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A nasal double DNA adjuvant system induces atheroprotective IgM antibodies via dendritic cell-B-1a B cell interactions

Hideki Yoshimatsu [a,b], Kosuke Kataoka [a,c,d,*], Kohtaro Fujihashi [e,f], Tatsuro Miyake [a,c], Yoshiaki Ono [a,b]

[a] Graduate School of Dentistry, Osaka Dental University, 8-1 Kuzuha Hanazono-cho, Hirakata-city, Osaka 573-1121, Japan
[b] Department of Special Care Dentistry, Osaka Dental University Hospital, 1-5-17 Otemae, Chuo-ku, Osaka-city, Osaka 540-0008, Japan
[c] Department of Preventive and Community Dentistry, Osaka Dental University, 8-1 Kuzuha Hanazono-cho, Hirakata-city 573-1211, Japan
[d] Department of Oral Health Science and Social Welfare, Graduate School of Biomedical Sciences, Tokushima University, 3-18-15 Kuramoto-cho, Tokushima-city, Tokushima 770-8504, Japan
[e] Division of Clinical Vaccinology, International Research and Development Center for Mucosal Vaccines, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan
[f] Department of Pediatric Dentistry, School of Dentistry, The University of Alabama at Birmingham, 1919 7th Avenue South, SDB 801A2, Birmingham, AL 35294-0007, USA

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ABSTRACT

We previously demonstrated that the dendritic cell (DC)-targeting nasal double DNA adjuvant system, which consists of a DNA plasmid expressing Flt3 ligand (pFL) and CpG oligodeoxynucleotide 1826 (CpG ODN), elicits specific immune responses to various antigens in the mucosal and systemic compartments. Here, we investigated, using phosphorylcholine (PC)-conjugated keyhole limpet hemocyanin (PC-KLH) as an antigen, whether the nasal double DNA adjuvant system induces protective immunity to atherosclerosis in apolipoprotein E-deficient (*ApoE* KO) mice. Further, we assessed the molecular and cellular mechanisms in the induction of anti-PC-specific immune responses. Nasal immunization with PC-KLH plus pFL and CpG ODN enhanced induction of PC-specific IgM in plasma, peritoneal fluids, and nasal washes when compared with mice administered PC-KLH alone. Of importance, these antibodies exhibited highly specific binding to the PC molecule, and dose-dependent binding to anti-T15 idiotype (AB1-2). Twelve weeks after the last immunization, the nasal double DNA adjuvant system with PC-KLH resulted in a reduction of atherogenesis in the aortic arch of *ApoE* KO mice. Therefore, we next assessed immunocytological mechanism to induce these antibodies. The nasal double DNA adjuvant system with PC-KLH resulted not only in significantly increased frequencies of $CD11c^+$ DCs in the spleen, peritoneal cavity (PEC), and nasopharyngeal-associated lymphoid tissues (NALT), but also significantly increased expression of a proliferation-inducing ligand and B-cell-activating factor by $CD11c^+$ DCs. In addition, the double DNA adjuvant system induced significantly increased numbers of B-1 B cells in the spleen, PEC, and NALT, and increased expression of transmembrane activator and calcium modulator and cyclophilin ligand interactor on $CD5^+$ $B220^+$ (B-1a) B cells. These findings demonstrated that the nasal double DNA adjuvant system with PC-KLH resulted in the induction of T15-like antibodies in the mucosal and systemic lymphoid tissues through interaction between DCs and B-1a B cells, and inhibited the progression of atherogenesis.

1. Introduction

Atherosclerosis constitutes a significant health impairment, given that atherosclerosis is a risk factor for cardiovascular disease, a leading cause of mortality and dysfunction worldwide. [1]. Atherosclerosis is a severe chronic disease characterized by inflammation of the arterial walls, which develops from the accumulation of low-density lipoprotein (LDL) and dying cells in the arterial inner membrane [2]. It is well-known that the uptake of oxidized LDL (OxLDL) by foamy macrophages results in fatty streak integra-

Abbreviations: APRIL, A proliferation-inducing ligand; BAFF, B cell activation factor of the TNF family; BAFF-R, BAFF receptor; BCMA, B cell maturation antigen; CpG ODN, CpG oligodeoxynucleotide; DC, dendritic cell; FL, Flt3 ligand; pFL, a DNA plasmid encoding FL; NALT, nasopharyngeal-associated lymphoid tissues; NWs, nasal washes; PC, phosphorylcholine; PC-KLH, PC-conjugated key limpet hemocyanin; PEC, peritoneal cavity; PFs, peritoneal fluids; SIgA, secretory IgA; TACI, Transmembrane activator and calcium modulator and cyclophilin ligand interactor.

* Corresponding author at: Department of Oral Health Science and Social Welfare, Graduate School of Biomedical Sciences, Tokushima University, 3-18-15 Kuramoto-cho, Tokushima 770-8504, Japan.
E-mail address: kataoka-k@tokushima-u.ac.jp (K. Kataoka).

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tion or vascular smooth cell proliferation, ultimately leading to arterial thrombosis [3]. Phosphorylcholine (PC) is the main component of LDL and OxLDL, and is known as an oxidation-specific epitope [4] that exhibits strong proinflammatory and atherosclerotic effects [5]. Epidemiological studies have demonstrated that natural immunoglobulin M (IgM) antibodies against PC derived from B-1 B cells [6] confer protection against cardiovascular disease [7]. On the other hand, low levels of anti-PC IgM antibodies have been shown to be associated with increased risk for cardiovascular disease [8,9]. In addition, the murine T15 idiotype, one kind of anti-PC IgM antibody, is involved in the decrease of atherosclerosis lesion formation through binding to pneumococcal antigens and OxLDL [10,11]. The atheroprotective effect of the T15 idiotype is due primarily to the blockade of OxLDL uptake by macrophages [5]. In previous work, we reported that nasal immunization of mice with the combination of PC-conjugated keyhole limpet hemocyanin (PC-KLH) and a plasmid encoding the Flt3 ligand cDNA (pFL) (which serves as an adjuvant) elicits PC-specific IgM and IgA antibody responses in plasma and bronchoalveolar lavage fluid. Furthermore, anti-PC antibodies reacted with anti-T15 idiotype antibody (AB1-2) in a dose-dependent manner, attenuating *Streptococcus pneumoniae* colonization of the lower and upper respiratory tracts of mouse [12].

B-1 B cells in the peritoneal and pleural cavity of mice differ from conventional B cells, exhibiting distinct surface phenotypes. B-1 B cells are known to constitutively and mainly secrete primarily T cell-independent IgM antibodies [13,14]; B-1 B cells are stimulated by direct recognition of B cell-activating molecules, such as the B cell activation factor of the TNF family (BAFF; also known as tumor necrosis factor ligand superfamily member 13B) and a proliferation-inducing ligand (APRIL), ligands that are derived from dendritic cells (DCs) [15]. It has been shown that transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI) and B cell maturation antigen (BCMA) on B cells are common receptors for BAFF and APRIL, such that TACI and BCMA contribute to B cell survival and maintenance [16,17], while also enhancing class switching recombination by B cells via the classical NFκB pathway [18]. It also has been reported that the overexpression of APRIL in apolipoprotein E-deficient (*ApoE* KO) mice results in the accumulation of peritoneal B-1a B cells and the induction of serum OxLDL-specific IgM antibodies [19]. Furthermore, BAFF-overexpression also increases the levels of plasma anti-OxLDL IgM antibodies [20] and contributes (through TACI activation) to anti-inflammatory effects in advanced atherosclerotic lesions [21]. Therefore, the interactions between APRIL/BAFF and TACI/BCMA are thought to be important for the differentiation of IgM^+ B cells into plasma cells, which provides a significant contribution to atheroprotection [22,23].

To preferentially induce antigen-specific antibody responses in various mucosal and systemic lymphoid tissues, mucosal vaccines require appropriate adjuvants [24,25]. In this regard, we have demonstrated that pFL as a nasal adjuvant preferentially expands $CD8^+$ DCs and subsequently induces antigen-specific mucosal immune responses mediated by IL-4, eliciting the production of $CD4^+$ T cells when mice are nasally immunized with a combination of ovalbumin (OVA) or bacterial antigen and pFL [26,27]. Furthermore, we have shown that nasal immunization with pFL combined with a CpG oligodeoxynucleotide (CpG ODN), as a double DNA adjuvant system, enhances mucosal and systemic immune responses to OVA [28], pneumococcal surface protein A [29], influenza virus hemagglutinin [30], and FimA of *Porphyromonas gingivalis* [31,32], via effects that are mediated by activation of DCs. In a recent study, we showed that nasal administration of FimA, a major subunit protein of *Porphyromonas gingivalis* fimbriae, in combination with pFL and CpG ODN effectively decreases *Porphyromonas gingivalis* infection in the respiratory tract; this efficacy is mediated through increased numbers of $CD8^+$ and $CD11b^+$ DCs and a balanced Th1- and Th2-type cytokine response in the mucosal tissues [33].

In the present study, we investigated whether the nasal double DNA adjuvant system, when combined with PC-KLH, induced anti-PC antibody responses, resulting in the protection of *ApoE* KO mice from atherosclerosis. Furthermore, we assessed the cellular and molecular interactions between DCs and B cells.

2. Materials and methods

2.1. Animals

Female *ApoE*-deficient spontaneously hyperlipidemic (C.KOR/StmSlc-Apoe^{shl}; *ApoE* KO) mice (8 weeks-old) on the BALB/c background were used for immunization. Animals were maintained at five per cage in horizontal laminar flow cabinets and were provided sterile food and water as part of a specific pathogen-free facility at Osaka Dental University. All animal experiments were conducted according to the guidelines provided by the "Guide for the Care and Use of Animals" of the Osaka Dental University, and were approved by the Committee on the Ethics of Animal Experiments of the Osaka Dental University (Approval Nos. 20–02009 and 20–02011).

2.2. Nasal immunization and sample collection schedule

Mice were immunized by the nasal route four times, at weekly intervals, with 10 μL of phosphate-buffered saline (PBS) containing 50 μg of PC-KLH (Biosearch, Petaluma, CA, USA) plus 50 μg of pFL and 10 μg of CpG ODN as double DNA adjuvant. As controls, mice were immunized nasally with 10 μL of PBS containing 50 μg of PC-KLH alone or double DNA adjuvant alone (50 μg of pFL and 10 μg of CpG ODN). All mice were immunized nasally under intraperitoneal anesthesia with hydrochloric acid medetomidine (0.3 mg/kg), midazolam (4 mg/kg), and butorphanol tartrate (5 mg/kg). Plasma, peritoneal fluids (PFs), and nasal washes (NWs) samples were collected 7 days after the last nasal immunization (Fig. 1) [26,33]. NWs were obtained by instillation of 1 mL of PBS three times into the posterior opening of the nasopharynx with a 30-gauge hypodermic needle [33].

2.3. Immune responses to PC

To assess PC-specific antibody levels in plasma, PFs, and NWs, each sample (each group, N = 20) was subjected to ELISA as described previously [12,26]. Briefly, 96-well microtest assay plates (BD BioSciences, Irvine, CA, USA) were coated with 5 μg/mL of PC-conjugated bovine serum albumin (PC-BSA) (Biosearch) in PBS. After blocking with 1% BSA in PBS, 2-fold serial dilutions of samples were added to each well. Following incubation of the plates overnight at 4 °C, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgM, IgG, or IgA antibodies (Southern Biotechnology Associates, Inc, Birmingham, AL) was added to the wells. The color reaction was developed for 15 min at room temperature using 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid). End point titer were expressed as the reciprocal $\log_2$ of the last dilution that gave an OD at 415 nm of 0.1 greater than background.

2.4. Enumeration of PC-specific antibody-forming cells (AFCs)

Mononuclear cells from the spleen and nasopharyngeal-associated lymphoid tissue (NALT) (each group, N = 20) were isolated aseptically by a mechanical dissociation method using gentle

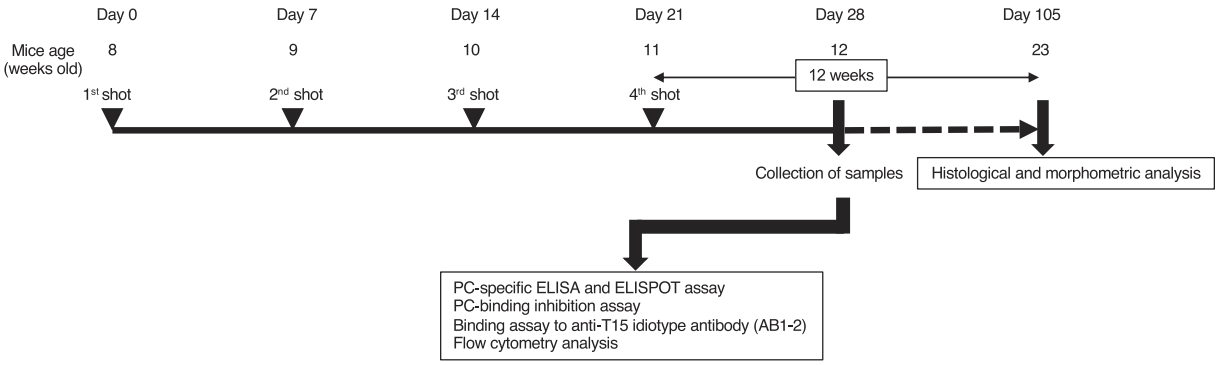

Fig. 1. Experimental schedule for nasal vaccination and for various assays. *ApoE* KO mice (8 weeks old, female) were immunized nasally, four times at weekly intervals, with PC-KLH (50 μg) alone, pFL (50 μg) and CpG ODN (10 μg) as double DNA adjuvant alone or PC-KLH (50 μg) plus pFL (50 μg) and CpG ODN (10 μg). Seven days after the last immunization, samples were harvested and the various assays and analyses were performed. Twelve weeks after the final nasal immunization, histological and morphometric analysis was performed.

teasing through stainless steel screens [26], and mononuclear cells from the peritoneal cavity (PEC) were extracted by perfusion as described previously [34]. The mononuclear cells were subjected to an enzyme-linked immunospot (ELISPOT) assay to determine the numbers of PC-specific antibody-forming cells (AFCs). Briefly, 96-well nitrocellulose plates (Millipore, Bedford, MA, USA) were coated with 100 μL/well of PC-BSA in PBS (5 μg/mL). The numbers of PC-specific AFCs were counted with the aid of a stereomicroscope as described previously [26].

2.5. PC-binding inhibition assay

To investigate the precise binding specificity of the anti-PC antibodies induced by nasal immunization with double DNA adjuvant plus PC-KLH to PC (N = 20), avidity was tested using hapten-free PC (in the form of phosphorylcholine chloride calcium salt tetrahydrate, PC-Cl) (Sigma-Aldrich, St. Louis, MO, USA), acetylcholine chloride (Ach-Cl) (Sigma-Aldrich), and O-phosphorylethanolamine (PE) (Sigma-Aldrich). PC-Cl and Ach-Cl were employed as potent inhibitors; PE was used as a negative control. Each of these compounds were pre-incubated with anti-PC antibodies as described previously [12,35]. Briefly, the concentration of anti-PC antibodies in the various samples from immunized mice were adjusted to a constant optical density (O.D. 1.0) by PC-specific ELISA. Serially diluted samples of 25 μL each subsequently were incubated with different concentrations of PC-Cl, Ach-Cl, or PE (again, 25 μL each) overnight at 4 °C, and 50 μL of the mixture then was added to a plate coated with PC-BSA (5 μg/mL, 100 μL/well). The plates were incubated with goat alkaline phosphatase (AP) - conjugated anti-mouse IgM and IgA antibodies (Southern Biotechnology Associates) and then were developed with *p*-nitrophenyl phosphoric acid disodium salt (PNPP; Invitrogen, Camarillo, CA, USA). The inhibition of the ability of anti-PC antibodies to bind to PC was calculated using the following formula: percent antibody binding = 100 × (OD_{405} of wells incubated with sample and inhibitors – OD_{405} of wells incubated without samples) / (OD_{405} of wells incubated with sample without inhibitor – OD_{405} of wells incubated without sample).

2.6. Specific binding to anti-T15 idiotype antibody (AB1-2)

In brief, 96-well microtest assay plates (BD Biosciences) were coated with 10 μg/mL AB1-2 (GeneTex, Irvine, CA, USA) in 0.05 M $NaHCO_3$/0.05 M Na_2CO_3 buffer (pH 9.6), and enzyme activity was subsequently blocked with 1% BSA in Tris-buffered saline (TBS; pH 7.4). Two-fold serially diluted samples (each group, N = 10) were added and incubated overnight at 4 °C. After washing the plates with PBS, AP-conjugated goat anti-mouse IgM and IgA antibodies were added to individual wells. The color reaction was developed for 30 min at room temperature with 100 μL/well of a PNPP solution containing 1 mM $MgCl_2$ (pH 9.4–9.6).

2.7. Flow cytometry analyses

Aliquots of mononuclear cells (1.0×10^6 cells) isolated from spleen, PEC, and NALT (each group, N = 10) were preincubated with anti-mouse CD16/32 monoclonal antibody (TruStain FcX PLUS antibody, Fc Block; BioLegend, San Diego, USA). For the detection of BAFF and APRIL expressing DCs, cells were stained with brilliant violet 421 (BV)-conjugated anti-mouse CD11c monoclonal antibody (Clone N418; BioLegend) and phycoerythrin (PE)-labeled anti-mouse APRIL monoclonal antibody (Clone A3D8; BioLegend), or PE-labeled anti-mBAFF/BLyS monoclonal antibody (Clone 121808; R&D Systems, Minneapolis, MN, USA). To determine the frequency of B-1a B cells, mononuclear cells were stained with fluorescein isothiocyanate (FITC) conjugated-anti-CD23 monoclonal antibody (Clone B3B4, BioLegend), BV-conjugated anti-B220 monoclonal antibody (Clone RA3-6B2; BioLegend) and allophycocyanin (APC)-labeled anti-CD5 monoclonal antibody (Clone 53–7.3; BioLegend). In some experiments, mononuclear cells isolated from various lymphoid tissues were incubated with anti-CD23 labelled microbeads (Miltenyi Biotec, Auburn, CA, USA). CD23 negative population was purified by using AutoMacs Pro Separator system (Miltenyi Biotec). Subsequently, aliquots of CD23 negative cells were preincubated with anti-mouse CD16/32 monoclonal antibody, and were then stained with BV-conjugated anti-mouse B220 monoclonal antibody, APC-conjugated anti-mouse CD5 monoclonal antibody, FITC-conjugated anti-mouse BCMA monoclonal antibody (Clone 161616; R&D Systems) and PE-conjugated anti-mouse TACI monoclonal antibody (Clone 8F10; BioLegend), or PE-conjugated anti-mouse BAFF-R monoclonal antibody (Clone 7H22-E16; BioLegend). These samples were added 7-AAD Viability Staining Solution (BioLegend), and subjected to flow cytometric analysis (FACSVerse®, BD Biosciences) as described previously [32]. Data were analyzed using FlowJo software, version 10.6.1 (Tree Star, Ashland, OR, USA).

2.8. Histological and morphometric analysis of atherosclerotic plaque accumulation

Twelve weeks after the last nasal immunization, mice (age; 23 weeks old, each group, N = 10) were euthanized and hearts were carefully dissected and removed. Half of the heart, including the aortic origin, was isolated and embedded in OCT compound

(Tissue-Tek®, Sakura Finetek, Torrance, CA, USA) in cryomolds and stored at −80 °C. The aorta samples were processed into serial frozen sections with a Leica CM 1850 Cryostat (Leica Microsystems, Nussloch, Germany). All cryosections (10 μm thicknesses) were obtained from the aortic root. Subsequently, staining with hematoxylin-eosin, and Oil Red-O (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was performed for visualization of lipid in plaques, as described previously [36,37]. The stained area was estimated thereafter using All-in-One microscopy (BZ-9000; KEYENCE Corporation, Tokyo, Japan), and values were analyzed with Image J 1.52 (NIH, Bethesda, MD, USA).

2.9. Statistical analysis

All mouse groups were compared to control mice via unpaired Mann-Whitney U tests or unpaired student's T test using Prism version 7 (GraphPad Software, Inc., La Jolla, CA, USA). p values of < 0.05 and 0.01 were considered statistically significant.

3. Results

3.1. PC-specific antibody responses in systemic and mucosal tissues of mice immunized with PC-KLH plus double DNA adjuvant

We first examined whether nasal immunization with PC-KLH plus double DNA adjuvant enhances PC-specific antibody responses in systemic and mucosal tissue. Mice administered nasal PC-KLH plus double DNA adjuvant showed significantly higher levels of PC-specific IgM antibody titers in plasma, PFs, and NWs as well as anti-PC IgA antibody in NWs when compared with those in mice administered nasal PC-KLH alone (Fig. 2). In contrast to PC-KLH plus double DNA adjuvant group, PC-specific IgM in plasma, PFs, and NWs, and IgA in NWs of mice given nasal PC-KLH or double DNA adjuvant alone were essentially the same as those of naïve mice (horizontal dotted line, Fig. 2). Similarly, PC-specific IgG antibody titers in plasma, PFs, and NWs of mice given PC-KLH with or without double DNA adjuvant were unchanged from the responses in naïve mice (Fig. 2). To support these findings, mice immunized nasally with PC-KLH plus double DNA adjuvant displayed high number of PC-specific IgM AFCs in spleen and PEC, and high numbers of PC-specific IgM and IgA AFCs in NALT (Fig. 3). These results clearly indicated that the nasal application of double DNA adjuvant with PC-KLH effectively enhanced PC-specific IgM antibody responses in systemic and mucosal lymphoid tissues and IgA antibody responses in mucosal lymphoid tissues. To evaluate the safety of this nasal vaccine, we monitored the body weight from the first day of vaccination to 12 weeks after the last vaccination (Day 105). The body weights of immunized mice nasally with PC-KLH plus double DNA adjuvant was not seen a significant difference when compared with that of non-immunized mice given PBS (**Supplementary Figure S1**).

3.2. PC-specificity of IgM or IgA antibodies induced by nasal double DNA adjuvant system

To investigate the functional properties of anti-PC IgM and IgA antibodies in plasma, PFs, and NWs elicited by nasal immunization with the double DNA adjuvant system plus PC-KLH, the inhibitory activities of the binding between each antibody and PC-BSA were

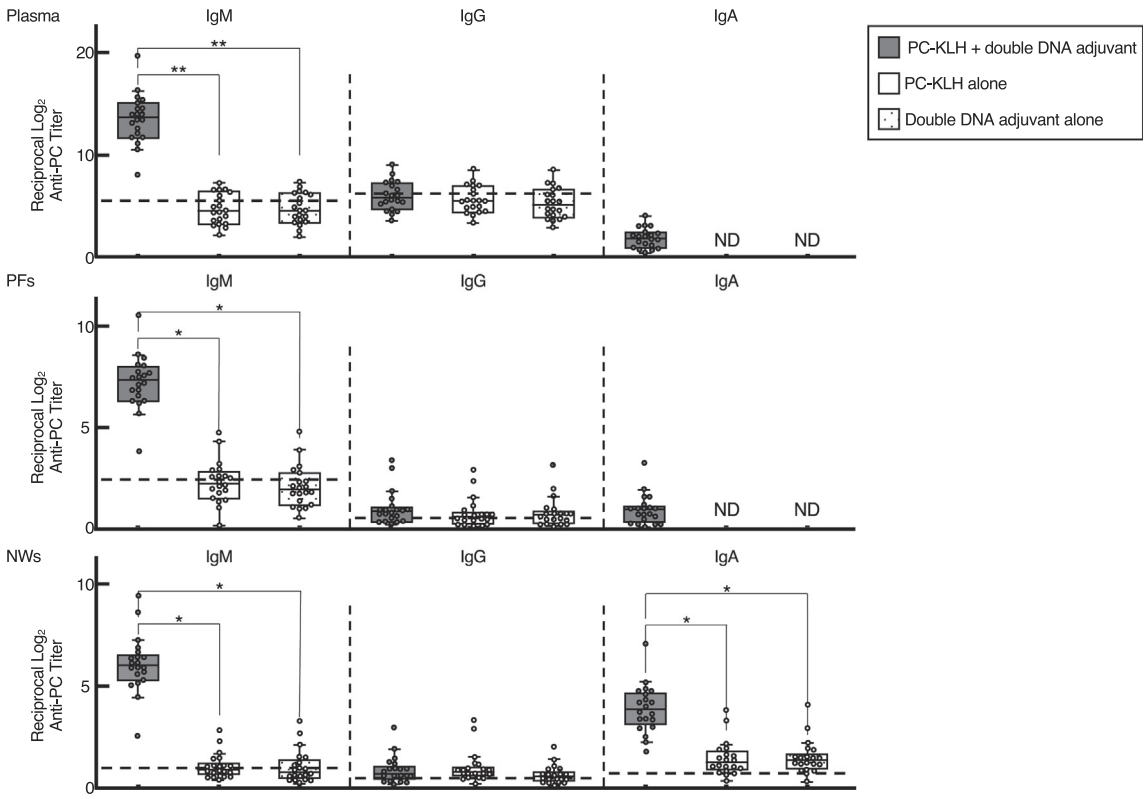

Fig. 2. PC-specific IgM, IgG, and IgA antibody responses in plasma, PFs, and NWs. *ApoE* KO mice (8 weeks old, female) were immunized nasally, four times at weekly intervals, with PC-KLH (50 μg) plus pFL (50 μg) and CpG ODN (10 μg) (closed column), PC-KLH (50 μg) alone (open column), pFL (50 μg) and CpG ODN (10 μg) as double DNA adjuvant alone (dotted column). Seven days after the last immunization, the levels of anti-PC IgM, IgG, and IgA antibodies in plasma, PFs, and NWs were determined by a PC-specific ELISA method. The values shown are the median and interquartile range (the first quartile – 1.5 IQR, the 3rd quartile + 1.5 IQR) of 20 mice in each experimental group. Mann-Whitney U test, $^{*}p < 0.05$ or $^{**}p < 0.01$ compared with mice group given PC-KLH alone or double DNA adjuvant alone. ND means that OD values were not detected. The dotted line indicates titers of samples from naïve mice.

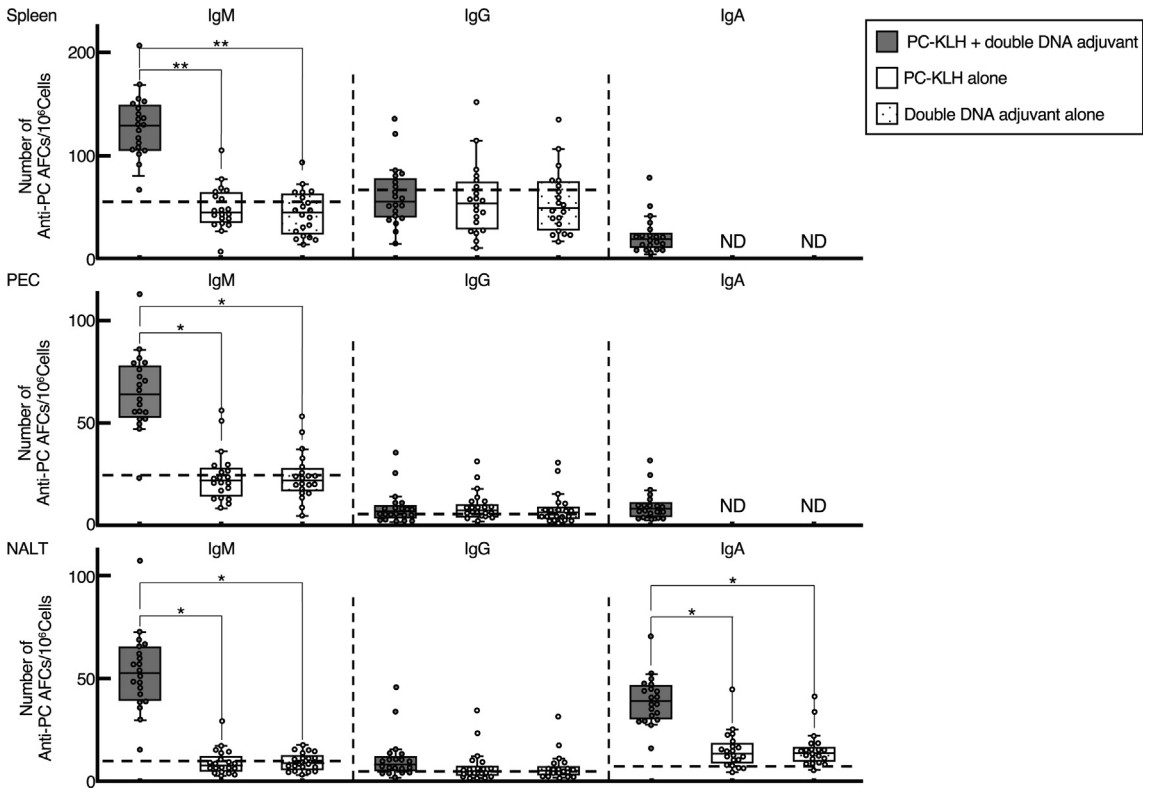

Fig. 3. Enumeration of PC-specific IgM, IgG and IgA AFCs in spleen, PEC, and NALT. Each group of *ApoE* KO mice (8 weeks old, female) was immunized nasally with PC-KLH plus double DNA adjuvant (closed column), PC-KLH alone (open column) or pFL (50 μg) and CpG ODN (10 μg) as double DNA adjuvant alone (dotted column), once weekly for four consecutive weeks. Seven days after the final immunization, mononuclear cells were isolated from the spleen, PEC, and NALT, and these cells then were subjected to PC-specific ELISPOT assay to determine the numbers of PC-specific IgM, IgG, and IgA AFCs. The values shown are the median and interquartile range (the first quartile – 1.5 IQR, the 3rd quartile + 1.5 IQR) of 20 mice in each experimental group. Mann-Whitney *U* test, $^{*}p < 0.05$ or $^{**}p < 0.01$ when compared with mice immunized with PC-KLH alone or double DNA adjuvant alone. The dotted line indicates cell counts of samples from naïve mice.

assessed by using PC-Cl, Ach-Cl, and PE. PC-Cl at concentrations of 10^{-3} M to 10^{-1} M showed significant inhibition of the interaction between IgM antibodies in plasma or PFs and PC-BSA compared to the effects seen with Ach-Cl and PE (Fig. 4). Furthermore, NWs IgM antibodies were inhibited significantly at PC-Cl at concentrations of 10^{-5} M to 10^{-1} M concentrations (Fig. 4). On the other hand, both Ach-Cl and PE resulted in significantly smaller inhibitory effects (Fig. 4). Interestingly, PC-Cl at concentrations of 10^{-5} M to 10^{-1} M significantly inhibited binding by IgA antibody in plasma, PFs, and NWs obtained from mice immunized by nasal vaccination with PC-KLH plus double DNA adjuvant; these effects with PC-Cl were dose dependent (Fig. 4) compared to those obtained with Ach-Cl and PE. Further, when anti-T15 monoclonal antibody (AB1-2) was added to our samples as the inhibitor, AB1-2 inhibited anti-PC antibodies to bind to PC at lower concentrations than those of PC-Cl (**Supplementary Figure S2**). In contrast, PC-Cl demonstrated essentially no inhibition of the binding to PC-BSA by plasma, PFs, and NWs from mice given nasal PC-KLH alone (data not shown). These findings indicated that the use of double DNA adjuvant as a mucosal adjuvant elicits anti-PC IgM and IgA antibodies that perceive the PC molecule in both the systemic and mucosal compartments.

3.3. Binding of systemic and mucosal antibodies to anti-T15 idiotype monoclonal antibody (AB1-2)

Given that the nasal administration of PC-KLH in combination with the double DNA adjuvant system elicited IgM and IgA antibodies that recognized PC molecules in both the systemic and mucosal compartments, we next examined the interactions between these antibodies and AB1-2, a monoclonal antibody against the T15 idiotype antibody that is specific for PC molecules. We found that IgM antibodies in plasma, PFs, and NWs obtained from mice administered with PC-KLH plus double DNA adjuvant, specifically bound to AB1-2 in a dose-dependent manner compared with the binding of IgM antibodies obtained from mice administered nasally with PC-KLH alone (Fig. 5). Similarly, IgA antibody in plasma and NWs, but not in PFs, of mice given PC-KLH with double DNA adjuvant displayed dose-dependent interactions with AB1-2 (Fig. 5). In contrast, essentially no binding of IgG antibodies was seen in either group of mice (Fig. 5). These results showed that IgM antibodies in the systemic and mucosal lymphoid tissues and IgA antibodies in plasma and NWs induced by PC-KLH plus pFL and CpG ODN are recognized by AB1-2, an anti-T15 idiotype monoclonal antibody, suggesting that PC-KLH plus double DNA adjuvant enhanced the induction of T15-like idiotype antibody in both the systemic and mucosal compartments.

3.4. APRIL/BAFF expression by $CD11c^{+}$ DCs of mice administered nasally with PC-KLH plus double DNA adjuvant

Since our previous studies showed that a combination of pFL and CpG ODN as a nasal adjuvant preferentially expands $CD11c^{+}$ DCs in both systemic and mucosal tissues [28,29,32], we initially assessed the frequencies of $CD11c^{+}$ DCs in the spleen, PEC, and NALT by flow cytometric analysis. Our results revealed that immunization with nasal PC-KLH plus double DNA adjuvant significantly increased the frequencies of $CD11c^{+}$ DCs in spleen, PEC, and NALT compared those obtained following immunization with PC-KLH alone (Fig. 6A).

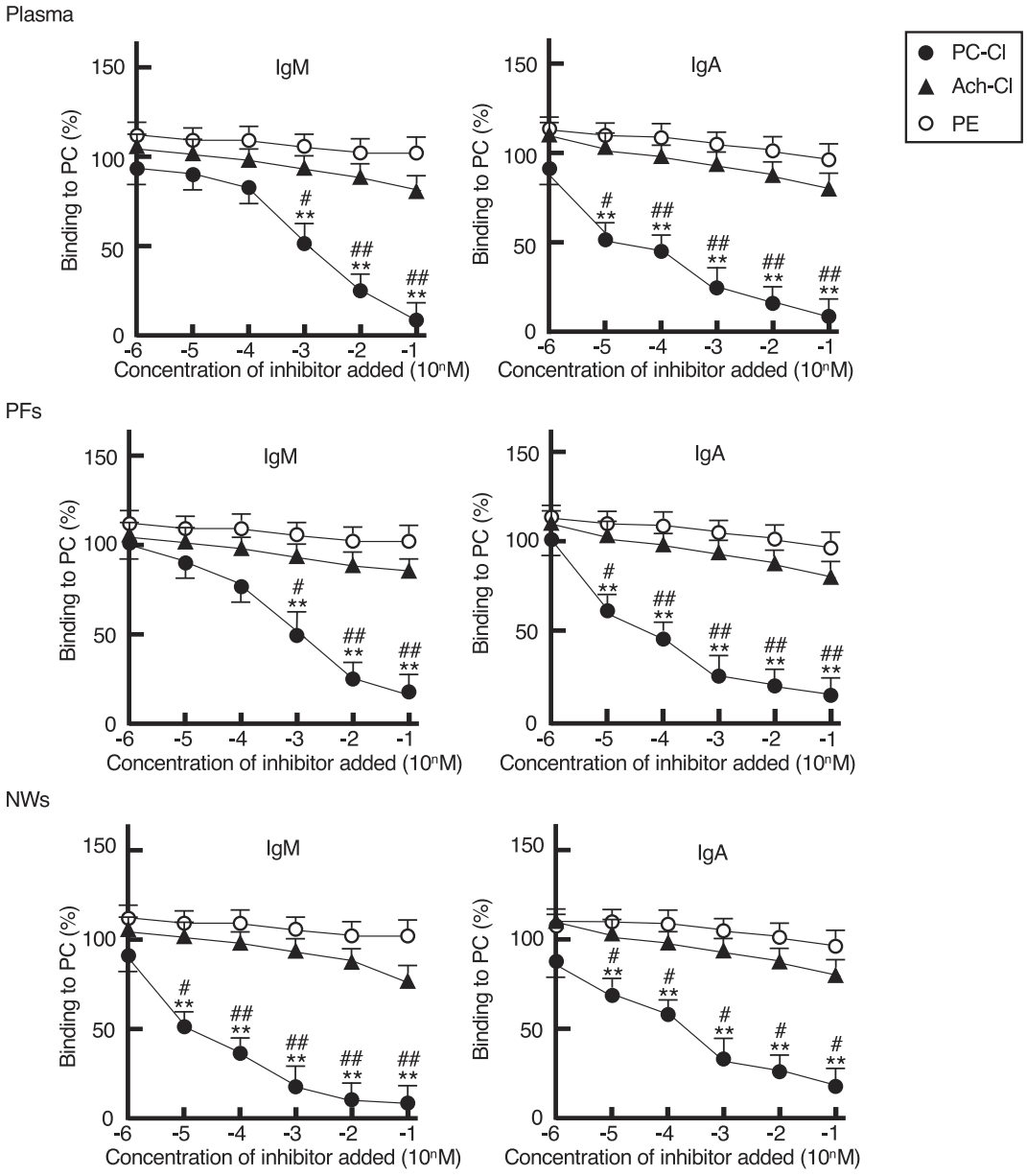

Fig. 4. Inhibition of binding to hapten-free PC. Seven days after the last immunization, plasma, PFs, and NWs samples were collected from *ApoE* KO mice administered nasally with PC-KLH plus double DNA adjuvant. The concentration of samples was adjusted to give an absorbance of 1.0 (OD at 405 nm) according to an ELISA assay using PC-BSA (5 μg/mL) as the antigen. An aliquot (25 μL) of each sample diluted with 1% BSA was pre-incubated with 25 μL of various concentrations of phosphorylcholine chloride (PC-Cl; closed circle), acetylcholine chloride (Ach-Cl; closed triangle), or O-phosphorylethanolamine (PE; open circle); the pre-incubated samples then were transferred to ELISA plate wells coated with PC-BSA. Binding activities of each sample were detected with goat AP-conjugated anti-mouse IgM and IgA antibodies, and binding attenuation was calculated using the following formula: Percent antibody binding = 100 × (OD_{405} of wells incubated with sample and inhibitors – OD_{405} of wells incubated without sample)/ (OD_{405} of wells incubated with sample without inhibitors - OD_{405} of wells incubated without sample). The values shown are the mean ± SE of 20 mice in each experimental group. Student's *t* test, $^{*}p < 0.05$ or $^{**}p < 0.01$ compared with PE as an inhibitor, and $^{\#}p < 0.05$ or $^{\#\#}p < 0.01$ when compared with Ach-Cl as an inhibitor.

We next examined the frequencies of APRIL and BAFF molecule expression based on lymphocyte gating (**Supplemetary Figure S4**) by DCs of spleen, PEC, and NALT, given that IgM antibodies are produced in a T-cell-independent manner [13,14] following direct stimulation by DC-derived BAFF and APRIL [15]. Increased frequencies of APRIL- and BAFF-expressing DCs were seen in the spleen, PEC, and NALT of mice nasally administered with PC-KLH and double DNA adjuvant compared to those in mice administered nasally with PC-KLH alone (Fig. 6B and C). Notably, DCs in PEC of mice administered nasally with PC-KLH plus double DNA adjuvant revealed more than 10-fold increases in APRIL and BAFF expression compared to those in mice administered nasally with PC-KLH alone (Fig. 6B and C). These results suggested that nasal immunization with PC-KLH plus double DNA adjuvant induces DC-mediated T cell-independent B cell activation in both systemic and mucosal tissues.

3.5. APRIL/BAFF receptor expression by B-1a B cells

Previous studies have reported that the T15 idiotype monoclonal antibody is produced primarily by B-1a cells derived from the PEC of mice [38]. Therefore, we next used flow cytometry to analyze the frequencies of B-1a B cells in spleen, PEC, and NALT after nasal immunization with PC-KLH plus a combination of pFL and CpG ODN. The population of $CD5^{+}$, $CD23^{-}$ and $B220^{+}$ (B-1a) B cells was significantly increased in the spleen, PEC, and NALT of

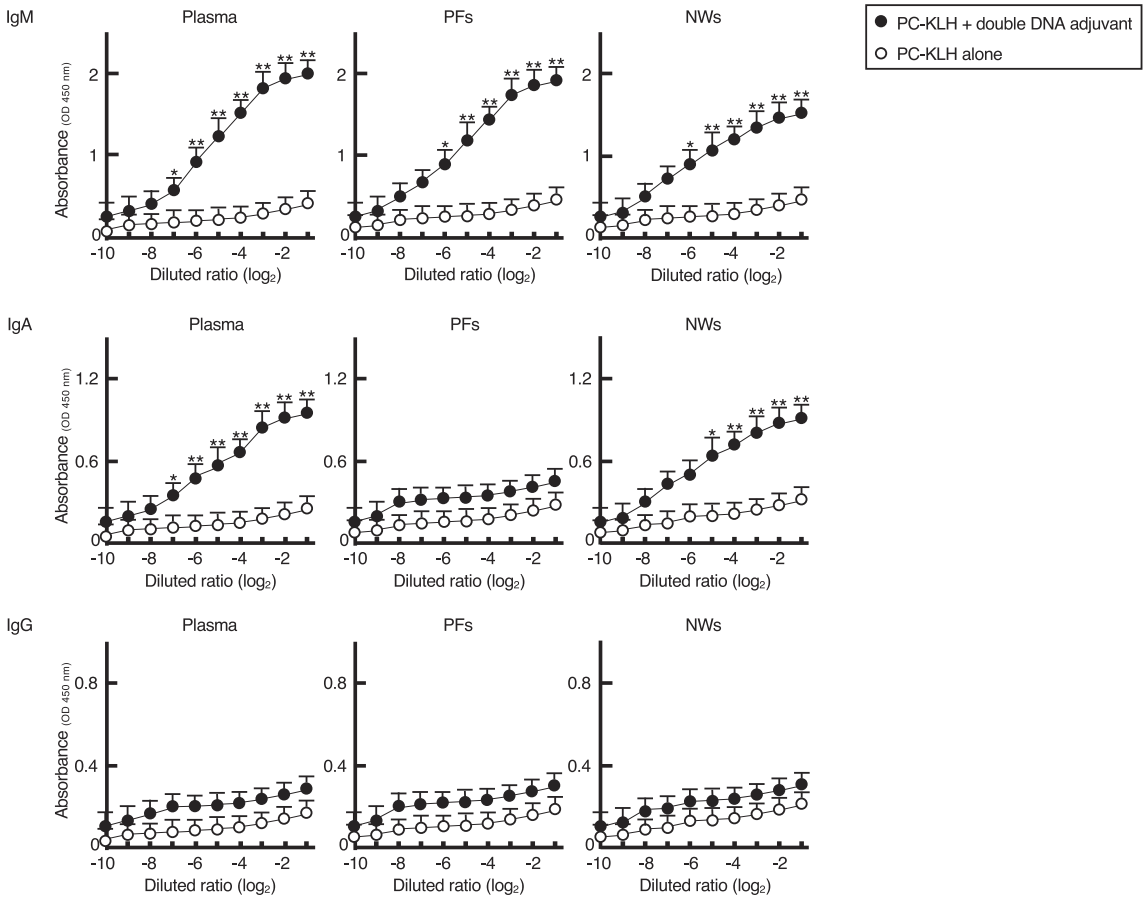

Fig. 5. Interaction between AB1-2 (anti-T15 idiotypic monolonal antibody) and PC-specific IgM, IgA, and IgG antibodies induced by nasal immunization with PC-KLH plus double DNA adjuvant. Plasma, PFs, and NWs were collected seven days after the last immunization. To determine the T15 dominance of the PC-specific IgM, IgA, and IgG antibody responses in the systemic and mucosal compartments, titers in each sample were determined by ELISA methods. The values shown are the mean ± SE of 10 mice in each experimental group. Student's *t* test $^{*}p < 0.05$ or $^{**}p < 0.01$ when compared with the mice immunized with PC-KLH alone.

mice immunized nasally with PC-KLH plus double DNA adjuvant compared to the populations in mice administered nasally with PC-KLH alone (Fig. 6D). On the other hand, no significant increase of $CD5^-$, $CD23^-$ and $B220^+$ B cells were seen in these lymphoid tissues. We next assessed the frequencies of TACI-, BAFF-R- or BCMA- (the receptors for APRIL and BAFF molecules) expressing B-1a B cells in the spleen, PEC, and NALT. Of interest, the frequencies of TACI-expressing B-1a B cells were markedly increased in spleen, PEC, and NALT of mice administered nasally with PC-KLH plus double DNA adjuvant (Table 1). In contrast, the expression levels of BAFF-R and BCMA by B-1a B cells were unchanged in the spleen, PEC, and NALT of mice given nasal PC-KLH with and without double DNA adjuvant (Table 1). These findings indicate that nasal administration of PC-KLH plus double DNA adjuvant preferentially elicits increased numbers of TACI-expressing B-1a B cells in the spleen, PEC, and NALT, most likely by contributing to the induction of the anti-PC IgM antibodies associated with the atheroprotective effect.

3.6. Impact on atherosclerotic plaque accumulation by the immunization with the nasal double DNA adjuvant plus PC-KLH

It has been reported that the T15 idiotype exerts an atheroprotective effect by blocking the uptake of OxLDL by macrophages [5]. Therefore, we next performed histochemical and histomorphometric analyses of the aortic sinus by Oil Red-O and hematoxylin-eosin staining using *ApoE* KO mice. Mice were nasally immunized with PC-KLH plus double DNA adjuvant, and atherosclerotic plaque accumulation was assessed 12 weeks after the final immunization. Significantly less atherosclerotic plaque accumulation observed in mice nasally administered with PC-KLH plus double DNA adjuvant than was seen in mice immunized with PC-KLH alone (Fig. 7A). Thus, the size of atherosclerotic plaques and necrosis core area in *ApoE* KO mice immunized with double DNA adjuvant plus PC-KLH was significantly smaller than those in mice administered nasally with PC-KLH alone (Fig. 7B and C). These results indicated that double DNA adjuvant may be an effective nasal adjuvant to provide prevention of atherosclerosis.

4. Discussion

In this study, we investigated whether a combination of pFL and CpG as a nasal adjuvant elicits functional PC-specific antibody responses for the prevention of atherosclerosis. We showed that nasal immunization of *ApoE* KO mice with PC-KLH plus double DNA adjuvant induced atheroprotective T15 idiotype-like antibody responses through elicitation of $CD11c^+$ DCs and $CD5^+$ B-1 B cells. To the best of our knowledge, this work is the first study to demonstrate that nasal vaccination with PC-KLH plus a combination of pFL and CpG ODN as nasal adjuvants has a potent atheroprotective function, an effect that is mediated by interactions between APRIL- and BAFF-expressing DCs and TACI-expressing B-1a B cells. Nasal vaccination targeting NALT that contains various immunocompetent cells, such as follicular B cells, T cells and DCs is thought to possess lots of advantages to elicit optimal protective immunity

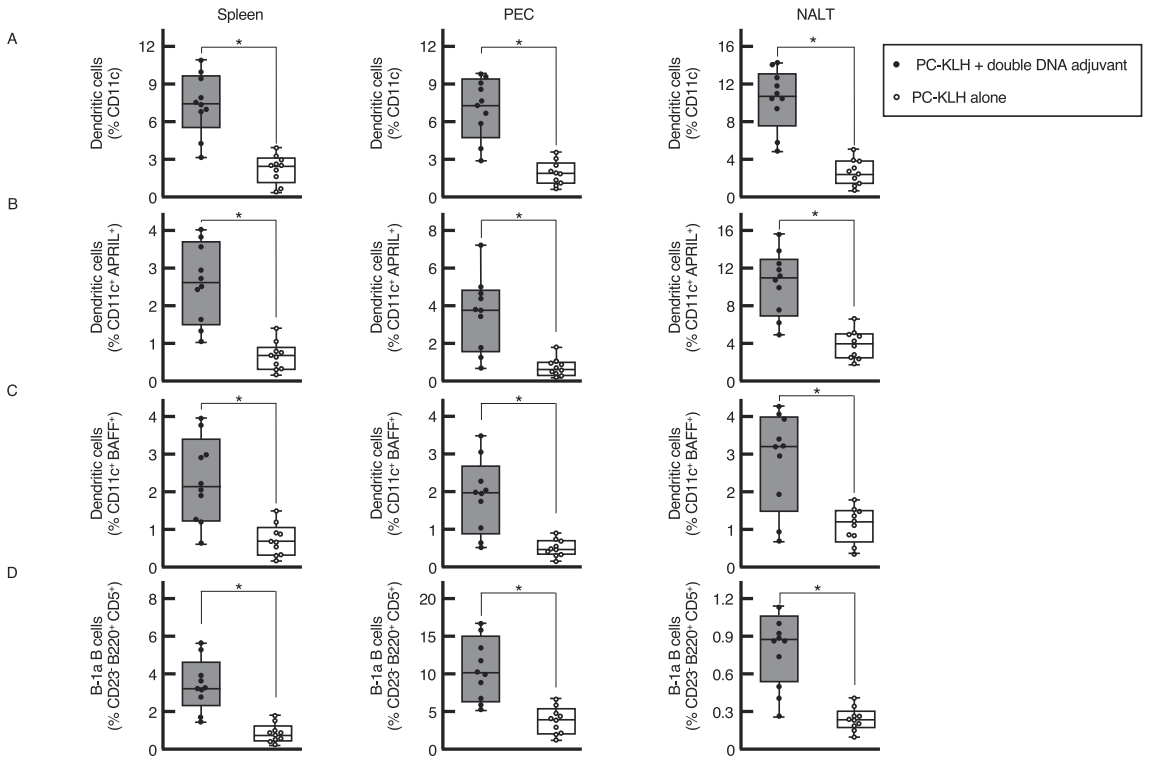

Fig. 6. Characterization of CD11c$^+$ DCs and CD23$^-$ B220$^+$ (B-1) B cells in spleen, PEC, and NALT of *ApoE* KO mice immunized nasally with PC-KLH plus double DNA adjuvant. Mice were immunized nasally, once weekly for four consecutive weeks, with PC-KLH plus double DNA adjuvant (n = 10) or PC-KLH alone (n = 10). Seven days after the last immunization, mononuclear cells from spleen, PEC, and NALT were stained with one of the following: BV-conjugated anti-CD11c monoclonal antibody **(A);** a combination of BV-conjugated anti-CD11c monoclonal antibody and PE-labelled anti-APRIL monoclonal antibody **(B)**; a combination of BV-conjugated anti-CD11c monoclonal antibody and PE-tagged anti-BAFF monoclonal antibody **(C);** a combination of FITC-conjugated anti-CD23 monoclonal antibody, BV-conjugated anti-B220 monoclonal antibody and APC-labeled anti-CD5 monoclonal antibody **(D)** were subjected to flow cytometric analysis by FACSVerse®. The values shown are the median and interquartile range (the first quartile – 1.5 IQR, the 3rd quartile + 1.5 IQR) of 10 mice in each experimental group. Mann-Whitney *U* test, $^*p < 0.05$ compared with PC-KLH alone.

Table 1
The frequencies of TACI-, BAFF-R-, or BCMA-expressing B cells in spleen, PEC, and NALT of *ApoE* KO mice nasally immunized with PC-KLH plus double DNA adjuvant (n = 10) or PC-KLH alone (n = 10).

Tissue	Nasal DA	Percentage of CD5$^+$, CD23$^-$, B220$^+$ (B-1a) B cells			Percentage of CD5$^-$, CD23$^-$, B220$^+$ B cells		
		TACI	BAFF-R	BCMA	TACI	BAFF-R	BCMA
NALT	+	* 16.2 (18.3, 8.8)	11.5 (18.2, 9.3)	11.3 (15.8, 5.8)	1.5 (2.6, 0.7)	1.6 (3.3, 0.7)	1.7 (2.9, 0.7)
	-	10.2 (12.8, 7.3)	11.2 (14.5, 8.6)	11.6 (16.7, 5.3)	1.4 (2.3, 0.7)	1.5 (3.4, 0.5)	1.6 (3.3, 0.3)
PEC	+	* 38.8 (50.2, 24.5)	16.5 (26.2, 11.7)	4.0 (8.8, 1.8)	4.1 (6.0, 1.8)	1.8 (3.25, 0.6)	1.1 (2.1, 0.4)
	-	23.3 (29.6, 18.8)	15.5 (25.9, 10.8)	3.0 (5.8, 0.9)	4.0 (5.3, 1.2)	1.6 (2.8, 0.5)	0.9 (2.3, 0.2)
Spleen	+	* 23.4 (29.8, 18.6)	7.5 (11.5, 4.5)	4.5 (8.2, 3.1)	3.2 (4.8, 0.8)	2.0 (3.5, 1.2)	1.6 (2.8, 0.3)
	-	12.9 (15.2, 9.3)	5.1 (10.9, 3.8)	4.4 (7.8, 2.4)	2.8 (4.6, 0.6)	2.1 (3.3, 0.8)	1.5 (3.2, 0.5)

The values shown are the median and interquartile range (the first quartile – 1.5 IQR, the 3rd quartile + 1.5 IQR) of 10 mice in each experimental group. Mann-Whitney *U* test, $^*p < 0.05$ when compared with nasal double DNA adjuvant (-) (PC-KLH alone).

in both mucosal and systemic immune compartments. Further, nasal delivery avoids the degradation of vaccine antigens caused by digestive enzymes or acids. In addition, it does not require a needle and trained medical personnel for delivery and thus causes less pain. However, mucosal vaccination including nasal vaccine requires adjuvants to effectively elicit systemic and mucosal immune responses. Based on these advantages, we hypothesize that this double DNA adjuvant system with PC-KLH effectively stimulates NALT DCs as antigen-presenting cells for the induction of follicular B and T cells in NALT.

Flt3 ligand (FL) is known as a hematopoietic growth factor that has emerged as a potential immunomodulator [39,40]; FL increases and enhances the DC population and the antigen-presenting activity [41,42]. We previously demonstrated that nasal administration of a plasmid expressing the *Fl* cDNA (pFL) plus pneumococcal surface protein (PspA) elicited increased numbers

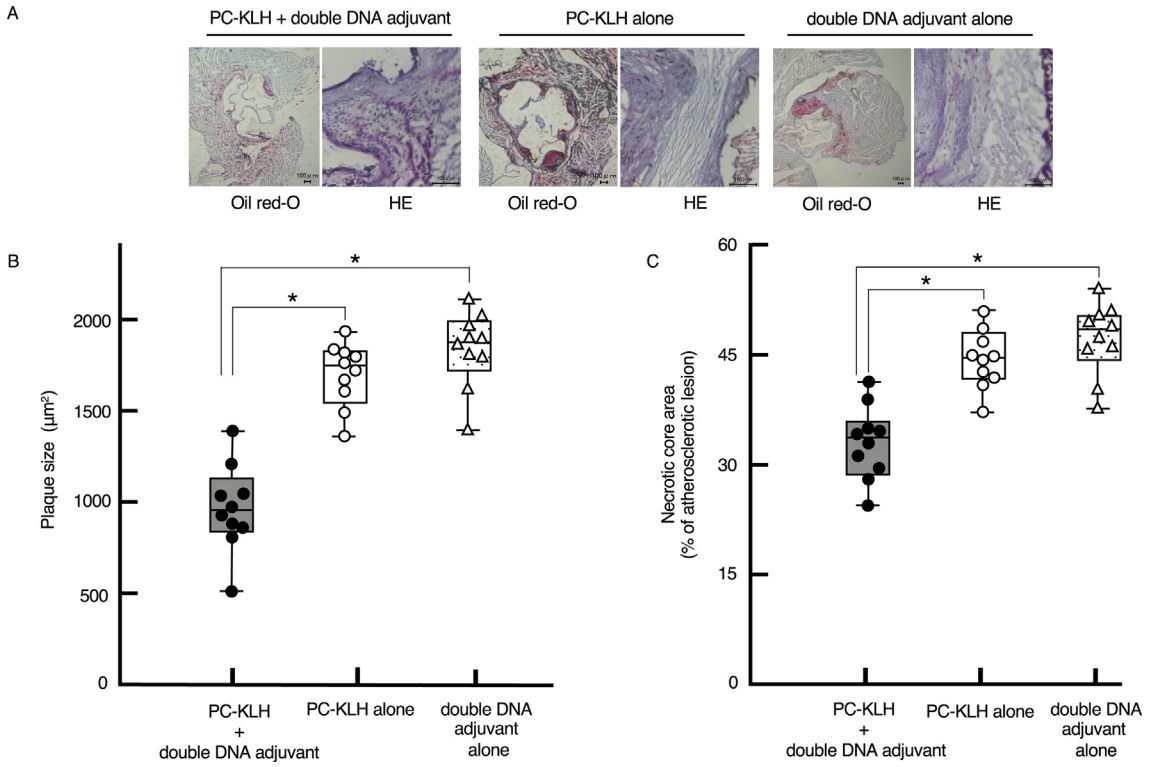

Fig. 7. Atherosclerotic plaques and necrotic core areas in the aortic arch of immunized *ApoE* KO mice. (A) *ApoE* KO mice were administered nasally with PC-KLH plus double DNA adjuvant, double DNA adjuvant alone or PC-KLH alone, four times in consecutive weeks (10 mice per group). Twelve weeks after the final vaccination, the aortic arches were removed and histomorphometric analysis was performed by the Oil red-O staining (left) and Hematoxylin0eosin staining (right) method. The picture represents typical aspect of the aortic arches for each group. **(B)** The plaque formation area (μm^2) and (C) necrotic cores area were calculated using the NIH ImageJ application. Data are expressed as the median and interquartile range (the first quartile – 1.5 IQR, the 3rd quartile + 1.5 IQR) of 10 mice in each experimental group. Mann-Whitney *U* test, $^*p < 0.05$ compared with PC-KLH alone.

of DCs and PspA-specific SIgA antibody responses in the naso-oral cavity [43]. In addition, when CpG ODN, a known TLR9 ligand, was added to pFL (double adjuvant), we observed potentiation of mucosal and systemic immune responses to OVA [28], influenza hemagglutinin antigen [30], *r*FimA [32] and PspA [29,44]; these effects were mediated by the expansion and activation of $CD11^+$ DCs in aged mice. Based on these findings, we hypothesized that nasal immunization with double DNA adjuvant plus PC-KLH would induce antigen-specific functional antibody responses in the systemic and mucosal compartments of ApoE-deficient mice. Since it has been shown that PC-KLH-specific antibody titers are well correlated with atheroprotection [22,23], we optimized nasal immunization protocol. Our results showed that 4 consecutive weeks of vaccination with PC-KLH plus double DNA adjuvant resulted in the maximum immune responses (data not shown). Indeed, nasal immunization, four times at weekly intervals, with PC-KLH plus double DNA adjuvant significantly enhanced PC-specific IgM antibody responses in plasma, PFs, and NWs of *ApoE* KO mice when compared to mice administered nasally with PC-KLH alone (Figs. 2 and 3). Of importance, we confirmed that IgM antibodies induced in plasma, PFs, and NWs showed high specificity for the PC molecule (Fig. 4). We previously showed that the nasal double DNA adjuvant system, when used in combination with various protein antigens, elicited long-lasting antigen-specific IgA and IgG antibody immune responses in the mucosal and systemic compartments [45]. Similarly, the present study showed that PC-specific IgM antibody titers in plasma and NWs were maintained at 12 months after the last immunization which most likely contributed to atheroprotection (**Supplementary Figure S3**). Since there were no significant differences on the frequencies of conventional B cells between mice administrated PC-KLH plus double DNA adjuvant and those administered PC-KLH alone (data not shown), we a predict that the conventional B cells may not involve in the induction of anti-PC IgM and IgA antibody responses. We are currently testing the potential involvement of conventional B cells for the induction of anti-PC antibodies by *in vitro* B cell-culture system. Natural IgM antibodies against PC or oxLDL have been shown to confer protection against cardiovascular disease via inhibition of oxLDL uptake by macrophages [7–10]. Therefore, we next examined the ability of PC-KLH plus double DNA adjuvant-induced antibodies to inhibit plaque accumulation on the aortic sinus in *ApoE* KO mice. Accumulation of plaque on the aortic sinus in mice administered nasally with PC-KLH plus double DNA adjuvant was inhibited and decreased compared to that in mice administered nasal PC-KLH alone (Fig. 7). To our knowledge, these findings are the first to demonstrate that the nasal double DNA adjuvant system can contribute to atheroprotective activities through induction of PC-specific IgM antibodies in the systemic and mucosal compartments.

It is well-known that the murine plasma T15 idiotype is an anti-PC IgM antibody and exhibits atheroprotective functions [6,10], and attenuation of plaque formation in *ApoE* KO mice [46]. It is thought that the atheroprotective action of the T15 idiotype is attributable primarily to blocking the uptake of OxLDL by macrophages, inhibiting inflammatory responses, and promoting apoptotic cell clearance [10]. In contrast, low plasma levels of T15 antibodies caused by lack of IL-5 are known to drive atherosclerosis in $LDLR^{-/-}$ mice [47], while decreased T15 antibodies levels in the aorta have been linked with increased atherosclerotic area in L-selectin-deficient $ApoE^{-/-}$ mice [48]. We previously also have

suggested that the T15 idiotype, which suppresses atherogenesis, is induced not only in the nasal cavity but also in plasma following nasal immunization with pFL plus PC-KLH [12]. The present study confirmed that the anti-PC IgM antibodies induced in *ApoE* KO mice by nasal immunization with PC-KLH plus double DNA adjuvant were of the T15 idiotype, as demonstrated using AB1-2, an anti-T15 idiotype monoclonal antibody. These results further support our conclusion that nasally administered PC-KLH plus double DNA adjuvant vaccine elicits atheroprotective IgM antibodies.

In contrast to the case with anti-PC IgM antibodies, there is limited evidence concerning the atheroprotective effects of PC-specific IgA antibodies. Instead, it has been suggested that serum anti-PC IgA may be associated with long-term cardiovascular disease risk [49]. Thus, PC-specific IgA induced by the nasal double DNA adjuvant system might have an opposing effect, instead promoting atherogenesis. Despite this prediction, we showed that IgA antibodies in plasma and NWs that were obtained from mice immunized nasally with PC-KLH plus double DNA adjuvant exhibited PC specificity and were T15 idiotype antibodies, given that these antibodies showed dose-dependent binding to the AB1-2 monoclonal antibody (Fig. 4). Since nasal administration of PC-KLH plus double DNA adjuvant induced high levels of anti-PC plasma IgM and minimal detectable levels of IgA antibodies (Fig. 2), we predict that anti-PC IgM antibodies play a major role in the prevention of atherogenesis. These data suggest that anti-PC IgA antibodies may provide a protective function countering the induction of atherosclerosis. Experiments to elucidate the precise roles of anti-PC antibodies in atherogenesis are currently underway in our laboratory.

To further examine the cellular and molecular mechanisms employed in the up-regulation of PC-specific immune responses, we characterized the subsets of DCs in the spleen, PEC, and NALT of immunized mice. Nasal immunization with PC-KLH plus double DNA adjuvant elicited higher frequencies of $CD11c^+$ DCs in spleen, PEC, and NALT in *ApoE* KO mice compared to those in mice immunized with PC-KLH alone (Fig. 6A). Since the T15 idiotype, PC-specific IgM antibodies are derived from B-1 B cells [6] by T cell-independent activation [13,14], we next assessed the expression levels of BAFF and APRIL [15] in DCs. Of interest, increased frequencies of APRIL- and BAFF-expressing DCs were seen in spleen, PEC, and NALT of mice immunized with nasally double DNA adjuvant plus PC-KLH compared to those in mice immunized with PC-KLH alone (Fig. 6B and C). BAFF-R, TACI, and BCMA are known to be common receptors for BAFF and APRIL, and expression of these receptors is required for the survival and maintenance of B cells [16,17] as well as for augmenting class-switching recombination through the classical NFκB pathway [18]. We therefore characterized B-1 B cell subsets and their expression levels of BAFF-R, TACI, and BCMA. Higher numbers of $CD5^+$ (B-1a) B cells were seen in spleen, PEC, and NALT of mice immunized nasally with PC-KLH plus double DNA adjuvant than in control mice (Fig. 6D). Further, B-1a B cells expressed higher levels of TACI, but not of BAFF-R and BCMA (Table 1). It has been reported that APRIL overexpression is associated with significant increases in both total and anti-OxLDL-specific plasma IgM antibodies, as well as increased incidences of plaque IgM accumulation, an outcome that is observed upon increases in the numbers of B-1a lymphocytes [19]. Another laboratory similarly has reported increased levels of plasma anti-OxLDL IgM antibodies following TACI activation in BAFF transgenic *ApoE* KO mice [20]. Furthermore, since BAFF exerts anti-inflammatory effects by its interactions with TACI-expressing B-1a B cells [20,21], BAFF neutralization is thought to aggravate atherosclerosis [21]. Taken together, these studies support our hypothesis that increased frequencies of APRIL/BAFF-expressing DCs and TACI-expressing B-1a B cells contribute to the induction of T15 idiotype-like antibody responses, thereby reducing atherosclerotic plaque accumulation.

Previous studies have shown that the anti-PC immune response is largely IgM, and IgM remains within the vascular system, where these antibodies appear to help remove OxLDL and to protect mice from atherosclerosis [5,10]. The titer of IgM antibodies with specificities to epitopes like malondialdehyde and PC are increased during atherogenesis, and these antibodies are associated with atheroprotective effects [50]. Anti-PC antibodies recognize not only bacterial PC but also PC moieties present on other molecules of diverse origins, including self-antigens (such as OxLDL), platelet-activating factor, and apoptotic cells [24]. Thus, the observed atheroprotective effects may represent the results of multiple pathways, including the ability to block the uptake of OxLDL by macrophages, suppression of inflammatory responses, and promotion of apoptotic cell clearance [5,51]. In this study, significantly increased levels of plasma anti-PC IgM antibodies were observed in mice immunized nasally with PC-KLH plus double DNA adjuvant. Our results suggest that anti-PC IgM antibodies, which bind to OxLDL, may attenuate the proatherogenic effect of oxidative agents; thus, immunization with PC-KLH plus double DNA adjuvant may potentiate atheroprotective effects.

Although it has been shown that B-1a B cells play greater roles in atheroprotection in murine studies [22,23], the role of B-1 B cells in the human immune system is poorly understood due to the lack of a defined human B-1 cell subset. It is a great benefit to develop the nasal vaccination strategy which stimulates B cells to produce atheroprotective antibodies in modern worldwide society. In this regard, our current study could facilitate understanding the cellular molecular mechanisms of induction of atheroprotective antibodies and subsequent effective nasal vaccine development in humans.

In summary, we demonstrated that nasal immunization with PC-KLH plus double DNA adjuvant elicits PC-specific IgM antibody responses in plasma and PFs of *ApoE* KO mice; production of these antibodies is mediated by DC-B-1a B cell interactions. Furthermore, we showed that PC-specific IgM antibodies are T15 idiotype-like antibodies, which play an atheroprotective role in *ApoE* KO mice. Thus, the double DNA adjuvant system with PC-KLH may be an effective nasal immunization strategy for reducing atherosclerotic plaque accumulation in the aortic sinus; this immunization strategy appears to enhance PC-specific immune responses, thereby preventing atherosclerosis.

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Author Contributions

KK and KF contributed to the conception, design, data analysis, and interpretation of the study, and drafted and critically revised the manuscript; TM contributed to the conception and interpretation of the study, and critically revised the manuscript; HY and YO contributed to the data acquisition and analysis, and critically revised the manuscript. All authors gave final approval and agreed to be accountable for all aspects of the work.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2022.01.027.

References

[1] Torres N, Guevara-Cruz M, Velázquez-Villegas LA, Tovar AR. Nutrition and atherosclerosis. Arch Med Res 2015;46(5):408–26. https://doi.org/10.1016/j.arcmed.2015.05.010.
[2] Galkina E, Ley K. Immune and inflammatory mechanisms of atherosclerosis. Annu Rev Immunol 2009;27(1):165–97. https://doi.org/10.1146/annurev.immunol.021908.132620.
[3] Holvoet P, Collen D. Oxidation of low density lipoproteins in the pathogenesis of atherosclerosis. Atherosclerosis 1998;137:33–8. https://doi.org/10.1016/s0021-9150(97)00305-5.
[4] Shaw PX, Hörkkö S, Chang M-K, Curtiss LK, Palinski W, Silverman GJ, et al. Natural antibodies with the T15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity. J Clin Invest 2000;105(12):1731–40. https://doi.org/10.1172/JCI8472.
[5] Que X, Hung M-Y, Yeang C, Gonen A, Prohaska TA, Sun X, et al. Oxidized phospholipids are proinflammatory and proatherogenic in hypercholesterolaemic mice. Nature 2018;558(7709):301–6. https://doi.org/10.1038/s41586-018-0198-8.
[6] Baldan A, Gonen A, Choung C, Que X, Marquart TJ, Hernandez I, et al. ABCG1 is required for pulmonary B-1 B cell and natural antibody homeostasis. J Immunol 2014;193(11):5637–48. https://doi.org/10.4049/jimmunol.1400606.
[7] Su J, Georgiades A, Wu R, Thulin T, de Faire U, Frostegård J. Antibodies of IgM subclass to phosphorylcholine and oxidized LDL are protective factors for atherosclerosis in patients with hypertension. Atherosclerosis. 2006;188(1):160–6. https://doi.org/10.1016/j.atherosclerosis.2005.10.017.
[8] Iseme RA, McEvoy M, Kelly B, Agnew L, Walker FR, Handley T, et al. A role for autoantibodies in atherogenesis. Cardiovasc Res 2017;113(10):1102–12. https://doi.org/10.1093/cvr/cvx112.
[9] Gigante B, Leander K, Vikström M, Baldassarre D, Veglia F, Strawbridge RJ, et al. Low levels of IgM antibodies against phosphorylcholine are associated with fast carotid intima media thickness progression and cardiovascular risk in men. Atherosclerosis 2014;236(2):394–9. https://doi.org/10.1016/j.atherosclerosis.2014.07.030.
[10] Binder CJ, Hörkkö S, Dewan A, Chang M-K, Kieu EP, Goodyear CS, et al. Pneumococcal vaccination decreases atherosclerotic lesion formation: molecular mimicry between *Streptococcus pneumoniae* and oxidized LDL. Nat Med. 2003;9(6):736–43. https://doi.org/10.1038/nm876.
[11] Vale AM, Kapoor P, Skibinski GA, Elgavish A, Mahmoud TI, Zemlin C, et al. The link between antibodies to OxLDL and natural protection against pneumococci depends on D_H gene conservation. J Exp Med 2013;210(5):875–90. https://doi.org/10.1084/jem.20121861.
[12] Baatarjav T, Kataoka K, Gilbert RS, Terao Y, Fukui M, Goto M, et al. Mucosal immune features to phosphorylcholine by nasal Flt3 ligand cDNA-based vaccination. Vaccine 2011;29(34):5747–57. https://doi.org/10.1016/j.vaccine.2011.05.097.
[13] Maruyama M, Lam K-P, Rajewsky K. Memory B-cell persistence is independent of persisting immunizing antigen. Nature 2000;407(6804):636–42. https://doi.org/10.1038/35036600.
[14] Baumgarth N. The double life of a B-1 cell: self-reactivity selects for protective effector functions. Nat Rev Immunol 2011;11(1):34–46. https://doi.org/10.1038/nri2901.
[15] Gronwall C, Vas J, Silverman G. Protective roles of natural IgM antibodies. Front Immunol 2012;3:66. https://doi.org/10.3389/fimmu.2012.00066.
[16] Mackay F, Schneider P. Cracking the BAFF code. Nat RevImmunol 2009;9(7):491–502. https://doi.org/10.1038/nri2572.
[17] Gross JA, Johnston J, Mudri S, Enselman R, Dillon SR, Madden K, et al. TACI and BCMA are receptors for a TNF homologue implicated in B-cell autoimmune disease. Nature 2000;404(6781):995–9. https://doi.org/10.1038/35010115.
[18] Castigli E, Scott S, Dedeoglu F, Bryce P, Jabara H, Bhan AK, et al. Impaired IgA class switching in APRIL-deficient mice. Proc Natl Acad Sci U S A 2004;101:3903–8. https://doi.org/10.1073/pnas0307348101.
[19] Bernelot Moens SJ, van Leuven SI, Zheng KH, Havik SR, Versloot MV, van Duivenvoorde LM, et al. Impact of the B cell growth factor APRIL on the qualitative and immunological characteristics of atherosclerotic plaques. PLoS One 2016;11: e0164690. doi: 10.1371/journal.pone.0164690.
[20] Jackson SW, Scharping NE, Jacobs HM, Wang S, Chait A, Rawlings DJ. BAFF overexpression reduces atherosclerosis via TACI-dependent B cell activation. J Immunol 2016;197:4529–34. https://doi.org/10.4049/jimmunol.1601198.
[21] Tsiantoulas D, Sage AP, Göderle L, Ozsvar-Kozma M, Murphy D, Porsch F, et al. B cell-activating factor neutralization aggravates atherosclerosis. Circulation 2018;138(20):2263–73. https://doi.org/10.1161/CIRCULATIONAHA.117.032790.
[22] Pattarabanjird T, Li C, McNamara C. B cells in atherosclerosis: Mechanisms and potential clinical applications. JACC Basic Transl Sci 2021;6(6):546–63. https://doi.org/10.1016/j.jacbts.2021.01.006.
[23] Tabares-Guevara JH, Villa-Pulgarin JA, Hernandez JC. Atherosclerosis: immunopathogenesis and strategies for immunotherapy. Immunotherapy 2021;13(14):1231–44. https://doi.org/10.2217/imt-2021-0009.
[24] Kurono Y, Yamamoto M, Fujihashi K, Kodama S, Suzuki M, Mogi G, et al. Nasal immunization induces Haemophilus influenzae-specific Th1 and Th2 responses with mucosal IgA and systemic IgG antibodies for protective immunity. J Infect Dis 1999;180(1):122–32. https://doi.org/10.1086/314827.
[25] Fujihashi K, McGhee JR, Kweon MN, Cooper MD, Tonegawa S, Takahashi I, et al. gamma/delta T cell-deficient mice have impaired mucosal immunoglobulin A responses. J Exp Med. 1996;183(4):1929–35. https://doi.org/10.1084/jem.183.4.1929.
[26] Kataoka K, McGhee JR, Kobayashi R, Fujihashi K, Shizukuishi S, Fujihashi K. Nasal Flt3 ligand cDNA elicits $CD11c^+$ $CD8^+$ dendritic cells for enhanced mucosal immunity. J Immunol 2004;172(6):3612–9. https://doi.org/10.4049/jimmunol.172.6.3612.
[27] Kataoka K, Fujihashi K. Dendritic cell-targetting DNA-based mucosal adjuvants for the development of mucosal vaccines. Expert Rev Vaccines 2009;8:1183–93. https://doi.org/10.1586/erv.09.80.
[28] Fukuiwa T, Sekine S, Kobayashi R, Suzuki H, Kataoka K, Gilbert RS, et al. A recombination of Flt3 ligand cDNA and CpG ODN as nasal adjuvant elicits NALT dendritic cells for prolonged mucosal immunity. Vaccine 2008;26:4849–59. https://doi.org/10.1016/j.vaccine.2008.06.091.
[29] Fukuyama Y, King JD, Kataoka K, Kobayashi R, Gilbert RS, Hollingshead SK, et al. A combination of Flt3 ligand cDNA and CpG oligodeoxynucleotide as nasal adjuvant elicits protective secretory-IgA immunity to *Streptococcus pneumoniae* in aged mice. J Immunol 2011;186(4):2454–61. https://doi.org/10.4049/jimmunol.1002837.
[30] Asanuma H, Zamri NB, Sekine S, Fukuyama Y, Tokuhara D, Gilbert RS, et al. A novel combined adjuvant for nasal delivery elicits mucosal immunity to Influenza in aging. Vaccine 2012;30(4):803–12. https://doi.org/10.1016/j.vaccine.2011.10.093.
[31] Kataoka K, Fukuyama Y, Briles DE, Miyake T, Fujihashi K. Dendritic cell-targeting DNA- based nasal adjuvants for protective mucosal immunity to Streptococcus pneumoniae. Microbiol Immunol. 2017;61(6):195–205. https://doi.org/10.1111/1348-0421.12487.
[32] Kobuchi K, Kataoka K, Taguchi Y, Miyake T, Umeda M. Nasal double DNA adjuvant induces salivary FimA-specific secretory IgA antibodies in young and aging mice and blocks *Porphyromonas gingivalis* binding to a salivary protein. BMC Oral Health 2019;19:188. https://doi.org/10.1186/s12903-019-0886-2.
[33] Kataoka K, Kawabata S, Koyanagi K, Hashimoto Y, Miyake T, Fujihashi K. Respiratory FimA-specific secretory IgA antibodies upregulated by DC-targeting nasal double DNA adjuvant are essential for elimination of *Porphyromonas gingivalis*. Front Immunol 2021;12:. https://doi.org/10.3389/fimmu.2021.634923634923.
[34] Kato C, Mikami M. Effect of aging on BCG immunostimulation of *Porphyromonas gingivalis* infection in mice. Biomed Res. 2011;32(1):45–54. https://doi.org/10.2220/biomedres.32.45.
[35] Tanaka N, Fukuyama S, Fukuiwa T, Kawabata M, Sagara Y, Ito H-o, et al. Intranasal immunization with phosphorylcholine induces antigen specific mucosal and systemic immune responses in mice. Vaccine 2007;25(14):2680–7. https://doi.org/10.1016/j.vaccine.2006.10.014.
[36] Wang J, Li P, Xu X, Zhang B, Zhang J. MicroRNA-200a inhibits inflammation and atherosclerotic lesion formation by disrupting EZH2-mediated methylation of STAT3. Front Immunol 2020;11:907. https://doi.org/10.3389/fimmu.2020.00907.
[37] Bernelot Moens SJ, van Leuven SI, Zheng KH, Havik SR, Versloot MV, van Duivenvoorde LM, et al. Impact of the B cell growth factor APRIL on the qualitative and immunological characteristics of atherosclerotic plaques. PLoS One 2016;11: e0164690. doi: 10.1371/journal.pone.0164690.
[38] Masmoudi H, Mota-Santos T, Huetz F, Coutinho A, Cazenave P-A. All T 15 id-positive antibodies (but not the majority of VHT15+ antibodies) are produced by peritoneal CD5+ B lymphocytes. Int Immunol. 1990;2(6):515–20. https://doi.org/10.1093/intimm/2.6.515.
[39] Edwan JH, Perry G, Talmadge JE, Agrawal DK. Flt-3 ligand reverses late allergic response and airway hyper-responsiveness in a mouse model of allergic inflammation. J Immunol 2004;172(8):5016–23. https://doi.org/10.4049/jimmunol.172.8.5016.
[40] Triccas JA, Shklovskaya E, Spratt J, Ryan AA, Palendira U, de St. Groth BF, et al. Effect of DNA-and *Mycobacterium bovis* BCG-based delivery of the Flt3 ligand on protective immunity to *Mycobacterium tuberculosis*. Infect Immun 2007;75:5368-75. doi: 10.1128/IAI.00322-07.
[41] Gilliland DG, Griffin JD. The role of Flt3 in hematopoiesis and leukeia. Blood 2002;100:1532–42. https://doi.org/10.1182/blood-2002-02-0492.

[42] Marakovsky E, Brasel K, Teepe M, Roux ER, Lyman SD, Shortman K, et al. Dramatic increase in the numbers of functionally mature dendritic cell subpopulations identified. J Exp Med 1996;184:1953–62. https://doi.org/10.1084/jem.184.5.1953.

[43] Fukuyama Y, King JD, Kataoka K, Kobayashi R, Gilbert RS, Hollingshead SK, et al. Secretory-IgA plays an important role in the immunity to *Streptcoccus pneumoniae*. J Immunol 2010;185:1755–62. https://doi.org/10.4049/jimmunol.1000831.

[44] Fukuyama Y, Ikeda Y, Ohori J, Sugita G, Aso K, Fujihashi K, et al. A molecular mucosal adjuvant to enhance immunity against pneumococcal infection in the elderly. Immune Netw 2015;15:9–15. https://doi.org/10.4110/in.2015.15.19.

[45] Fukuiwa T, Sekine S, Kobayashi R, Suzuki H, Kataoka K, Gilbert RS, et al. A combination of Flt3 ligand cDNA and CpG ODN as nasal adjuvant elicits NALT dendritic cells for prolonged mucosal immunity. Vaccine 2008;26 (37):4849–59. https://doi.org/10.1016/j.vaccine.2008.06.091.

[46] Cesena FHY, Dimayuga PC, Yano J, Zhao X, Kirzner J, Zhou J, et al. Immune-modulation by polyclonal IgM treatment reduces atherosclerosis in hypercholesterolemic apoE$^{-/-}$ mice. Atherosclerosis 2012;220(1):59–65. https://doi.org/10.1016/j.atherosclerosis.2011.10.002.

[47] Binder CJ, Hartvigsen K, Chang MK, Miller M, Broide D, Palinski W, et al. IL-5 links adaptive and natural immunity specific for epitopes of oxidized LDL and protects from atherosclerosis. J Clin Invest 2004;114:427–37. https://doi.org/10.1172/JCI1200420479.

[48] Gjurich BN, Taghavie-Moghadam PL, Ley K, Galkina EV. L-selectin deficiency decrease aortic B1a and B_{reg} subsets and promotes atherosclerosis. Thomb Haemost 2014;112:803–11. https://doi.org/10.1160/TH13-10-0865.

[49] Kankaanpaa J, Sampi M, Bloigu R, Wang C, Akhi R, Kesaniemi YA, et al. IgA antibodies to phosphorylcholine associate with long-term cardiovascular diseases risk. Atherosclerosis. 2018;269:294–300. https://doi.org/10.1016/j.atherosclerosis.2017.12.010.

[50] Weismann D, Hartvigsen K, Lauer N, Bennett KL, Scholl HPN, Issa PC, et al. Complement factor H binds malondialdehyde epitopes and protect from oxidative stress. Nature 2011;478:76–81. https://doi.org/10.1038/nature10449.

[51] Tsiantoulas D, Sage AP, Mallat Z. Binder CJ. Targeting B cells in atherosclerosis closing the gap from bench to beside. Arterioscler Thromb Vasc Biol 2015;35:296-302. doi: 10.1161/ATVBAHA.114.303569.