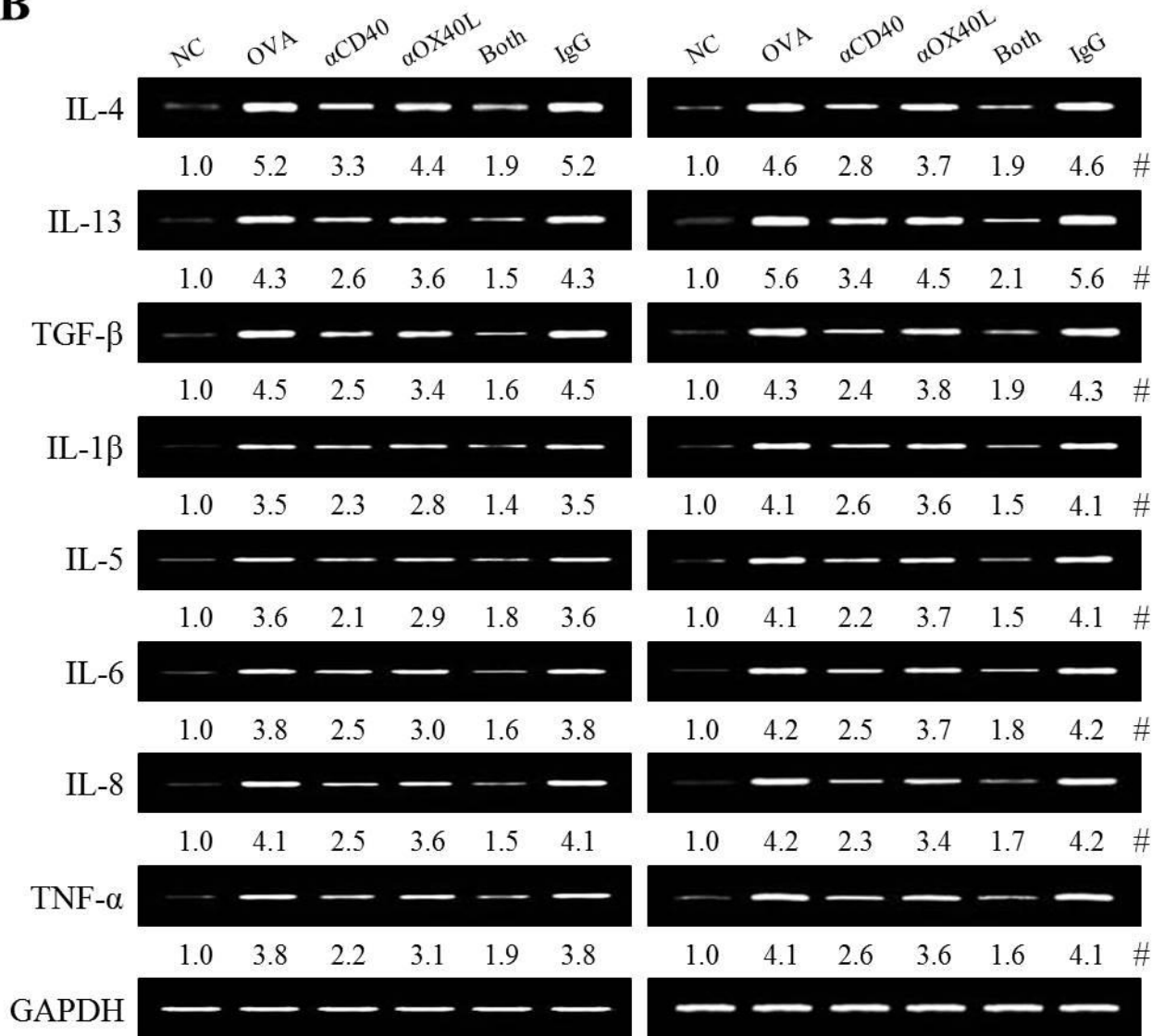
CD19

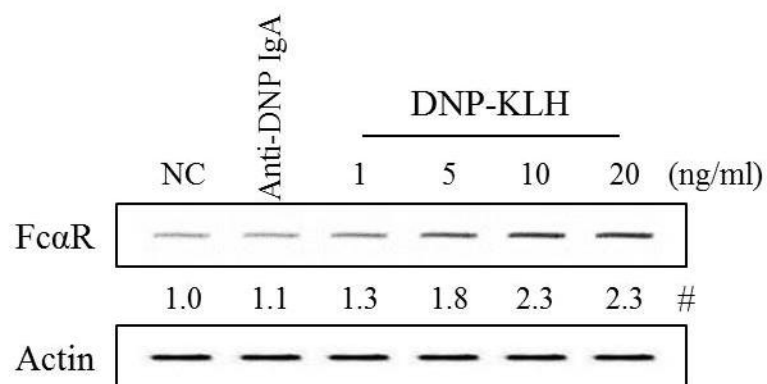
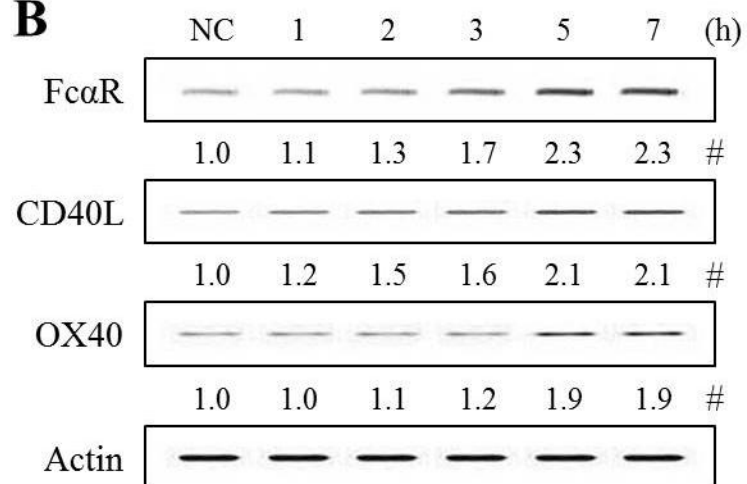
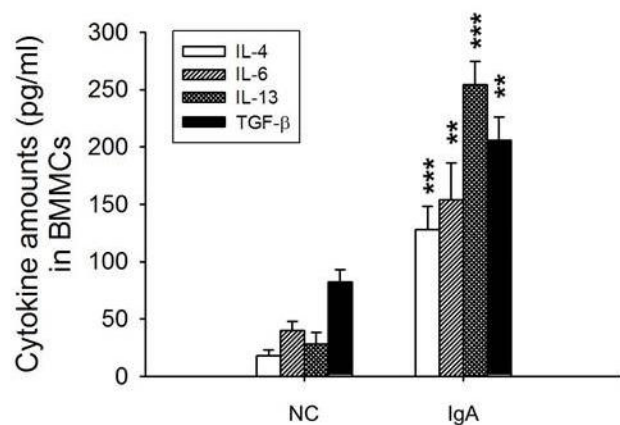
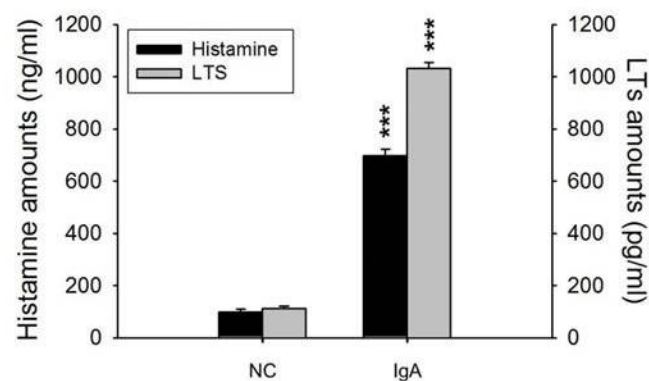
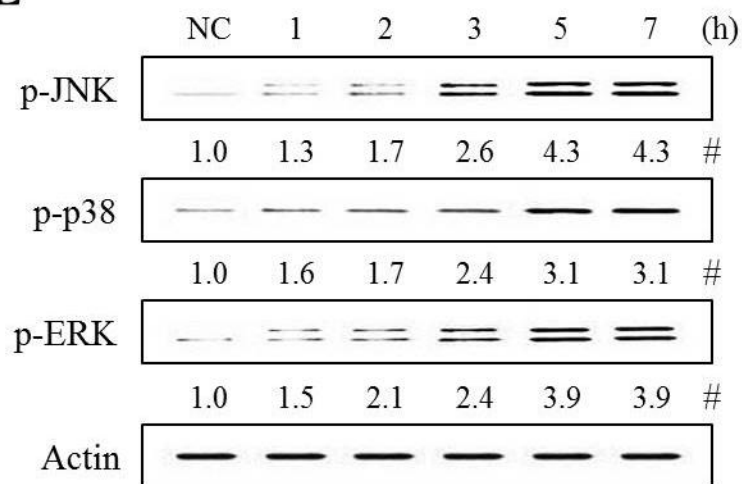
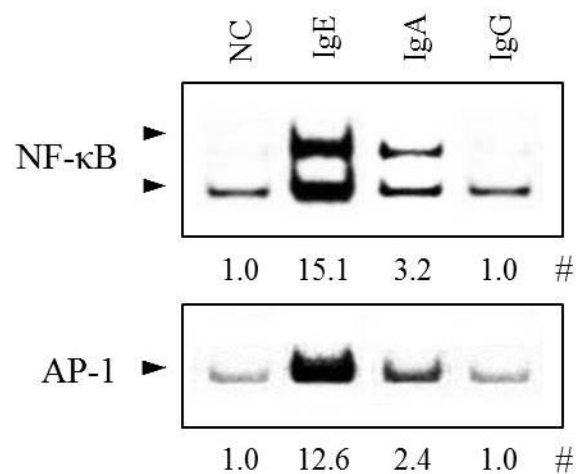
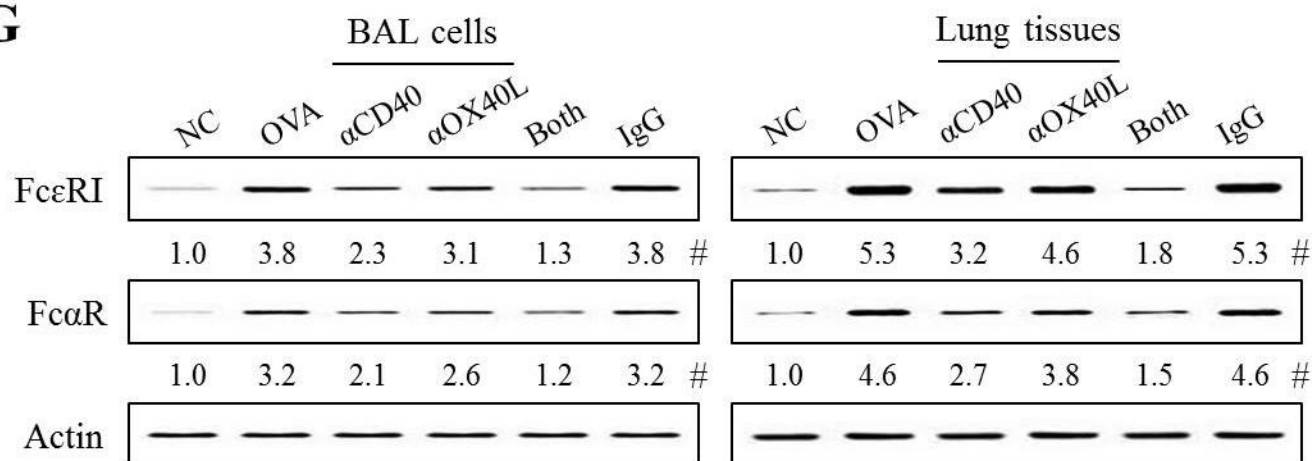
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 0 ± 0.1 25 ± 2.7 11 ± 1.9 20 ± 2.1 5 ± 1.2 25 ± 2.2





A**B**

A**B****C****D****E****F****G**

Supplemental materials

IgE and IgA produced by OX40-OX40L or CD40-CD40L interaction in B cells-mast cells re-activate Fc ϵ RI or Fc γ RI on mast cells in mouse allergic asthma

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Materials and Methods

2.4. Bronchoalveolar lavage (BAL) fluid and May-Grünwald–Giemsa staining

Immediately after blood collection, thoracic cavities were carefully opened. After tracheas were exposed and cannulated at the upper part of the trachea, BAL fluid was collected by lavaging twice with 1 ml and then 0.8 ml PBS (85-90% of the lavage volume was recovered). Lavaged samples from each mouse were kept on ice, and were centrifuged at $400 \times g$ for 5 min at 4 °C. After centrifugation, pellets were resuspended in 100 μ l PBS, and total viable cell numbers were counted by Trypan blue exclusion using a hemocytometer. BAL cells were adjusted to a concentration of 5×10^4 cells/ml in PBS. For cytospin preparations, cells were centrifuged at $400 \times g$ for 3 min using a Cytospin III (Shandon, Pittsburgh, PA), and were stained with Diff-Quik for different cell counting using standard morphological criteria (Hong et al., 2013a).

To confirm numbers of mast cells, BAL cells were obtained from Cytospin, and lung tissues were fixed in 4% paraformaldehyde embedded in paraffin, and cut at 3 μ m. BAL cells or lung sections were stained with May-Grünwald-Giemsa solution. Briefly, BAL cells or lung sections fixed in methanol for 2-3 min were stained with May-Grünwald solution for 15 min, and then dipped in Giemsa solution for 10 min. After washing with running tap water, dried slides were covered with mounting solution and confirmed under a microscope. The number of mast cells were quantified in 5 sites of $200 \times 200 \mu$ m areas (5 areas/each slide $\times$ 8 mice/each group = 40 areas) (Hong et al., 2013a).

2.5. Immunohistochemistry and Immunofluorescence

Lung sections (3 μ m) were deparaffinized in xylene and washed in ethanol.

Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 5 min. Slides were treated with citrate buffer using microwave to antigen retrieval, and then blocked with 1% BSA in PBS for 1 h. The slides were incubated for overnight with a primary Ab against CD19 (1:100). After that, the slides were incubated with HRP-conjugated goat anti-mouse (1:1000) for 1 h, and then washed with PBS, followed by staining with diaminobenzidine (DAB). The slides were counterstained with hematoxylin and finally mounted using aqueous mounting medium, and then examined under a light microscopy (Axiophot, Carl Zeiss, Oberkochen, Germany) (Hong et al., 2013a).

For immunofluorescence, lung sections (3 μ m) were deparaffinized with xylene and washed in ethanol. Slides were blocked with 1% BSA in PBS for 1 h and incubated with the Abs against mMCP5, CD19, IgE or IgA (diluted 1:25) in a blocking solution for overnight. The slides were treated with the corresponding FITC or TEXAS-Red-conjugated anti-IgG for 1 h at room temperature. Washing between steps in each stage was performed for 2~3 min. After washing, the slides were mounted using an aqueous mounting medium, and examined using a LSM 5 EXCITER confocal microscope (Carl Zeiss, Oberkochen, Germany) (Hong et al., 2013a).

2.6. Western blot analysis

BAL cells, BMMCs, B cells (1×10^6 cells) or lung tissues (50 mg/50 μ l) were suspended in a low-salt lysis buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 2 μ g/ml aprotinin, 2 μ g/ml leupeptin] and allowed to swell on ice for 30 min. The cells or tissues were then homogenized using a Polytron homogenizer (Kinematica, Littau, Switzerland). After centrifugation, supernatants obtained

from cells or tissue homogenates were analyzed by 10% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose membranes. The membranes were washed with PBS containing 0.1% Tween 20 (PBST), and then blocked for 1 h in 5% skim milk in PBST. After the membranes were washed with PBST, they were incubated for 1 h at room temperature with primary Abs against actin, tryptase, B220, CD19, CD40, CD40L, OX40, TAK, MEKK1, mMCP5, OX40L, TRAF2, TRAF6, MAPKs, as well as p-TAK, p-MEKK1, p-ERK, p-JNK, p-p38 diluted with PBST (1:1000). Membranes were washed with PBST and treated with HRP-conjugated goat anti-mouse or HRP-conjugated rabbit anti-goat IgG or HRP-conjugated goat anti-rabbit IgG (diluted to 1:5,000~1:10,000) in PBST for 1 h. After washing, the protein bands were visualized by ECL using a chemiluminometer (Amersham Biosciences, Buckinghamshire, UK) (Hong et al., 2013a).

2.7. Measurement of total and OVA-specific serum IgE and IgA level

To obtain serum, blood samples collected by cardiac puncture immediately after anesthesia were centrifuged at $550 \times g$ for 30 min after clotting at room temperature. *In vitro*, the supernatants (200 μ l) were isolated from media of B cells co-cultured with BMMCs and of B cells stimulated with CD40L (soluble mouse recombinant CD40L) or OX40 (OX40 purified anti-CD134 Ab; LEATTM) agonist. Amounts of IgE or IgA in the sera, BAL fluid or the supernatants (total IgE and IgA) were analyzed using total and specific IgE ELISA kit and total and specific IgA ELISA kit, respectively (Hong et al., 2013a). Briefly, for measurement of total, OVA-specific IgE, or IgA in sera, BAL fluid or the supernatants, 96-well microplates were coated with purified rat anti-mouse IgE or IgA for overnight, and treated with mouse sera, BAL fluid or supernatants, followed by adding total IgE or IgA or

biotinylated OVA specific Ab which is prepared with a horseradish peroxidase-conjugated goat anti-biotin Ab, respectively. Mouse standard IgE or IgA was used for calibrating the assay. Reactions were read using an ELISA plate reader at 450 nm. The lowest detection limit for total and OVA-specific IgE or IgA was better than 2.1 ng/ml and 1.5 ng/ml, 3.5 ng/ml and 5 ng/ml, respectively (Hong et al., 2013a).

2.8. Immunoassay for cytokines, histamine and leukotrienes (LTs)

In the BAL fluid, supernatants obtained from lung tissue homogenates ($20,000 \times g$, 10 min) or from media of B cells stimulated with CD40L or OX40 agonist, amounts of cytokines were determined using an ELISA kit according to manufacturer's methods. Standard curves were generated using different concentrations of each of the recombinant cytokines. The lowest detection limit for IL-4 was better than 31.3 pg/ml, for IL-6 was 7.8 pg/ml, and for IL-13 and TGF- β was 15.6 pg/ml.

The amounts of histamine or LTs in BAL fluid or each supernatant were determined using a histamine ELISA kit and LTs ELISA kit, respectively. The concentrations of histamine or LTs were calculated using the standard curves generated by their specific standards. LTs were analyzed using analysis tools on the Cayman Chemical website (<http://www.caymanchem.com/app/template/analysis%2CEIA.vm/a/z>). The results for histamine or LTs are expressed by ng/ml or pg/ml, respectively. The lowest detection limits for histamine or LTs was 2.5 ng/ml and 7.8 pg/ml, respectively (Hong et al., 2013a).

2.9. Culture and activation of bone marrow-derived mast cells (BMMCs)

Bone marrow cells obtained from femurs and tibias of mice were cultured in RPMI-1640 supplemented 50% WEHI-3B conditioned media for 5 weeks. The medium was changed at every 2~3 days during cultured periods, and the cells were transferred to new plates to separate them from other adherent cells (Hong et al., 2013a). The purity for BMMCs using May-Grünwald-Giemsa staining was more than 95% of total cells.

2.11. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from BMMCs, B cells, BAL cells (1×10^6 cells) or lung tissues (50 mg/500 μ l), and then EMSA were performed as described previously (Kim et al., 2011).

Ten μ l of a mixture of NF- κ B (5'-AGT TGA GGG GAC TTT CCC AGG C-3', 3'-TCA ACT CCC CTG AAA GGG TCC G-5') or AP-1 (5'-CGC TTG ATG AGT CAG CCG GAA-3', 3'-GCG AAC TAC TCA GTC GGC CTT-5') oligonucleotide (1.75 pmol/ μ l), T4 polynucleotide kinase 10X buffer, [γ - ^{32} P]ATP (10 μ Ci; 3,000 Ci/mmol), nuclear-free water, and T4 polynucleotide kinase (5-10 U/ μ l) were incubated at 37 °C for 30 min. After adding 89 μ l Tris-EDTA (TE) buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA], unincorporated nucleotides were removed from the DNA probe by chromatography through a G-25 spin column. The nuclear extract (4 μ l) and gel shift binding 5X buffer (6 μ l) [20% glycerol, 5 mM MgCl₂, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50mM Tris-HCl (pH 7.5), 0.25 mg/ml poly (dI-dC)] were incubated at room temperature for 10 min, and then 20-30 fmol of ^{32} P-labeled NF- κ B or AP-1 oligonucleotide (9 μ l) was added, and incubated at room

temperature for 20 min. After stopping the reaction, 1 µl of 10X gel loading buffer was added to each reaction. Reaction mixtures (20 µl) were electrophoresed on 6% polyacrylamide gels, and the gels were analyzed using FLA-2000 (FUJIFILM, Tokyo, Japan).

2.12. Reverse-transcriptase-polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated from BAL cells (1×10^6 cells) or lung tissues (50 mg/500 µl) using Trizol reagent. RT-PCR was performed in a final volume of 50 µl using a amfiRivert one-step RT-PCR kit in an automated thermal cycler (BIOER Technology, Hangzhou, China). PCR assays were performed for 35 cycles. PCR products were analyzed using 1% agarose gel containing ethidium bromide (EtBr) (Hong et al., 2013a).

The primer sequences used were as follows: IL-4 sense, 5-TCG GCA TTT TGA ACG AGG TC-3; IL-4 anti-sense, 5-GAA AAG CCC GAA AGA GTC TC-3; IL-13 sense, 5-CAG CTC CCT GGT TCT CTC AC-3; IL-13 anti-sense, 5-CCA CAC TCC ATA CCA TGC TG-3; TGF-β sense, 5-CTC TCC ACC TGC AAG ACC AT-3; TGF-β anti-sense, 5-CTG CCG TAC AAT TCC AGT GA-3; IL-1β sense, 5-TGG TGT GTG ACG TTC CCA TT-3; IL-1β anti-sense, 5-CCG ACA GCA ACG AGG CTT TT-3; IL-5 sense, 5-ATG GAG ATT CCC ATG AGC AC-3; IL-5 anti-sense, 5-GTC TCT CCT CGC CAC ACT TC-3; IL-6 sense, 5-TCT TCA TGG TCG GCT TTT CT-3; IL-6 anti-sense, 5-TGA TTG TAC CAC ACC GTG CT-3; IL-8 sense, 5-CAA ACC TTT CCA CCC CAA AT-3; IL-8 anti-sense, 5-ATT GCA TCT GGC AAC CCT AC-3; TNF-α sense, 5-TTA TCT CTC AGC TCC ACG CC-3; TNF-α anti-sense, 5-TGC GCA CTG AAA GCA TGA TC-3; GAPDH sense, 5-GAT GCA GGG ATG ATG TTC TG-3; GAPDH anti-sense, 5-GTG AAG GTC GGT AAC GG-3.

Supplementary figure legends

Fig. 1. Effects of anti-CD40 and anti-OX40L Abs on the expression of surface molecules, levels of IgE and IgA, release of mediators or recruitment of inflammatory cells into BAL fluid or lung tissues of OVA-challenged mice. C57BL/6 mice were divided into six groups (eight mice/group), as follows; NC (negative control) or OVA groups (mice were sensitized and nebulized with PBS or OVA, respectively), α CD40, α OX40L, α CD40 plus α OX40L or IgG Ab (used as a positive control) groups [OVA mice were pretreated with anti-CD40 blocking Ab (Clone HM40-3), anti-OX40L blocking Ab (Clone RM134L), anti-CD40 plus anti-OX40L Abs or IgG once daily 30 min before OVA challenge for 3 days]. Mice were sensitized with 20 μ g/200 μ l OVA adsorbed in 1 mg/50 μ l of aluminum hydroxide gel adjuvant delivered by i.p. injection on days 1 and 15, and then challenged nasally with 5 % OVA in PBS for 30 min once daily for 3 days (from day 22 to day 24) using a nebulizer. NCs were sensitized and nebulized with PBS. All mice were killed on day 25. Expression of surface molecules was determined in the protein extracts isolated from lung tissues by Western blot. Levels of IgE/IgA in BAL fluid, or amounts of cytokines, histamine and LTs in the supernatant isolated from lung tissue homogenates were determined by ELISA. BAL cells separated using cytopsin were stained with Diff-Quick for counting by standard morphological criteria. (A) and (B) Expression of CD40 and CD40L, and OX40 and OX40L, respectively. #, Numbers below bands are mean values ($n = 4$) of the ratio of band densities of surface molecules versus those of control and actin as determined by four independent experiments. (C) and (D) Total and OVA-specific IgE and IgA levels in BAL fluid. (E)-(G) Amounts of cytokines related to Ig class switching, histamine and LTs, respectively, in lung tissues. (H) Numbers of individual inflammatory cells recruited in BAL fluid. ^{***}, $P < 0.001$

versus NC mice. ⁺, $P < 0.05$; ⁺⁺, $P < 0.01$; ⁺⁺⁺, $P < 0.001$ versus OVA mice.

Fig. S2. Effects of anti-CD40 and anti-OX40L Abs on the co-localization of mast cells and B cells in the lung tissues of OVA-challenged mice. Experimental details of the animal models of allergic asthma, treatments with blocking Abs, and the definition of all symbols used are provided in Fig. 1 legend of text. Co-localization of mast cells (mMCP5) and IgG TEXAS-Red as an isotype control Ab for B cells, IgG FITC as an isotype control Ab for mast cells and CD19 or mMCP5 and CD19 in lung tissues was determined by immunofluorescence. Degrees of immunofluorescence (yellow) developed by co-localization were quantified by counting numbers of cells at 5 sites of area 200 μm x 200 μm under a microscope (5 areas/slide x 8 mice/each group = 40 areas). Values (means $\pm$ S.E.M.) are shown in the merge box below. (A) Co-localization of mMCP5 (green) and isotype IgG TAXAS-Red. (B) Co-localization of isotype IgG FITC and CD19 (red). (C) Co-localization (yellow) of mMCP5 and CD19 (red). IgG Ab in right column was used a positive control instead of blocking Abs. Original magnification in NC (negative control) was x400 and the scale bars were 100 μm .

Fig. S3. Time courses for expression of CD40-CD40L or OX40-OX40L, or time courses and dose responses for anti-CD40 Ab or anti-OX40L Ab pretreatment in the B cells co-cultured with BMMCs. BMMCs (1×10^6 cells) were activated with 10 ng/ml DNP-HSA and 0.1 $\mu\text{g/ml}$ anti-DNP IgE Ab (referred to as act-BMMCs), and B cells (1×10^6 cells) were activated with 0.5 $\mu\text{g/mL}$ anti-mouse IgM Ab (referred to as act-B cells) for 48 h. B cells and BMMCs were co-cultured for the time periods indicated. Anti-CD40 Ab, anti-OX40L Ab or

both Abs were pretreated with time periods or concentration indicated before B cell activation. Expression of surface molecules were determined in protein extracts isolated from BMMCs or B cells separated from co-culture by Western blot. (A) Time courses for expression of CD40-CD40L or OX40-OX40L in B cells or BMMCs separated after co-culture. (B) Time courses and dose responses for anti-CD40 Ab pretreatment on expression of CD40-CD40L in B cells or BMMCs after co-culture. (C) Time courses and dose responses for anti-OX40L Ab pretreatment on expression of OX40-OX40L in B cells or BMMCs after co-culture. The histogram of densitometry results shows means $\pm$ S.E.M. ($n = 4$) of the ratios of the band densities of surface molecules versus the control and actin of four independent experiments. co, co-culture. ^{***}, $P < 0.001$ versus NC (negative control).

Fig. S4. Dose responses or time courses for CD40L or OX40 agonist on the expression of signaling molecules in B cells, or expression of CD40L and OX40 protein by CD40L or OX40 siRNA in B cells. B cells (1×10^6 cells) were stimulated with CD40L or OX40 as an agonist (CD40L-sit- or OX40-sti-B cells) for concentration or the time periods indicated. Transfection for CD40L or OX40 on B cell surface was carried out according to the manufacture's method. Expression of TRAFs, MEKK1 and TAK1, MAP kinases, activities of NF- κ B and AP-1, or MAP kinases were determined in protein extracts isolated from B cells using Western blot and EMSA, respectively. (A) Dose responses for CD40L or OX40 agonists on expression of TRAF2 or TRAF6 in B cells. (B) Time courses for CD40 or OX40L agonist on expression and activities of signaling molecules in B cells. (C) Time courses for expression of MAP kinases in B cells. Initial time for expression of MAP kinases was 30 min. (D) Time courses for CD40L or OX40 agonist on the activities of NF- κ B and

AP-1. (E) Expression of CD40L or OX40 in CD40L- and OX40-sti-B cells, respectively, after transfection of CD40L or OX40. #, Numbers below bands are mean values ($n = 4$) obtained as described in Supplementary Fig. 1 legend.

Fig. S5. Effects of anti-CD40 and anti-OX40L Abs on the activities of NF- κ B/AP-1 or mRNA expression of inflammatory cytokines in BAL cells and lung tissues of OVA-challenged mice. Experimental details for animal models of allergic asthma, treatment with blocking Abs and definition of all symbols used was described in Supplementary Fig. 1 legend. Activities of NF- κ B and AP-1 or expression of cytokine mRNA was determined in the nuclear or mRNA extracts isolated from BAL cells or lung tissues using EMSA and RT-PCR, respectively. (A) Activities of NF- κ B and AP-1. (B) Expression of cytokine mRNA. IgG Ab was used as a positive control instead of blocking Abs. #, Numbers below bands are mean values ($n = 4$) obtained as described in Supplementary Fig. 1 legend.

Fig. S6. Dose responses and time courses for expression of Fc α RI and activities of signaling molecules or mediator release in the Fc α RI-Ag-mediated BMMCs or effects of anti-CD40 and anti-OX40L Abs on the expression of Fc ϵ RI or Fc α RI in the BAL cells of OVA-challenged mice. BMMCs (1×10^6 cells) were sensitized/activated with DNP-KLH/anti-DNP IgA (10 μ g/ml/10 μ g/ml) for Fc α RI-mediated res for the time periods indicated or 5 h. Experimental details for animal models of allergic asthma, treatment with blocking Abs and definition of all symbols used were described in Supplementary Fig. 1 legend. Expression of Fc ϵ RI, Fc α RI, CD40L and OX40, MAP kinases or activities of NF- κ B/AP-1 were

determined in the protein or nuclear extracts isolated from BMMCs, BAL cells using Western blot and EMSA, respectively. Amounts of cytokines, histamine or LTs were determined using ELISA. (A) Dose responses for DNP-KLH (Ag) on Fc α RI expression in BMMCs. (B) Time courses for DNP-KLH (Ag) on the expression of Fc α RI, CD40L or OX40 in BMMCs. (C) Amounts of cytokines related to Ig class switching in the Fc α RI-mediated BMMCs. (D) Amounts of histamine and LTs released in the Fc α RI-mediated BMMCs. (E) The time courses for expression of MAP kinases in Fc α RI-mediated BMMCs. (F) Activities of NF- κ B/AP-1 in the IgE-mediated or IgA-mediated BMMCs. (G) Expression of Fc ϵ RI or Fc α RI after blocking Abs pretreatment in BAL cells and lung tissues (*in vivo*). #, Numbers below bands are mean values ($n = 4$) obtained as described in Supplementary Fig. 1 legend. IgG was used as positive control instead of blocking Abs. IgE, DNP-HSA/anti-DNP IgE Ab reaction; IgA, DNP-KLH/anti-DNP IgA Ab reaction. **, $P < 0.01$; ***, $P < 0.001$ versus NC mice.