maturation and then relocate to the T cell areas of PPs. Further, $CCR6^+$ DCs in the small intestinal lamina propria migrate into mesenteric lymph nodes (MLNs) after capturing luminal Ags [10]. A second major chemokine receptor CCR1 is expressed by $CD11b^+$ DCs in the dome region of PPs [11]. The epithelial cells covering PPs produce the CCR1 ligand CCL9 which regulates $CD11b^+$ $CD11c^+$ DC recruitment [11]. Antigen uptake in the lungs also leads to DC recruitment. In this regard, knockout of CCR2 resulted in impaired pulmonary DC activation with diminished inflammation [12]. Recent studies have shown that CCR7 plays a key role in migration of local DCs into CLNs following sublingual immunization [13].

Taken together, these studies indicate that it is important to characterize the chemokine receptor expression by Ad-FL-induced $CD11b^+$ DCs in NALT which ultimately leads to the induction of Ag-specific immune responses.

Materials and Methods

Mice

Young adult 6- to 8- week (wk) old C57BL/6 mice were purchased from the Frederick Cancer Research Facility (National Cancer Institute, NIH, Frederick, MD). $CCR5^{-/-}$, $CCR6^{-/-}$ and CD11c-DTR mice on a C57BL/6 background were obtained from The Jackson Laboratory (Bar Harbor, ME). Upon arrival, all mice were transferred to microisolators, maintained in horizontal laminar flow cabinets, and provided sterile food and water in a specific-pathogen-free animal facility at the University of Alabama at Birmingham (UAB) Immunobiology Vaccine Center (IVC). All mice used in these experiments were free of bacterial and viral pathogens. All experiments involving mice were performed in accordance with both NIH and the UAB Institutional Animal Care and Use Committee (IACUC) guidelines. The UAB IACUC gave specific approval for all procedures involving mice; animal protocol number 110908212.

Preparation of Chimera Mice by Bone Marrow Transplantation

C57BL/6 mice were irradiated (1000 rads), and injected intravenously with a mixture of bone marrow cells from CD11c-DTR and C57BL/6 (CD11c-DTR/C57BL/6), CD11c-DTR and $CCR5^{-/-}$ (CD11c-DTR/$CCR5^{-/-}$), or CD11c-DTR and $CCR6^{-/-}$ (CD11c-DTR/$CCR6^{-/-}$) mice 6 h after irradiation (Figure S1). Six weeks after bone marrow transplantation, recipient mice were nasally immunized three times at weekly intervals with OVA plus Ad-FL as adjuvant. Diphtheria toxin (DT) (100 ng/mouse; Sigma-Aldrich, St. Louis, MO) was injected into CD11c-DTR/C57BL/6 chimera, CD11c-DTR/$CCR5^{-/-}$ chimera and CD11c-DTR/$CCR6^{-/-}$ chimera mice as well as CD11c-DTR mice via the intraperitoneal route 6 h before each nasal immunization.

Preparation of the Adenovirus Vector

Replication-incompetent adenovirus vectors expressing FL (Ad-FL) and firefly luciferase (Ad-Luc), respectively, were constructed through homologous recombination in *Escherichia coli* using the AdEasy system [4]. The vectors used in our experiments contained transgene cassettes driven by the human CMV promoter placed in the E1-deleted region of an adenoviral vector backbone. Thus, the recombinant Ad-FL was constructed by inserting the murine FL cDNA into an early region (E1). Expression of cDNA was driven by the human CMV immediate gene promoter and terminated by the polyadenylation sequence, poly (A), of SV40. The viruses were propagated in the Ad-packaging cell line, human embryonic kidney (HEK) 293 cells (Microbix Biosystems), and purified by twice repeating a CsCl density gradient centrifugation step followed by dialysis against PBS with 10% glycerol. The Ad vectors were titrated by plaque assay and stored at −80°Celsius (C) until used.

Nasal Immunization and Sample Collection

Mice were nasally immunized three times at weekly intervals with 3 μl per nostril of PBS containing 1×10^8 PFU of Ad-FL and 100 μg of OVA (Sigma-Aldrich). Plasma, saliva and nasal washes (NWs) were collected on day 21. Saliva was obtained from mice following intraperitoneal injection of 100 μg of pilocarpine hydrochloride (Sigma-Aldrich). NWs were collected by gently flushing the NPs with 1 ml of Ringer's lactate (Abbot Laboratories, North Chicago, IL).

OVA-specific ELISA

OVA-specific Abs in plasma, saliva and NWs were determined by ELISA as previously described [3,4,14]. Briefly, 96-well Falcon microtest assay plates (BD Biosciences, San Jose, CA) were coated with 1 mg/ml of OVA in PBS. After blocking with 1% bovine serum albumin (BSA) (Sigma-Aldrich) in PBS, 2-fold serial dilutions of samples were added and incubated overnight at 4°C. Horse radish peroxidase (HRP)-labeled goat anti-mouse μ, γ or α heavy chain-specific Abs (Southern Biotechnology Associates, Birmingham, AL) were added to individual wells. The color reaction was developed for 15 min at room temperature with 100 μl of 1.1 mM 2, 2′-azino bis (3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS; EMD Biosciences, La Jolla, CA). Endpoint titers were expressed as the reciprocal $\log_2$ of the last dilution that gave an optical density at 415 nm of 0.1 greater than background.

OVA-specific ELISPOT

Mononuclear cells from the spleen, NPs and SMGs were isolated as described previously [4,14,15,16,17]. Cells were subjected to an ELISPOT assay to determine the numbers and isotype of OVA-specific Ab-forming cells (AFCs). Briefly, 96-well nitrocellulose plates (Millititer HA; Millipore, Billerica, MA) were coated with 1 mg/ml of OVA for analysis of anti-OVA-specific AFCs. The numbers and isotype of OVA-specific AFCs were quantified using a CTL-ImmunoSpot® analyzer (Cellular Technology Limited, Shaker Heights, OH).

Flow Cytometry Analysis

To characterize the phenotype of DCs, mononuclear cells ($0.2–1\times10^6$) from spleens, CLNs, NALT, NPs and SMGs were isolated one week after the last immunization. The cells were stained with FITC-conjugated anti-CD11b, PE-labeled anti-CCR5, allophycocyanin-tagged anti-CCR6 or -CCR7 and biotinylated anti-CD11c mAbs followed by PerCP-Cy5.5-streptavidin (BD Biosciences). In order to confirm CD11c staining for DCs, cells were incubated with an Ab cocktail consisting of FITC-conjugated anti-CD3 (145-2C11), -CD49b (DX5), -NK1.1 (PK136) and -Ig (goat anti-mouse), and biotinylated anti-CD11c mAb. In some experiments, mononuclear cells were incubated with FITC-conjugated anti-CD11b, PE-labeled anti-CD11c, allophycocyanin-tagged anti-B220 and biotinylated anti-CD8 (BD Biosciences) mAbs followed by PerCP-Cy5.5-streptavidin. These samples were subjected to flow cytometry (FACS Calibur; BD Biosciences) for cell subset analysis.

CCL3, CCL4, CCL5 and CCL20 Production

Mononuclear cells from NALT, NPs and SMGs were isolated three days after the second immunization (day 10) or one week

after the last immunization (day 21), and cultured for 5 days. The supernatants from individual wells were then subjected to the respective chemokine-specific ELISA kits (R&D Systems), according to the manufacturer's instructions.

Statistical Analysis

The results are presented as the mean ± one standard error of the mean (SEM). Groups of C57BL/6 mice nasally immunized with OVA plus Ad-FL were compared with mice nasally immunized with OVA and Ad-Luc using an unpaired Mann-Whitney *U* test with Statview software (Abacus Concepts, Cary, NC) designed for Macintosh computers. In some experiments, $CCR5^{-/-}$ or $CCR6^{-/-}$ mouse groups nasally immunized with OVA plus Ad-FL were compared with identically immunized, normal C57BL/6 mice. DT treated, CD11c-DTR/$CCR5^{-/-}$ or CD11c-DTR/$CCR6^{-/-}$ mouse groups given nasal OVA plus Ad-FL were compared with identically immunized, DT treated, CD11c-DTR/C57BL/6 chimera mice. Values of $p<0.05$ or $p<0.01$ were considered significant.

Results

Nasal OVA and Ad-FL Induces Increased Numbers of $CCR5^+$ and CCR6,$^+$ $CD11b^+$ DC Subsets in Nasal-oral Lymphoid Tissues

Our previous studies showed that $CD11b^+$ DCs in mucosal effector tissues originate from NALT after nasal delivery of Ag and Ad-FL. This $CD11b^+$ DC subset was shown to express high levels of CD11c molecules but were negative for F4/80, CD3, CD49b, NK1.1 and Ig. Our initial experiments in the present study assessed the frequencies of $CD11b^+$ DCs expressing the chemokine receptors CCR5, CCR6 and CCR7 in mucosal and peripheral lymphoid tissues of mice given nasal OVA plus Ad-FL as mucosal adjuvant. Early expansion of $CCR5^+$ and $CCR6^+$ $CD11b^+$ DC populations were seen in NALT and CLNs of C57BL/6 mice (Figure 1A and 1B). In this regard, the highest numbers of $CCR5^+$ $CCR6^+$ $CD11b^+$ DCs were noted in NALT five days after the first nasal immunization with OVA plus Ad-FL (Figure 1A), and the numbers of $CCR5^+$, $CCR6^+$ $CD11b^+$ DCs in CLNs peaked three days after the second immunization (day 10) (Figure 1B). $CCR5^+$, $CCR6^-$ $CD11b^+$ DCs showed a similar pattern of kinetics in NALT and CLNs (Figure 1A and 1B). Of interest, $CCR5^+$, $CCR6^+$ $CD11b^+$ DC subsets in NPs and SMGs developed later and increased in a time-dependent manner (Figure 1C and 1D). In contrast, $CCR5^+$, $CCR6^-$ $CD11b^+$ DCs showed an earlier expansion in NPs and SMGs (Figure 1C and 1D). Of importance, although CCR7-expressing $CD11b^+$ DC subsets were seen in NALT, CLNs, NPs or SMGs, the frequency of this DC subset did not increase significantly (Figure 1A–D). These results show that nasal OVA and Ad-FL induces increased numbers of $CCR5^+$ and $CCR6^+$ $CD11b^+$ DC subsets in NALT, and suggest the possible migration of these DC subsets into the effector lymphoid tissues through CLNs and the systemic circulation.

CCL3, CCL4, CCL5 and CCL20 Expression in Mice Nasally-immunized with OVA Plus Ad-FL

We next assessed expression levels of CCL3, CCL4 and CCL5 (all ligands for CCR5) and CCL20 (ligand for CCR6) in both nasal-oral inductive and effector tissues of mice nasally immunized with OVA plus Ad-FL. Increased levels of all three CCR5 ligands were seen in the NALT of mice nasally immunized with OVA plus Ad-FL at day 10; however, these responses had returned to basal levels by day 21 (Figure 2). A similar pattern of CCL3 and CCL5 production was noted in the NPs (Figure 2). Of importance, NPs contained increased levels of both CCR5- and CCR6-ligands (CCL4 and CCL20) at day 21 (Figure 2). The SMGs revealed elevated levels of CCL3 and CCL5 at day 10 when compared with chemokine levels at day 0 (Figure 2). Further, CCL3, CCL4 and CCL20 production was significantly upregulated in the SMGs at day 21 after nasal immunization (Figure 2). These results suggest that CCL3, CCL4 and CCL5 as well as CCL20 play important roles in the recruitment of CCR5- and CCR6-expressing DCs for the induction of mucosal immune responses.

Decreased Numbers of DCs Occur in Nasally Immunized CCR5 Gene-deficient ($CCR5^{-/-}$) and CCR6 Gene-deficient ($CCR6^{-/-}$) Mice

We next investigated the frequency of DCs in various mucosal inductive and effector tissues of $CCR5^{-/-}$ or $CCR6^{-/-}$ mice given nasal OVA plus Ad-FL. The numbers of $CD11c^+$ DCs were decreased in NALT, CLNs, NPs, SMGs and spleens of both $CCR5^{-/-}$ and $CCR6^{-/-}$ mice given nasal OVA plus Ad-FL seven days after the last immunization when compared with identically immunized C57BL/6 mice (Table 1). Importantly, the numbers of $CD11b^+CD11c^+$ DCs were significantly decreased in NALT, CLNs, NPs and SMGs of both $CCR5^{-/-}$ and $CCR6^{-/-}$ mice given nasal OVA plus Ad-FL on day 21 when compared with identically immunized C57BL/6 mice (Table 1 and Figure 3A). Interestingly, at an earlier time point (day 10), reduced numbers of $CD11b^+CD11c^+$ DCs were seen in the NALT and CLNs of $CCR5^{-/-}$ and $CCR6^{-/-}$ mice. Of interest, $CCR5^{-/-}$ but not $CCR6^{-/-}$ mice showed significantly reduced numbers of $CD11b^+CD11c^+$ DCs in the NPs and SMGs at day 10 (Figure 3B). Although reduced frequencies of $CD8^+$ or $B220^+$ $CD11c^+$ DCs were also seen in $CCR5^{-/-}$ and $CCR6^{-/-}$ mice, the levels of reduction were not as significant as that seen in the CD11b population (Table 1). These results suggest that both CCR5 and CCR6 play key roles in the increase of $CD11b^+$ DCs in mucosal lymphoid tissues.

$CCR5^{-/-}$ and $CCR6^{-/-}$ Mice Immunized with OVA plus Ad-FL Fail to Undergo OVA-specific IgA Ab Responses

We next assessed the effects of loss of CCR5 and CCR6 expression on OVA-specific Ab responses after nasal immunization with OVA plus Ad-FL. Immune responses were analyzed in plasma, NWs and saliva one week after the last immunization. Loss of neither CCR5 nor CCR6 had an effect on plasma IgG or IgM, or IgG anti-OVA Abs in NWs; however, IgA anti-OVA Ab responses were abrogated in both plasma and external secretions (Figure 4A). Nasal immunization with OVA plus Ad-Luc resulted in essentially no anti-OVA IgA Ab responses in either $CCR5^{-/-}$ or $CCR6^{-/-}$ mice or in normal C57BL/6 mice (data not shown). These findings were also supported at the cellular level, where OVA-specific IgM and IgG AFCs were essentially identical in C57BL/6, $CCR5^{-/-}$ and $CCR6^{-/-}$ mice (Figure 4B). In marked contrast, the numbers of OVA-specific IgA AFCs were essentially absent in spleen, SMGs and NPs of both $CCR5^{-/-}$ and $CCR6^{-/-}$ mice given nasal OVA plus Ad-FL when compared with identically immunized, normal C57BL/6 mice (Figure 4B). Despite the lack of OVA-specific IgA Ab responses in $CCR5^{-/-}$ and $CCR6^{-/-}$ mice, these mice maintained normal levels of total IgA levels in both plasma, nasal washes and saliva (data not shown). These results clearly show that both CCR5 and CCR6 are essential molecules involved in the selective induction of Ag-specific mucosal SIgA and plasma IgA Ab responses. However, it

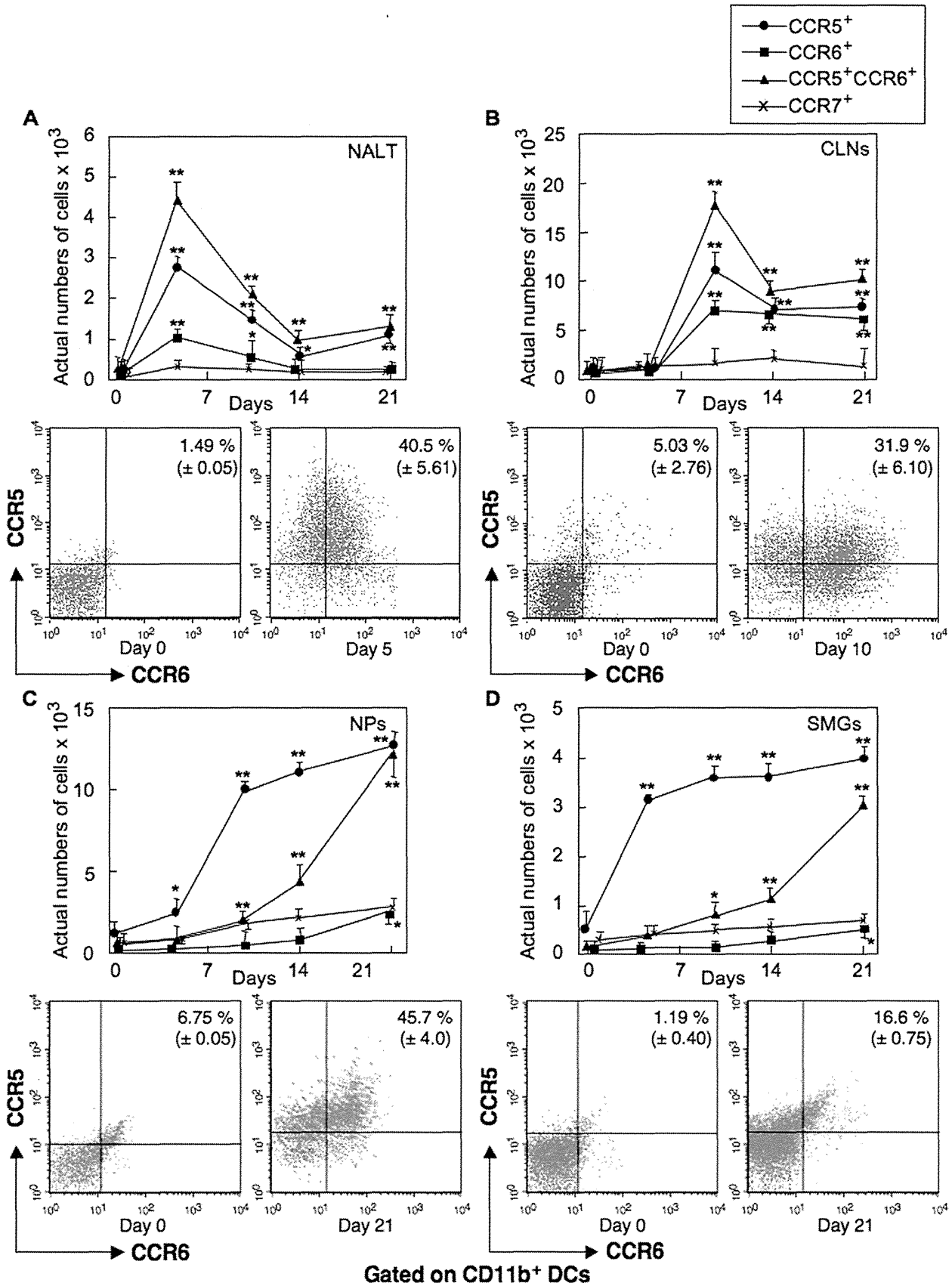
CCR5+
CCR6+
CCR5+CCR6+
CCR7+
A
NALT
Actual numbers of cells x 10^3
Days
B
CLNs
1.49 % (± 0.05)
40.5 % (± 5.61)
5.03 % (± 2.76)
31.9 % (± 6.10)
CCR5
CCR6
Day 0
Day 5
Day 10
C
NPs
D
SMGs
6.75 % (± 0.05)
45.7 % (± 4.0)
1.19 % (± 0.40)
16.6 % (± 0.75)
Day 21
Gated on CD11b+ DCs

Figure 1. Kinetics of chemokine receptor expression by CD11b$^+$ DCs. Mice were nasally immunized weekly for three consecutive weeks with OVA plus Ad-FL (A-D). Five, 10, 14 and 21 days after the initial immunization, mononuclear cells (MNCs) from NALT (A), CLNs (B), NPs (C), and SMGs (D) were stained with FITC-conjugated anti-CD11b, PE-labeled anti-CCR5, allophycocyanin-tagged anti-CCR6 or -CCR7 and biotinylated anti-CD11c mAbs followed by PerCP-Cy5.5-streptavidin. CD11c$^+$ and CD11b$^+$ cells were gated and their CCR expression was analyzed by FACSCalibur®. The values shown are the mean of the actual numbers of cells ± SEM taken from five separate experiments (two samples/experiment) with a total of 10 samples in each experimental group. * $p<0.05$, ** $p<0.01$ when compared with day 0.
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does not distinguish the specific cell types which contribute to IgA-mediated mucosal and systemic immune responses.

Potential Roles of CCR5/CCR6 Double Positive DCs for the Induction of OVA-specific IgA Ab Responses

The increased expression of CCR5 and CCR6 by CD11c$^+$ DCs in nasal-oral mucosal lymphoid tissues certainly implicate this cell type in supporting IgA isotype-specific Ab responses; however direct proof is lacking. In order to more directly probe the role of CCR5$^+$ and/or CCR6$^+$ DCs in mucosal immunity, CD11c-DTR and normal bone marrow chimeras (CD11c-DTR/C57BL/6), CD11c-DTR and CCR5$^{-/-}$ chimeras (CD11c-DTR/CCR5$^{-/-}$) or CD11c-DTR and CCR6$^{-/-}$ chimeras (CD11c-DTR/CCR6$^{-/-}$) as well as CD11c-DTR mice were employed (Figure S1). When CD11c-DTR mice, CD11c-DTR/CCR5$^{-/-}$ and CD11c-DTR/CCR6$^{-/-}$chimera mice were treated with DT 6 h prior to nasal immunization with OVA plus Ad-FL, whole DCs, CCR5- or CCR6-expressing DCs were transiently depleted in mice; however, these mice contained intact CCR5- or CCR6-expressing T cells. Chimera mice containing normal C57BL/6 bone marrow cells (CD11c-DTR/C57BL/6) with DT treatment were employed as a positive control group. CD11c-DTR, CD11c-DTR/CCR5$^{-/-}$ and CD11c-DTR/CCR6$^{-/-}$ mice all lacked OVA-specific SIgA Ab responses in saliva, NWs or plasma when compared with those responses seen in controls (Figure 5A). It is important to note that all chimera mouse groups without DT treatment showed essentially the same levels of anti-OVA IgA and IgG Ab responses as normal C57BL/6 mice when they were immunized with OVA plus Ad-FL (Figure 5B). Further, significantly reduced numbers of IgA AFCs were seen in SMGs and NPs of chimera mice or CD11c-DTR mice treated with DT

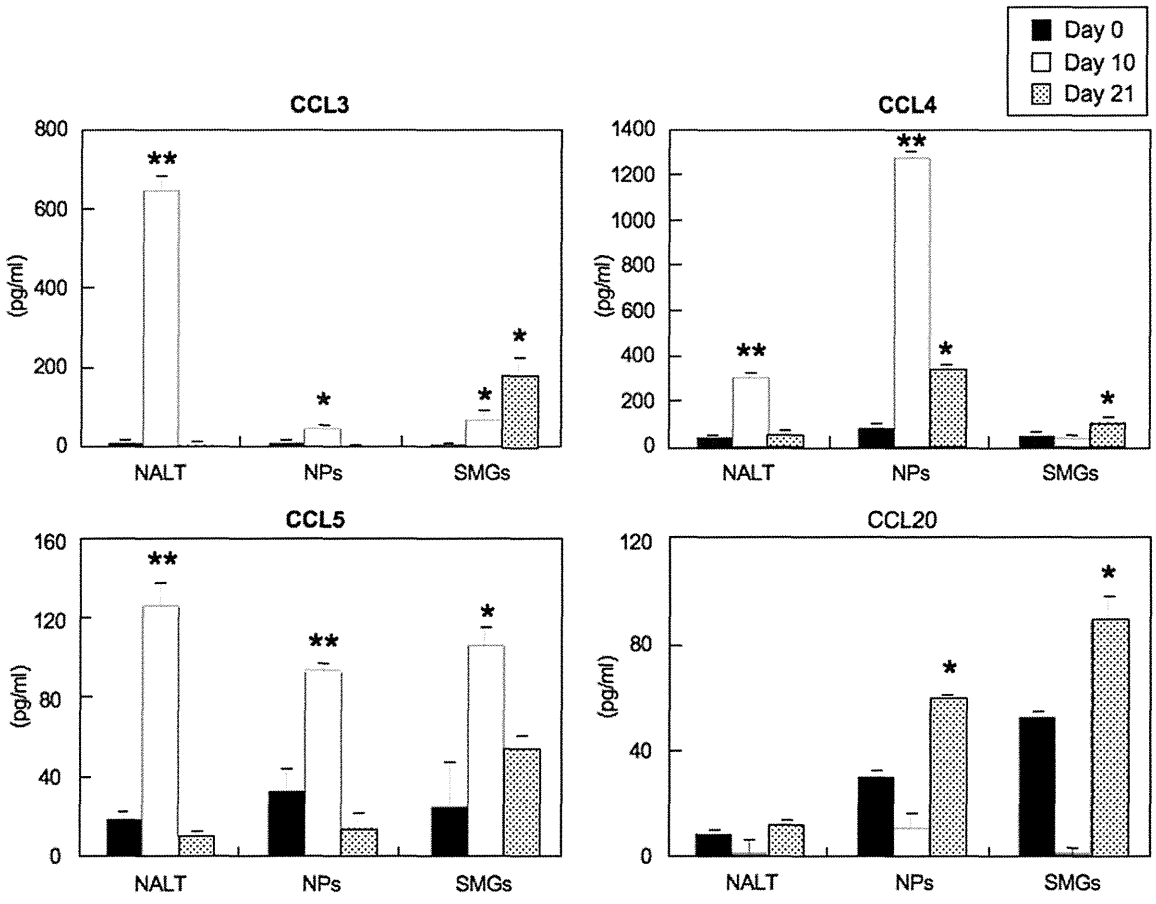

Figure 2. CCL3, CCL4, CCL5 and CCL20 synthesis in various mucosal tissues. Mice were nasally immunized weekly for either two or three consecutive weeks with OVA plus Ad-FL. Three days after the second immunization (day 10) or one week after the last immunization (day 21), MNCs were taken from NALT, NPs and SMGs, and cultured *in vitro* for 5 days. Culture supernatants were harvested after 5 days of incubation and analyzed for the respective chemokine by ELISA. The values shown are the mean ± SEM taken from five separate experiments with a total of 25 mice in each experimental group. * $p<0.05$, ** $p<0.01$ when compared with naïve mice (day 0).
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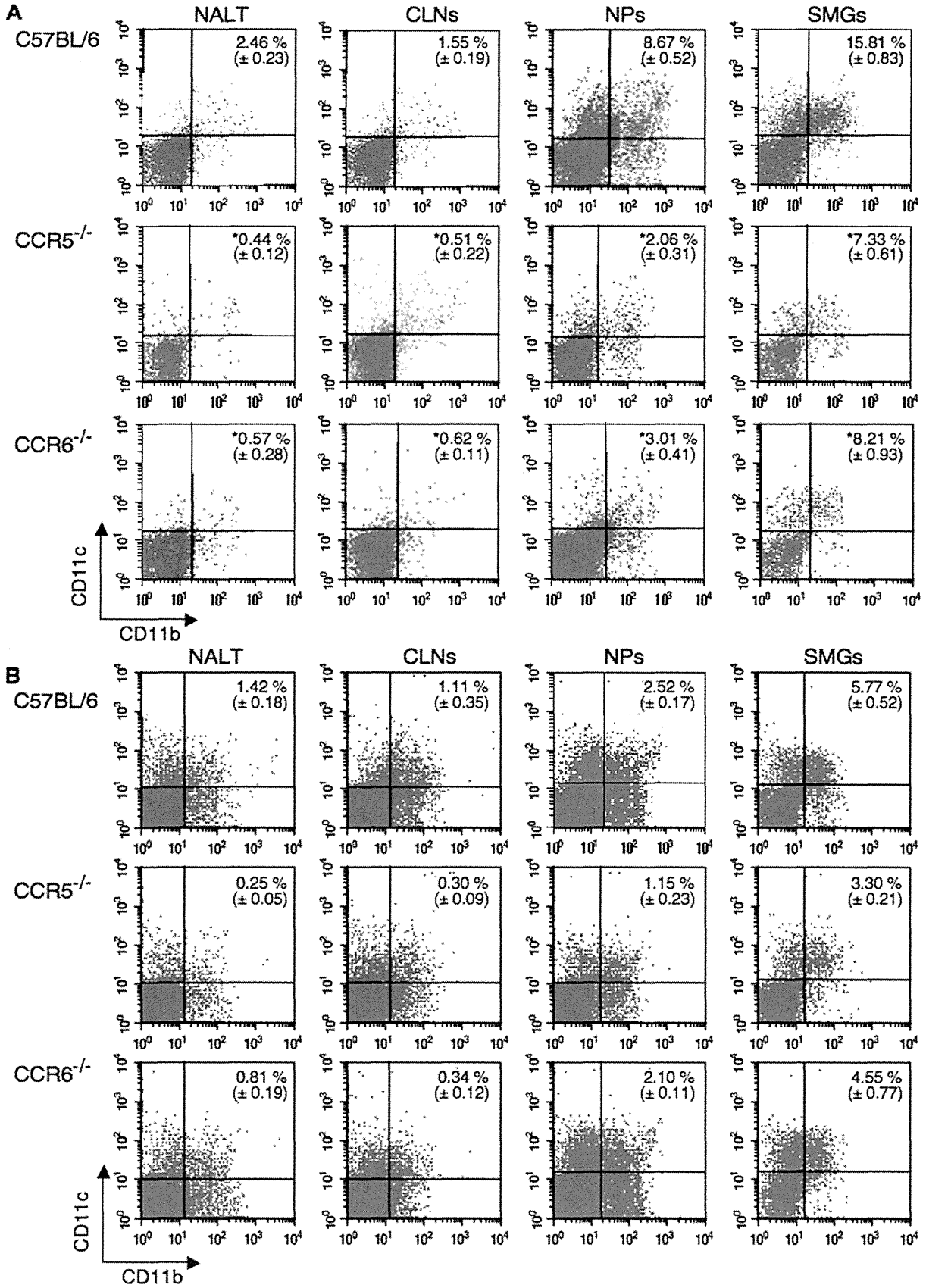
A
NALT
CLNs
NPs
SMGs
C57BL/6
2.46 % (± 0.23)
1.55 % (± 0.19)
8.67 % (± 0.52)
15.81 % (± 0.83)
CCR5-/-
*0.44 % (± 0.12)
*0.51 % (± 0.22)
*2.06 % (± 0.31)
*7.33 % (± 0.61)
CCR6-/-
*0.57 % (± 0.28)
*0.62 % (± 0.11)
*3.01 % (± 0.41)
*8.21 % (± 0.93)
CD11c
CD11b
B
NALT
CLNs
NPs
SMGs
C57BL/6
1.42 % (± 0.18)
1.11 % (± 0.35)
2.52 % (± 0.17)
5.77 % (± 0.52)
CCR5-/-
0.25 % (± 0.05)
0.30 % (± 0.09)
1.15 % (± 0.23)
3.30 % (± 0.21)
CCR6-/-
0.81 % (± 0.19)
0.34 % (± 0.12)
2.10 % (± 0.11)
4.55 % (± 0.77)
CD11c
CD11b

Figure 3. Comparison of the frequency of CD11b$^+$ DCs in mucosal and peripheral lymphoid tissues of normal, CCR5$^{-/-}$ and CCR6$^{-/-}$ C57BL/6 mice. Mice were nasally immunized weekly for three consecutive weeks with OVA plus Ad-FL. (A) One week after the last immunization or (B) three days after the second immunization (day 10), MNCs were taken from NALT, CLNs, NPs and SMGs, and stained with FITC-conjugated anti-CD11b and PE-labeled anti-CD11c. All samples were subjected to flow cytometry analysis with a FACSCalibur®. After lymphocyte gating, (A) 10,000 cells were assessed whereas (B) 50,000 cells were taken for the analyses since the frequencies of CD11b$^+$ DCs at day 10 samples were limited. The results represent the mean values ± SEM from three separate experiments with a total of 15 mice in each experimental group and are taken from three separate experiments. The profiles represent typical results and are taken from one of three separate experiments. *$p<0.05$ when compared with normal C57BL/6 mice.
doi:10.1371/journal.pone.0060453.g003

(Figure 5C). In contrast, CD11c-DTR/C57BL/6 mice showed significantly increased levels of OVA-specific IgA Ab responses (Figure 5A and 5C). Similarly, C57BL/6 mice treated weekly with DT injections revealed high levels of anti-OVA IgA Ab responses when mice were nasally immunized with OVA plus Ad-FL (data not shown). The levels of anti-OVA IgM and IgG Ab responses in plasma and AFC responses in spleens from these mice were essentially the same as those seen in control mice (Figure 5A and 5B). These results suggest that CCR5- and CCR6- expressing DCs, notably CCR5/CCR6 double positive DCs, play a key role in the induction of OVA-specific IgA Ab responses when mice were nasally immunized with OVA plus Ad-FL as mucosal adjuvant.

Discussion

The present study has focused on the specific chemokine receptors which are associated with DCs following nasal delivery of Ag plus the DC-inducing adjuvant Ad-FL. Our findings are the first to show that both CCR5 and CCR6 expression by mucosal DCs (CCR5$^+$ CCR6$^+$ double positive DCs) are essential molecules for potential DC migration and subsequent induction of mucosal and peripheral IgA Ab responses.

It is well accepted that DCs express multiple chemokine receptors and respond to a variety of chemokine ligands [18,19]. Thus, chemokine receptors expressed by mucosal DCs play central roles in their relocation within mucosal inductive tissues and their

Table 1. Comparison of the subpopulation of CD11c$^+$ DCs in mucosal and peripheral lymphoid tissues of mice given nasal OVA plus Ad-FL[a].

Tissue	Mice	Actual Numbers of Cells in Total Lymphocytes (x 10^4)	Actual Numbers of DC subsets inTotal Lymphocytes (x 10^4)		
		CD11c [b, d]	CD8$^+$ CD11c$^+$ [c, d]	CD11b$^+$ CD11c$^+$ [c, d]	B220$^+$ CD11c$^+$ [c, d]
NALT	Naïve	2.55 (±1.73)	0.26 (±0.02)	0.62 (±0.03)	1.52 (±0.03)
	C57BL/6	8.10 (±1.49)	0.77 (±0.01)	3.25 (±0.02)	4.40 (±0.03)
	CCR5$^{-/-}$	2.89 (±1.45) **	0.27 (±0.04) **	0.91 (±0.05) **	1.71 (±0.02) **
	CCR6$^{-/-}$	2.75 (±1.07) **	0.27 (±0.02) **	0.93 (±0.03) **	1.54 (±0.04) **
CLNs	Naïve	41 (±8.1)	8.7 (±0.19)	10.6 (±0.21)	16.6 (±0.18)
	C57BL/6	170 (±35.5)	34.9 (±0.89)	66.7 (±1.42)	67.2 (±0.67)
	CCR5$^{-/-}$	86 (±31.9) **	18.0 (±1.89) **	18.5 (±1.31) **	32.1 (±1.79) **
	CCR6$^{-/-}$	86 (±17.2) **	16.0 (±0.79) **	23.9 (±0.76) **	35.4 (±0.83) **
NPs	Naïve	6.9 (±1.1)	1.65 (±0.03)	3.18 (±0.03)	0.94 (±0.03)
	C57BL/6	25.7 (±0.8)	6.03 (±0.02)	16.2 (±0.03)	3.21 (±0.01)
	CCR5$^{-/-}$	10.8 (±0.43) **	2.70 (±0.01) **	4.34 (±0.01) **	1.43 (±0.02) *
	CCR6$^{-/-}$	11.1 (±0.92) **	2.77 (±0.02) **	5.29 (±0.05) **	1.48 (±0.03) *
SMGs	Naïve	17.8 (±2.48)	2.19 (±0.03)	6.31 (±0.04)	5.14 (±0.02)
	C57BL/6	65.8 (±10.9)	8.81 (±0.21)	42.9 (±0.68)	14.1 (±0.38)
	CCR5$^{-/-}$	29.6 (±2.58) **	4.44 (±0.03) **	12.4 (±0.01) **	7.22 (±0.05) **
	CCR6$^{-/-}$	25.5 (±2.19) **	3.69 (±0.03) **	12.9 (±0.16) **	5.52 (±0.16) **
Spleen	Naïve	144 (±30)	16.6 (±0.15)	48.2 (±0.51)	61.2 (±0.63)
	C57BL/6	384 (±52.3)	51.9 (±2.25)	166 (±1.99)	158 (±1.41)
	CCR5$^{-/-}$	189 (±33.4) **	20.2 (±0.30) **	72.3 (±0.77) **	76.5 (±1.04) **
	CCR6$^{-/-}$	158 (±46.6) **	18.1 (±1.35) **	57.2 (±1.26) **	65.8 (±1.77) **

[a]Mice were nasally immunized weekly for three consecutive weeks with OVA plus Ad-FL.
One week after the last immunization, mononuclear cells from NALT, CLNs, NPs, SMGs and spleen were stained with a combination of the respective mAbs and subjected to flow cytometry analysis by FACSCalibur®.
[b]Mononuclear cells were stained with PE- labeled anti-CD11c mAb.
[c]Mononuclear cells were stained with FITC-conjugated anti-CD11b, PE-labeled anti-CD11c, allophycocyanin-tagged anti-B220 and biotinylated anti-CD8 mAbs followed by PerCP-Cy5.5-streptavidin.
[d]*The values shown are the mean ± SEM of five independent experiments. Each experimental group consisted of five mice.*
*$p<0.05$,
**$p<0.01$ when compared with C57BL/6 mice nasally immunized with OVA plus Ad-FL.
doi:10.1371/journal.pone.0060453.t001

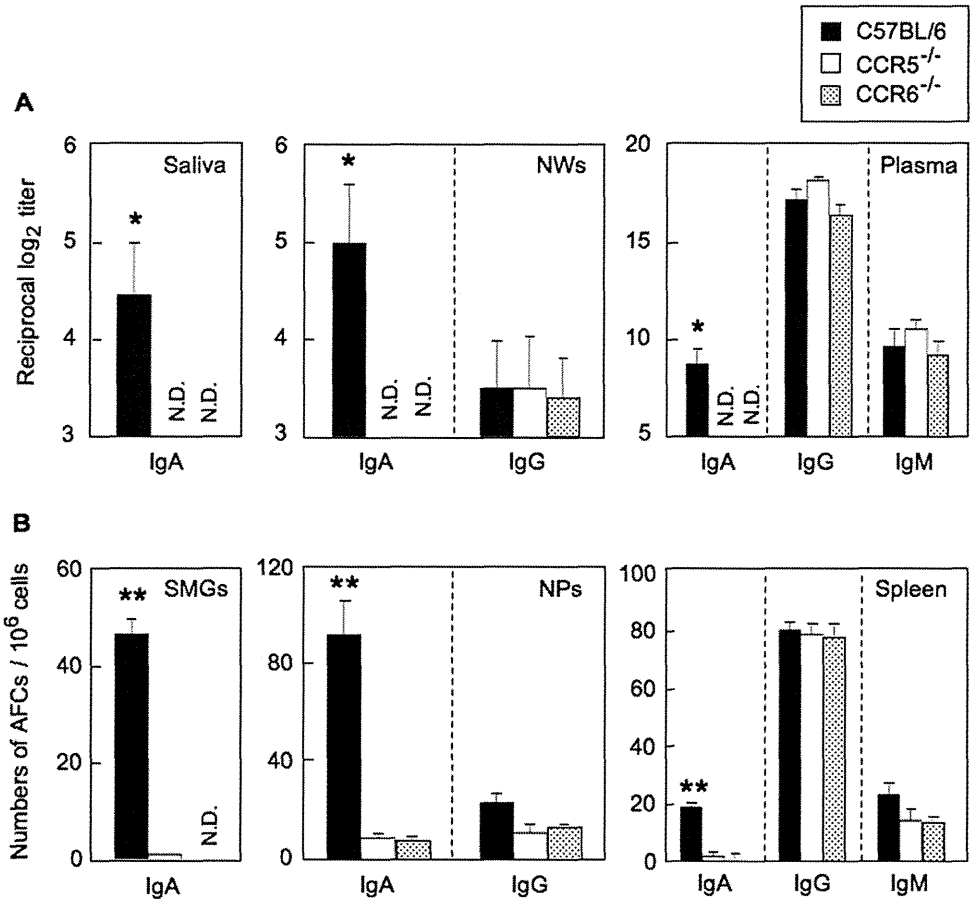

Figure 4. OVA-specific Ab responses in external secretions, plasma and AFC responses in mucosa-associated lymphoid tissues of nasally immunized normal, CCR5$^{-/-}$ or CCR6$^{-/-}$ C57BL/6 mice. Each mouse group was nasally immunized three times at weekly intervals with OVA plus Ad-FL. (A) Seven days after the last immunization, levels of SIgA anti-OVA Abs in saliva, SIgA and IgG Abs in NWs, and IgA, IgG and IgM Abs in plasma were determined by OVA-specific ELISA. (B) MNCs from NPs, SMGs and spleens were isolated 7 days after the last immunization and subjected to an OVA-specific ELISPOT assay to determine the numbers of IgA, IgG and IgM AFCs. The values shown are the mean ± SEM taken from five separate experiments with a total of 25 mice in each experimental group. The ELISA and ELISPOT data for spleen represented the Ab responses from 25 individual mice. MNCs from SMGs and NPs were pooled from 2 or 3 mice and subjected to OVA-specific ELISPOT assay. N.D. means that O.D. values were not detected. *$p<0.05$, **$p<0.01$ when compared with normal C57BL/6 mice nasally immunized with OVA plus Ad-FL.
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migration into mucosal effector sites [6,8,9,11,12,13]. For example, CD11b$^+$, CD11c$^+$, CD8$^-$ immature myeloid-type DCs in Peyer's patches (PPs) expressed either CCR1 or CCR6 for directing their migration toward the subepithelial dome region [6,11]. In this regard, CCL20 and CCL9, the ligands for these two receptors, were produced by the follicle-associated epithelium [6,11]. CCR7-expressing, mature lymphoid-type CD8$^+$ CD11c$^+$ DCs were preferentially found in the T cell zone of PPs, showing that CCR7 expression is important for the relocation of DCs from the subepithelial area to the parafollicular T cell area [6]. Moreover, it has been shown that CCR7 expression by Ag-processing DCs in the intestinal lamina propria and sublingual mucosa are essential for their migration into MLNs or CLNs, respectively [7,13]. In contrast, our present study clearly showed essentially no increase in CCR7$^+$ CD11b$^+$ DCs after nasal delivery of OVA plus Ad-FL. On the other hand, increased numbers of CCR5/CCR6-expressing CD11b$^+$ DCs were induced in a time-dependent manner beginning in NALT, next in CLNs followed by mucosal effector tissues. Thus, CCR5-expressing CD11b$^+$ DCs were increased in both SMGs and NPs. It is possible that increased numbers of these two subsets of DCs in NPs and SMGs may be attributable to migration of CCR5- and CCR5/CCR6-positive CD11b$^+$ DCs to their ligand-expressing tissues. To support this, increased levels of CCL3, CCL4 and CCL5 production in NPs and high levels of CCL5 synthesis in SMGs were seen at day 10, which attracted CCR5-expressing CD11b$^+$ DCs at this early time point. On the other hand, higher levels of CCL4 and CCL20 by day 21 in NPs and SMGs (but not in NALT) of mice played a key role leading to the increased numbers of CCR5/CCR6-expressing CD11b$^+$ DCs in these tissues. Since both CCR5$^{-/-}$ and CCR6$^{-/-}$ mice showed significantly reduced numbers of CD11b$^+$ DCs in NPs and SMGs, it is most likely that both CCR5 and CCR6 expression are essential for NALT DCs to migrate into CCL4- and CCL20-producing mucosal effector tissues. Although it is possible that double positive CCR5$^+$/CCR6$^+$ DCs can express either receptor even in these knockout conditions and reach mucosal effector sites, marked reductions in the numbers of CD11b$^+$ DCs suggest that a deficiency of either CCR5 or CCR6 leads to a loss of double positive CCR5$^+$/CCR6$^+$ DCs in the SMGs and NPs. Further, it has been shown that CCR6 is a key molecule for the

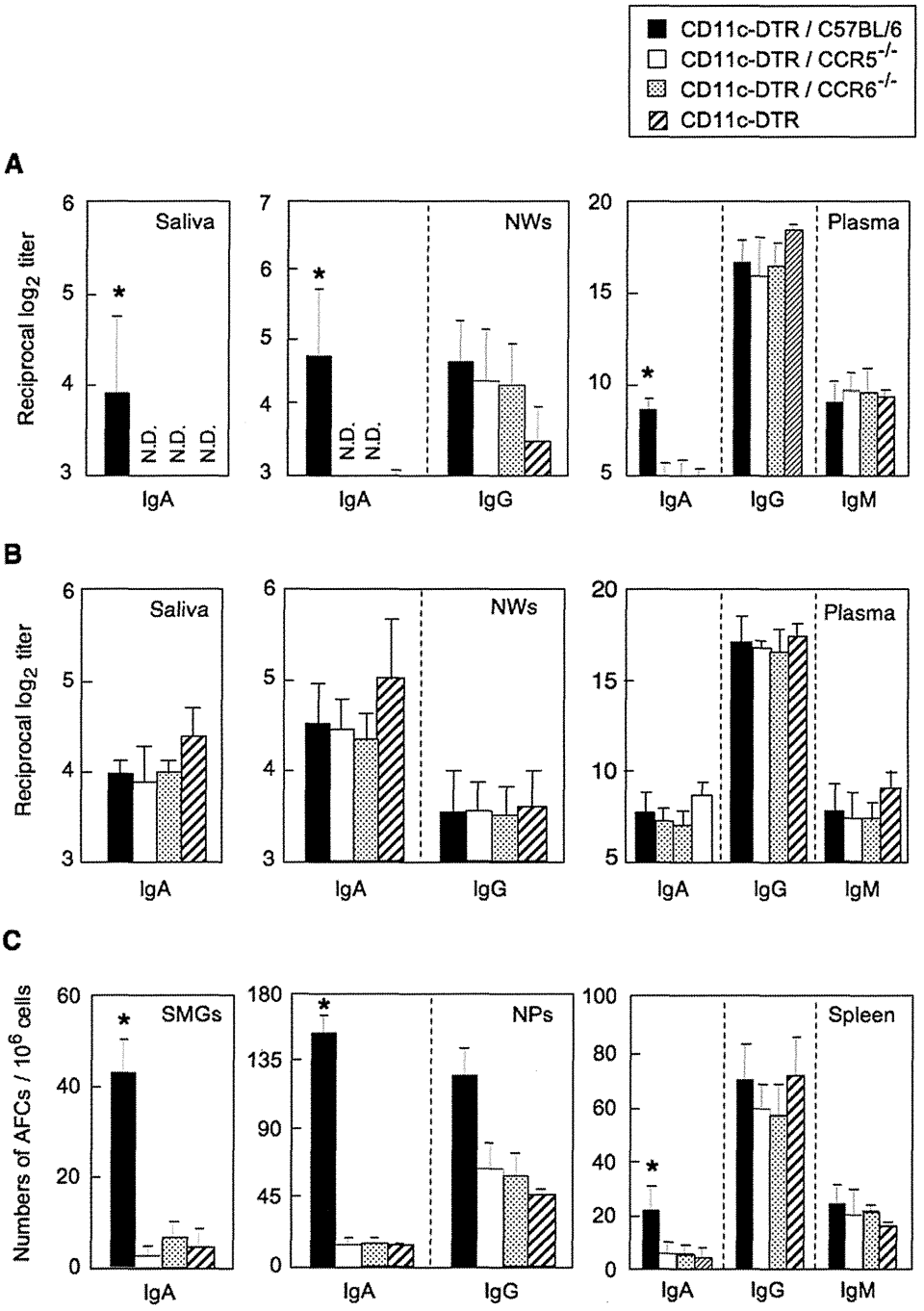

Figure 5. OVA-specific Ab responses in systemic and mucosa-associated lymphoid tissues of chimera mice which lack CCR5- or CCR6-expressing DCs (Figure S1). (A and C) CD11c-DTR, CD11c-DTR/C57BL/6, CD11c-DTR/CCR5$^{-/-}$ and CD11c-DTR/CCR6$^{-/-}$ mice were injected with DT via the intraperitoneal route 6 h before each nasal immunization (three times at weekly intervals with OVA plus Ad-FL). CD11c-DTR/C57BL/6 chimera mice given the DT injection served as positive controls. (B) CD11c-DTR/C57BL/6, CD11c-DTR/CCR5$^{-/-}$, CD11c-DTR/CCR6$^{-/-}$ and normal C57BL/6 were nasally immunized three times at weekly intervals with OVA plus Ad-FL without DT injection. (A and B) Seven days after the last immunization, levels of SIgA anti-OVA Abs in saliva, SIgA and IgG Abs in NWs, and IgA, IgG and IgM Abs in plasma were determined by OVA-specific ELISA. (C) MNCs from NPs, SMGs and spleens were isolated 7 days after the last immunization and subjected to OVA-specific ELISPOT assay to determine the numbers of IgA, IgG and IgM AFCs. The values shown are the mean ± SEM taken from five separate experiments with a total of 25 mice in each experimental group. The data for controls were obtained from two separate experiments which consisted of 6 mice for each experiment. The ELISA and ELISPOT data for spleen represented the Ab responses from 12 individual mice for controls and 25 individual mice for experimental groups. MNCs from SMGs and NPs were pooled from 2 or 3 mice and subjected to OVA-specific ELISPOT assays. N.D. means that O.D. values were not detected. (A and C) *$p<0.05$ when compared with DT treated, CD11c-DTR/C57BL/6 chimera mice nasally immunized with OVA plus Ad-FL. (B) No significant differences were detected when compared with normal C57BL/6 mice.
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induction and regulation of mucosal immune responses by recruiting DCs as well as T and B cells into mucosal effector tissues where allergic responses, active immunity or chronic inflammation occurs [20,21,22]. Thus, the CCR6 molecule is one of the essential homing markers for DC migration into mucosal effector sites. Although roles for CCR5 expression in mucosal homing remain to be elucidated, one recent study has shown that adenovirus-specific $CD4^+$ T cells expressed higher levels of CCR5 which enhanced their migration to the gut mucosa [23]. Furthermore, others showed that CCR5 plays a role in DC recruitment in Flt3-L-treated mice [24]. Based upon these studies, it is likely that Ad-FL as nasal adjuvant preferentially induced CCR5 expression which served as a mucosal effector tissue-homing molecule. To this end, we are currently testing whether single positive, $CCR5^+/CCR6^-$ and $CCR5^-/CCR6^+$ DC subsets differentiate into the double positive DC subset.

Loss of OVA-specific IgA Ab responses in $CCR5^{-/-}$ and $CCR6^{-/-}$ mice indicates that DCs expressing both CCR5 and CCR6 are key players for the induction of upper respiratory tract mucosal SIgA Ab responses when Ag is delivered by the nasal route. However, it is still possible that lack of mucosal immune responses in $CCR6^{-/-}$ mice is due to the nature of this mutant mouse strain. In this regard, CCR6 is selectively expressed by memory T cells, B cells and DCs, and appears to be involved in the initiation of memory immune responses. Further, $CCR6^{-/-}$ mice have been shown to either lack or have reduced numbers of $CD11b^+$ DCs in the subepithelial dome region of PPs [5,25]. Indeed, our findings showed that this DC population was reduced in the oral-nasopharyngeal mucosal tissues as well. In addition, other studies reported that orally immunized $CCR6^{-/-}$ mice fail to support Ag-specific SIgA Ab responses [5,26]. Furthermore, $CCR6^{-/-}$ mice showed impaired M cell development, a cell type that plays a key role in Ag uptake for the induction of mucosal immunity. In contrast to $CCR6^{-/-}$ mice, $CCR5^{-/-}$ mice have intact $CD11b^+$ DCs and normal M cell development. However, it has been reported that CC-chemokines differentially enhanced mucosal and systemic Ab responses. Thus, CCL4 as nasal adjuvant resulted in selective upregulation of Ag-specific SIgA but not IgG and IgM Ab responses [27]. Taken together, these results indicate that CCR5 expression by nasal DCs is essential both for the increased numbers of DCs and for their support of Ag-specific SIgA Ab responses in the upper respiratory tract and oral cavity.

In order to provide more direct roles for CCR5- and CCR6-expressing DCs in the regulation of mucosal IgA immune responses, we constructed chimera mice by mixed bone marrow transplantation from CD11c-DTR and $CCR5^{-/-}$ or $CCR6^{-/-}$ mice. By injecting DT prior to each nasal immunization, these chimera mice became deficient in CCR5- or CCR6-expressing DCs. Impaired OVA-specific mucosal IgA Ab responses were seen in both CD11c-DTR/CCR5 and CD11c-DTR/CCR6 mice. If single positive CCR5 or CCR6 DCs play a role, either CD11c-DTR/CCR5- or CD11c-DTR/CCR6-chimera mice should show intact Ag-specific IgA Ab responses. Further, we showed that CCR5/CCR6 double positive DCs are increased in mucosal inductive and effector tissues after nasal immunization. Since $CCR5^+/CCR6^-$ DCs are also increased in mucosal effector tissues, it is possible that both $CCR5^+/CCR6^-$ and double positive $CCR5^+/CCR6^+$ DC subsets may be required for the induction of mucosal SIgA Ab responses. Nevertheless, our results clearly show that CCR5/CCR6 double positive nasal DCs play an essential role in the induction of OVA-specific IgA Ab responses. In contrast, OVA-specific systemic IgM and IgG Ab responses in these chimera mice were essentially the same as those responses in normal mice given nasal OVA plus Ad-FL. These findings suggest that different types of APCs in the nasal mucosa are responsible for systemic IgM and IgG but not mucosal IgA Ab responses. Indeed, it has been suggested that some DCs in the intestinal lamina propria extend their dendrites between epithelial cell junctions into the lumen for taking-up Ags (intraepithelial DCs) for the initiation of Ag-specific systemic IgG Ab responses [28,29,30,31]. Similar types of intraepithelial DCs were also reported in the upper respiratory tract [32]. In addition, M cells are found in the lungs and on the bronchial bifurcation of the airways. Airway challenge with *Mycobacterium tuberculosis* resulted in the entry of this pathogen via these lung M cells [33]. Further, others have also noted the presence of M cells in the nasal cavity itself which are capable of taking up both particulate and soluble antigens delivered by the nasal route [34]. Thus, not only DCs but also other immune cells in the lamina propria of the nasal cavity play an essential role in the induction of Ag-specific systemic IgM and IgG Ab responses. Another potential explanation for impaired IgA but intact systemic IgM and IgG Ab responses in chimera mice is that DCs must be present when vaccines are administered via the mucosal route. Indeed, DT-treated CD11c-DTR mice given nasal OVA plus Ad-FL resulted in essentially no anti-OVA IgA but intact IgM and IgG Ab responses. The absence of DCs may lead to the majority of Ag and adjuvant transversing into the systemic compartment. Since DC depletion by DT is transient, recovered systemic DCs could handle circulating systemic Ags for induction of IgG Ab responses, whereas mucosal DCs would miss this opportunity to recognize Ag and adjuvant after recovery. To support this, our group has shown that a mucosal DC targeting immunization strategy is essential for the induction of Ag-specific IgA Ab responses in mucosal effector sites [14,16,17,35,36]. It is also possible that CCR5/CCR6 double positive $CD11b^+$ DCs directly regulate Ag-specific IgA Ab responses by inducing $sIgA^+$ B cells to differentiate into IgA producing plasma cells. Indeed it has been shown that APRIL-expressing mucosal DCs interact with TACI expressing B cells to induce IgA Ab responses [37,38]. Further, retinoic acid (RA)-producing mucosal DCs play key roles in mucosal T and B cell migration for the induction of Ag-specific mucosal immunity [39–41]. In addition, others showed that follicular DCs (FDCs) in PPs directly induce IgA CSR by PP B cells via RA and BAFF and APRIL pathways [42]. In this regard, we are currently investigating potential cellular and molecular mechanisms whereby CCR5/CCR6 double positive $CD11b^+$ DCs express BAFF and APRIL and produce RA for the induction of Ag-specific SIgA Ab responses.

In summary, the present study showed that Ad-FL as a nasal adjuvant induced $CD11b^+$ DCs which expressed increased levels of CCR5 and CCR6 for their subsequent migration into CCL3- and CCL20- producing mucosal effector tissues. In this regard, lack of these chemokine receptors failed to allow $CD11b^+$ DC recruitment and subsequent induction of Ag-specific SIgA Ab responses in oral-nasopharyengeal effector tissues. Defining the cellular and molecular mechanisms for mucosal DC recruitment will help provide a better understanding of how mucosal immune responses are initiated including immunity, inflammation and tolerance. Our present findings clearly support a strategy for the development of a nasal immunization protocol which recruits DCs for vaccine uptake and their subsequent increase in mucosal effector sites where protective SIgA Ab responses could be induced for host protection.

Supporting Information

Figure S1 Preparation of chimera mice by bone marrow transplantation. C57BL/6 mice were irradiated (1000 rads), and injected intravenously with a mixture of bone marrow cells from CD11c-DTR and C57BL/6 (CD11c-DTR/C57BL/6), CD11c-DTR and CCR5$^{-/-}$ (CD11c-DTR/CCR5$^{-/-}$), or CD11c-DTR and CCR6$^{-/-}$ (CD11c-DTR/CCR6$^{-/-}$) mice 6 h after irradiation. Diphtheria toxin (DT) (100 ng/mouse) was injected into CD11c-DTR/C57BL/6 chimera, CD11c-DTR/CCR5$^{-/-}$ chimera and CD11c-DTR/CCR6$^{-/-}$ chimera mice via the intraperitoneal route 6 h before each nasal immunization. (TIF)

Author Contributions

Conceived and designed the experiments: YF SS KK JK YY HK JRM Kohtaro Fujihashi. Performed the experiments: YF DT KA RSG YT Keiko Fujihashi. Analyzed the data: YF DT KA RSG YT Keiko Fujihashi Kohtaro Fujihashi. Contributed reagents/materials/analysis tools: JD MY. Wrote the paper: YF JRM Kohtaro Fujihashi.

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NUTRITION AND THE DIGESTIVE SYSTEM

Nutritional components regulate the gut immune system and its association with intestinal immune disease development

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Abstract

The gut is equipped with a unique immune system for maintaining immunological homeostasis, and its functional immune disruption can result in the development of immune diseases such as food allergy and intestinal inflammation. Accumulating evidence has demonstrated that nutritional components play an important role in the regulation of gut immune responses and also in the development of intestinal immune diseases. In this review, we focus on the immunological functions of lipids, vitamins, and nucleotides in the regulation of the intestinal immune system and as potential targets for the control of intestinal immune diseases.

Introduction

The intestinal mucosa is the largest surface area of the body and is constantly exposed to a vast array of microbes and dietary materials. To withstand this harsh environment, the gastrointestinal tract is equipped with a highly organized mucosal immune system that creates and maintains an immunologically dynamic and harmonized homeostasis between the host and the external environment.[1] The immunological components of the gut not only induce protective immunity against pathogenic microorganisms, but also immunologically ignore beneficial nonself antigens (Ags) such as nutritional materials and commensal bacteria. Thus, gut immune system orchestrates both active and quiescent immune responses and plays a central role in creating and maintaining immunologic homeostasis in the gut. Therefore, normal functioning of the gut immune system and integrity of the epithelial barrier are essential for preventing invasion by pathogenic and commensal microorganisms but at the same time preventing the development of intestinal immune diseases (e.g. inflammatory bowel diseases and food allergies).[1]

Nutritional components derived from the diet or synthesized *de novo* are essential environmental factors for the development, maintenance, and regulation of gut immune responses. Indeed, deficient or inappropriate nutritional intake increases the risk of infectious, allergic, and inflammatory diseases.[2] Accumulating evidence has revealed the immunological functions of nutritional molecules such as vitamins, lipids, and nucleotides. In this review, we first describe the unique features of the gut immune system and then examine its regulation by nutritional molecules and its association with the development of intestinal immune diseases.

Intricate immune system in the gut for maintenance of immunological homeostasis

To protect the vast surfaces of mucosal tissues, higher mammals have evolved a unique mucosal immune system. The surfaces of mucosal tissues are covered by single- or multiple-layered epithelium and are in direct contact with the outer environment.[3] In the gastrointestinal tract, the epithelial layer consists of several subsets of intestinal epithelial cells (ECs) including M cells, goblet cells, Paneth cells, enteroendocrine cells, and columnar ECs. Each of these cells has unique functions, for example, goblet cells secrete mucus, Paneth cells produce anti-microbial peptides such as α-defensins, and other ECs produce β-defensin. All of these components are of central importance in host defense as physical, chemical, and immunological barriers.[4] In addition to ECs, another major resident cell component of the mucosal epithelium is intraepithelial lymphocytes (IELs). IELs consist mainly of various T cell subsets, primarily those expressing the γδ T cell receptor, and they bi-directionally interact with ECs to maintain normal homeostasis.[5]

The gut immune system has many organized lymphoid structures, which can be separated into inductive and effector sites on

the basis of their anatomical and functional properties.[6] In the gastrointestinal tract, the inductive sites are the gut-associated lymphoid tissues (GALT) (e.g. Peyer's patches [PPs], isolated lymphoid follicles, and colonic patches).[7] The GALT contains T cell- and B cell-enriched regions, which harbor large numbers of surface immunoglobulin A $(IgA)^+$ B cells.[7] In the follicle-associated epithelium, the PP M cells take up Ags from the lumen of the intestinal mucosa and transport them to underlying dendritic cells (DCs) on a subepithelial dome region.[7] The DCs carry the Ags into the T cell region and subsequently germinal centers in the GALT or via draining lymphatics into the mesenteric lymph nodes, for the initiation of T and B cell responses.

After emigration from the inductive tissues (e.g. PPs), primed lymphocytes traffic into the lamina propria, where they further differentiate into effector cells such as IgA-producing plasma cells (PCs), regulatory T (Treg) cells, and Th17 cells. The ECs again become central players in subsequent event by transporting polymeric IgA via the polymeric immunoglobulin receptor.[8]

These immunological networks in the gut allow the orchestration of both active and quiescent immune responses for immunosurveillance and immunologic homeostasis in the gut.

Sphingosine 1-phosphate (S1P) regulates cell trafficking in gut immunity and immune diseases

It is generally accepted that nutritional materials are involved in immune regulation. Lipids, after their conversion into lipid mediators, are among the major nutritional components involved in the regulation of intestinal immune responses, and imbalances in lipid mediator signaling pathways contribute to disease induction and resolution phases in inflammation, autoimmunity, allergy, cancer, atherosclerosis, hypertension, metabolic syndrome, and degenerative diseases.[9,10]

Among many lipid mediators, S1P is essential for the trafficking and activation of immunocompetent cells.[11] S1P is a metabolite of sphingomyelin from both the host cell plasma membrane and diet.[12] Sphingomyelin is degraded into ceramide by alkaline sphingomyelinase and subsequently to sphingosine by ceramidase. Sphingosine is then phosphorylated to generate S1P by sphingosine kinases.[11] S1P is formed in most cells, but is simultaneously irreversibly degraded by S1P lyase or dephosphorylated by S1P phosphatases.[11] Therefore, S1P levels are extremely low in most tissues but high in the blood and lymph because of the lack of S1P degrading activity of erythrocytes, platelets, and lymphatic endothelial cells; the difference creates an S1P gradient between these types of tissues.[13,14] Cells expressing S1P receptors sense the S1P gradient and traffic toward high concentrations of S1P.

Among five closely related S1P receptors, the type 1 S1P receptor ($S1P_1$) is preferentially expressed by lymphocytes and thus determines lymphocyte emigration from and retention in the lymphoid tissue.[15] Naïve lymphocytes express high levels of $S1P_1$, and their activation is associated with downregulation of this receptor. However, $S1P_1$ expression recovers in fully differentiated activated lymphocytes. These dramatic changes in $S1P_1$ determine whether the lymphocytes are retained in the lymphoid tissues or emigrate from them into the blood or lymph circulation.

We and others have shown that S1P regulates the innate and acquired phases of gut immune responses and the development of intestinal immune diseases (reviewed in Reference[12]). For instance, S1P regulates the trafficking of B cells in the PPs and subsequent intestinal IgA production.[16] In the PPs, B cells differentiate into IgA^+ plasmablasts. During B cell differentiation in the PPs, the B cells change their expression of $S1P_1$; high expression is noted on immunoglobulin M^+ naïve B cells but is downregulated during class switching to IgA. The low level of $S1P_1$ allows newly formed IgA^+ B cells to be retained in the PPs so that they can differentiate into IgA^+ plasmablasts. The IgA^+ plasmablasts show recovery of $S1P_1$ expression, resulting in their emigration from the PPs.[16] In agreement with this finding, when mice were treated with the immunosuppressant FTY720 to induce downregulation of $S1P_1$ expression,[17] IgA^+ plasmablasts selectively accumulated in the PPs, and their population was decreased in the lamina propria.[16] As a result, FTY720-treated mice showed reduced intestinal IgA responses against orally administered protein Ag.[16] We have also reported that IgA PCs originated from peritoneal cavity, along with unique subsets of IELs require S1P for their trafficking into the intestine.[18–20]

Several lines of evidence have indicated that S1P plays a key role in the development of intestinal immune diseases. In an ovalbumin-induced murine food allergy model,[21] we found that activated T cells migrate into the colon, where they produced large amounts of Th2 cytokines such as interleukin (IL)-4 and IL-5. Our subsequent study has demonstrated that trafficking of pathogenic T cells from the systemic compartments into the colon is mediated by S1P; thus, infiltration of activated T cells into the colon of allergic mice is inhibited by treatment with FTY720 (Fig. 1).[22] In addition, infiltration or proliferation of mast cells, effector cells in the development of food allergy, in the colon is prevented by treatment with FTY720 (Fig. 1).[22] Similar effects of FTY720 on trafficking of pathogenic cells in the development of intestinal inflammation have been reported in some experimental intestinal inflammation models (e.g. IL-10-deficient mice, dextran sulphate sodium treatment, and T cell transfer models).[23–25] Collectively, these findings suggest that in addition to the physiological role of S1P–$S1P_1$ axis in the optimal supplementation of immunocompetent cells to the intestine, it also participates mainly in the development of intestinal immune diseases (e.g. allergy and inflammation) at the stage of pathogenic cell trafficking into the colon, which is a potential target for prevention and treatment of these intestinal immune diseases.

Immunological functions of vitamins in the versatile intestinal immunity

Vitamins are organic compounds that we cannot synthesize in sufficient quantities, and that therefore need to be supplied from the diet or commensal bacteria. Some of these vitamins are water-soluble (e.g. vitamin B family and vitamin C) whereas others are hydrophobic (e.g. vitamins A, D, E, and K). Both hydrophilic and hydrophobic vitamins and their metabolites have diverse functions in many biological events, including immunological regulation (Fig. 2). Indeed, vitamin deficiency results in high susceptibility to infection and immune diseases.[26]

Accumulating evidence has revealed the molecular and cellular mechanisms of vitamins underlying regulation of the immune system. The biggest breakthrough was the discovery of the function of vitamin A in regulating the tissue-tropism of lymphocytes

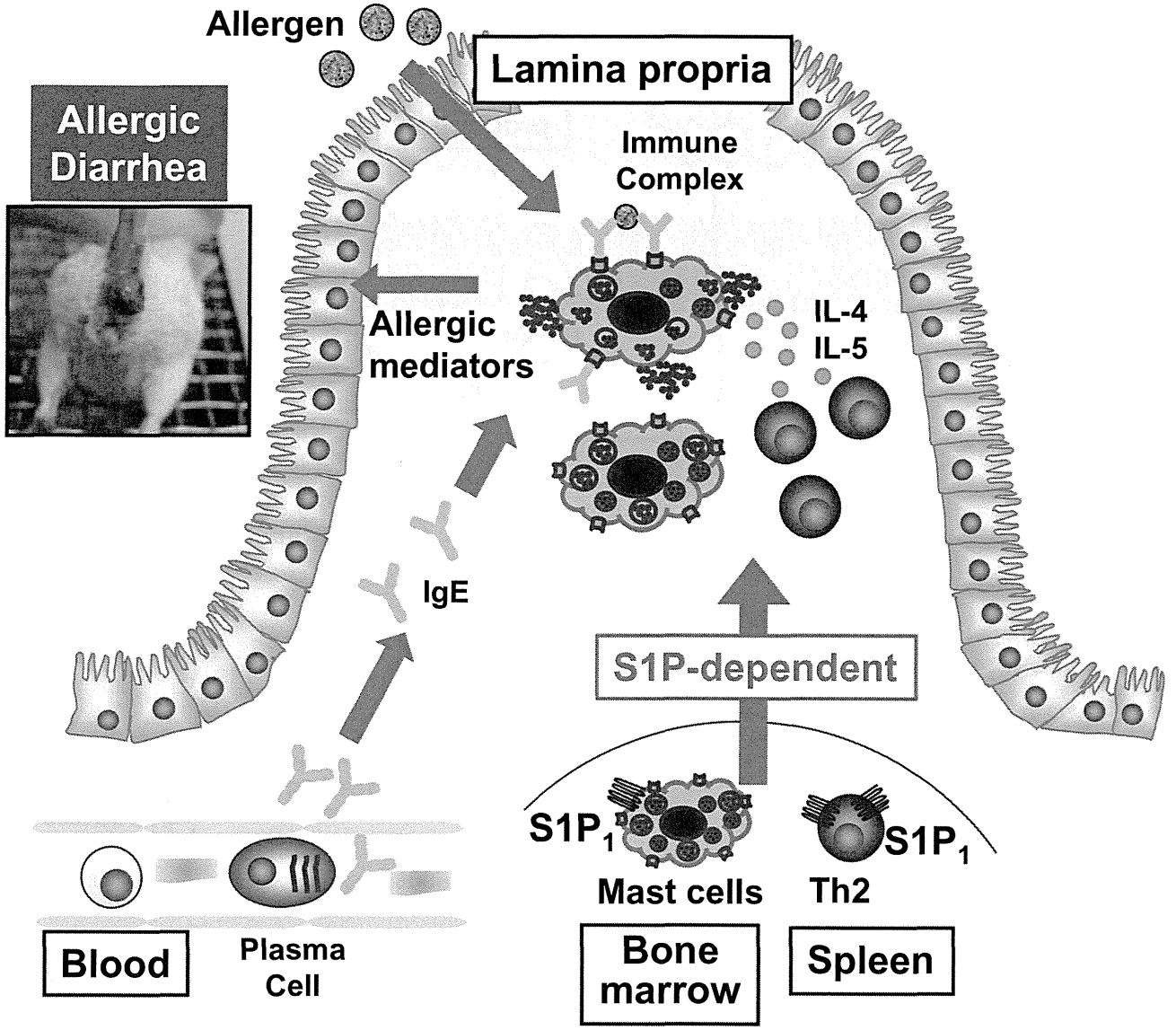

Figure 1 Involvement of sphingosine 1-phosphate in the development of allergic responses. Intestinal allergy is developed by several steps, including the production of allergen-specific immunoglobulin E (IgE) production, Th2 cell infiltration and activation (e.g. interleukin [IL]-4 and IL-5 production), mast cell accumulation and activation via cross-linking among IgE, allergen, and IgE receptor (FcεR1). During these steps, activated T cells and mast cells use sphingosine 1-phosphate (S1P) in their trafficking or growth in the colon.

activated in the gut (reviewed in Reference[27]). Vitamin A is obtained from the diet as all-trans-retinol, retinyl esters, or β-carotene and is metabolized into retinol or retinoic acid (RA) in the tissues.[28] Immunologically, RA induces the expression of $\alpha_4\beta_7$ integrin and the chemokine receptor CCR9 on both T and B cells.[29,30] Because both $\alpha_4\beta_7$ integrin and CCR9 are key molecules in lymphocyte homing into the gut, activated lymphocytes in the presence of RA tend to traffic into the intestinal lamina propria (Fig. 2). In agreement with this, vitamin A-deficient mice show a lack of T cells and IgA PCs in the intestine.[29,30]

RA plays an important role in determining not only the gut-tropism of lymphocytes activated in the intestine but also T cell differentiation. RA potentiates Treg induction with inhibition of Th17 differentiation *ex vivo* (Fig. 2).[31–33] Retinaldehyde dehydrogenase, a key enzyme converting vitamin A into RA, is uniquely expressed on gut-associated DCs, especially $CD103^+$ migratory DCs and ECs.[29,34] Thus, vitamin A metabolism by intestinal DCs and ECs plays a pivotal role in both T cell differentiation and subsequent cell trafficking to maintain the immunological homeostasis in the gut.

Recent studies have revealed the immunological role of vitamin B9 (also known as folate or folic acid) in the maintenance of Treg cells. Vitamin B9 is a water-soluble vitamin derived from both diet and commensal bacteria; the pathways for its *de novo* synthesis are

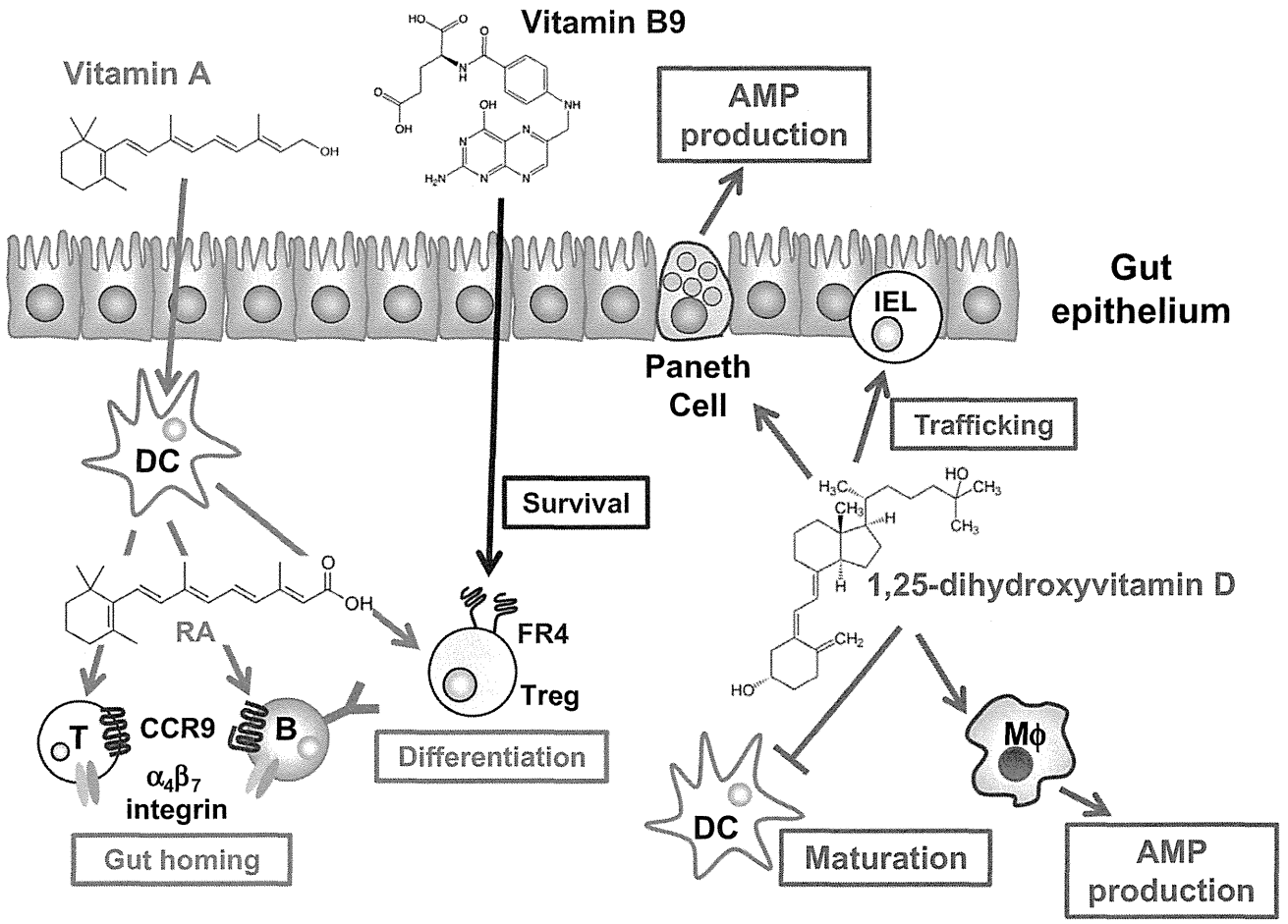

Figure 2 Vitamin-mediated immune regulations in the gut. Vitamin A from diet is converted into retinoic acid (RA) by dendritic cells (DCs). B and T cells primed by DCs in the presence of RA express gut homing molecules ($\alpha_4\beta_7$ integrin and CCR9). RA also promotes the differentiation of naïve T cells into regulatory T (Treg) cells. Upon the differentiation into the Treg cells, Treg cells start to express folate receptor 4 (FR4), a receptor for vitamin B9. The vitamin B9–FR4 axis is required for the survival of Treg cells. Vitamin D acts on Paneth cells on the epithelial layer and macrophages (MΦ) and aids production of antimicrobial peptides (AMPs). It also promotes the trafficking of intraepithelial lymphocyte (IEL) population and inhibits the maturation of DCs.

absent in mammals.[35] The biological functions of vitamin B9 are basically synthesis, replication, and repair of nucleotides for DNA and RNA to maintain cell proliferation and survival.[36] From an immunological perspective, Yamaguchi *et al.*[37] reported that folate receptor 4, one type of vitamin B9 receptor, is highly expressed on the surfaces of Treg cells, implicating the specific function of vitamin B9 on Treg cells. Moreover, we recently reported that Treg cells could differentiate from naïve T cells, but not survive, in the absence of vitamin B9 *in vitro* and *in vivo*, which was associated with the reduced expression of anti-apoptotic molecules (e.g. Bcl-2).[38] Because Treg cells are essential for maintaining immunological quiescence, mice deficient in vitamin B9 have increased susceptibility to intestinal inflammation.[39] These findings collectively suggest that vitamin A is required for the induction of Treg cells and that subsequent maintenance of the differentiated Treg cells is mediated by vitamin B9 (Fig. 2).

In addition to modulating lymphocytes, vitamins regulate innate immunocompetent cells. For example, vitamin D enhances the production of the antimicrobial peptide cathelicidin by intestinal Paneth cells,[40] stabilizes tight-junction structures in ECs,[41] and enhances homing of the IEL population in the gut (Fig. 2).[42] Consistent with these findings, mice lacking vitamin D receptors have increased bacterial loads in the intestine and show intestinal inflammation.[42,43] In addition, vitamin D receptors and CYP27B1, a vitamin D-activating enzyme, are induced in macrophages or DCs upon their activation (Fig. 2). In macrophages, intracrine synthesis of an active form of vitamin D, 1,25-dihydroxyvitamin D, promotes their antibacterial response to infection.[44] Intracrine 1,25-dihydroxyvitamin D in DCs inhibits their maturation, which in turn results in impaired T cell activation.[45] 1,25-dihydroxyvitamin D also acts extrinsically on T cells. 1,25-dihydroxyvitamin D3 inhibits T cell differentiation into interferon-γ-, IL-17-, or IL-21-producing inflammatory T cells but promotes the differentiation of Treg cells.[46] These versatile functions of vitamin D have led to its use in the control of infectious and inflammatory diseases.[47,48]

Nucleotides act as danger signals to induce the inflammatory responses

In addition to vitamins and lipids, our diet also contains sizable amounts of nucleotides. Dietary nucleotides have various effects on the immune responses such as protection from bacterial infections[49] and immune regulations.[50]

With dietary nucleotides, there is an abundance of extracellular nucleotides in the intestinal lumen, mainly in the form of adenosine triphosphate (ATP). Several lines of evidence have demonstrated that extracellular ATP acts as a danger signal to induce inflammatory responses. Therefore, stimulation of macrophages and DCs by ATP induces the production of inflammatory cytokines, which can consequently lead to the development of asthma, contact hypersensitivity, or graft-versus-host disease.[51–53] ATP is also involved in the development of intestinal inflammation through the induction of Th17 cells via intestinal DC activation.[54] In the intestinal lumen, extracellular ATP is catalyzed by ATP-hydrolyzing enzymes, such as ectonucleoside triphosphate disphosphohydrolases preferentially expressed on intestinal ECs.[55] A recent study demonstrated that mice lacking ecto-nucleoside triphosphate disphosphohydrolases had elevated levels of ATP in the intestinal lumen and consequently high numbers of Th17 cells in the intestinal lamina propria.[56] We recently reported that, in addition to inducing Th17 cells, ATP directly stimulates mast cells in the intestine.[57] Among immunocompetent cells in the intestine (e.g. DC, T and B cells, macrophages, and ECs), mast cells express the highest levels of P2X7 purinoceptor (one type of receptor for extracellular ATP). ATP-mediated stimulation of mast cells results in the production of inflammatory cytokines (e.g. IL-1β and tumor necrosis factor-α), chemokines (e.g. CCL1), and lipid mediators (e.g. leukotriene B4), and thus, inhibition of this pathway by blocking antibody led to the prevention of intestinal inflammation (Fig. 3).[57]

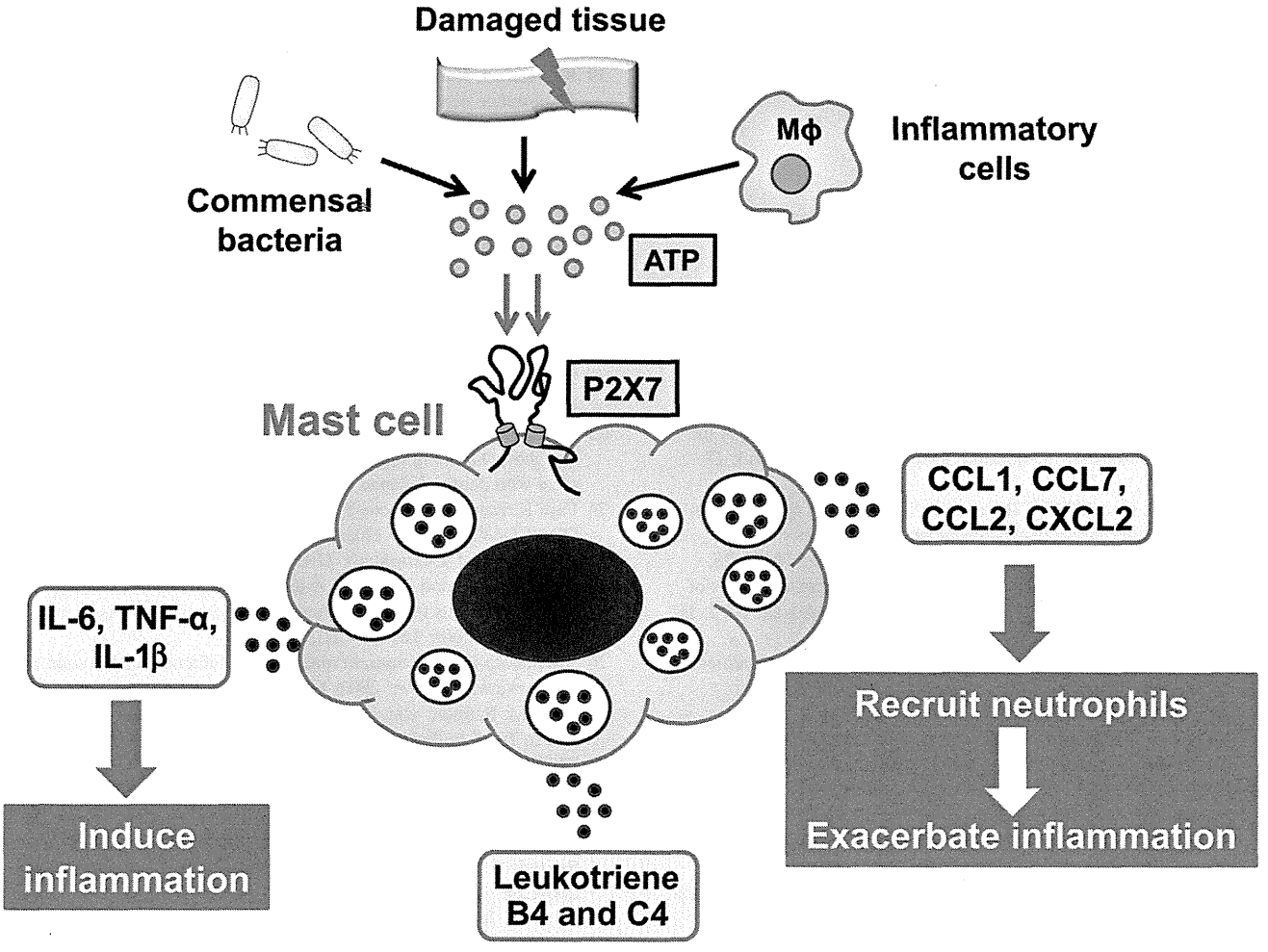

Figure 3 Extracellular adenosine triphosphate (ATP) acts as pro-inflammatory nucleotide through mast cell activation. Extracellular ATP derived from damaged tissues, commensal bacteria, and inflammatory cells can act as danger signals in the intestine. Colonic mast cells express the highest levels of P2X7 purinoceptors, a receptor for extracellular ATP. ATP-mediated activation of mast cells through P2X7 purinoceptors results in the production of inflammatory cytokines (e.g. interleukin [IL]-6, tumor necrosis factor [TNF]-α, IL-1β), chemokines (e.g. CCL1, CCL7, CCL2, CXCL2), and lipid mediators (leukotriene B4 and C4), which in turn leads to the induction and exacerbation of intestinal inflammation.

Conclusion

Immunological homeostasis and immunosurveillance in the gut are achieved by both innate and acquired unique immune systems. Many nutritional components play important roles in the development and smooth functioning of the gut immune system in both the innate and the acquired phase. Further elucidation of the intricate system by which nutrients regulate mucosal immunity by nutrition will allow us to develop functional nutritional materials for controlling the intestinal immune system and thus preventing intestinal immune diseases.

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Robo4 is an effective tumor endothelial marker for antibody-drug conjugates based on the rapid isolation of the anti-Robo4 cell-internalizing antibody

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Regular Article

VASCULAR BIOLOGY

Robo4 is an effective tumor endothelial marker for antibody-drug conjugates based on the rapid isolation of the anti-Robo4 cell-internalizing antibody

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Key Points

- First therapeutic application that targets Robo4 on the tumor blood vasculature
- High-throughput screening system to isolate cell-internalizing monoclonal antibodies useful to develop effective antibody-drug conjugates

Monoclonal antibodies (mAbs) that are internalized into cells are a current focus in the development of antibody-drug conjugates (ADCs). We describe a phage display–based high-throughput screening system to rapidly isolate cell-internalizing mAbs. We simultaneously examined the cell-internalizing activities of several hundred independent mAbs and successfully isolated cell-internalizing mAbs against the tumor endothelial markers Roundabout homolog 4 (Robo4) and vascular endothelial growth factor receptor 2 (VEGFR2). Tumor accumulation of mAbs with high cell-internalizing activity was significantly higher than that of mAbs with low cell-internalizing activity. Furthermore, the antitumor effects of ADCs of mAbs with high cell-internalizing activity were significantly stronger than those of mAbs with low cell-internalizing activity. Although anti-VEGFR2 therapy caused a significant loss of body weight, anti-Robo4 therapy did not. These findings indicate that cell-internalizing activity plays an important role in the biodistribution and therapeutic effects of ADCs. Further, Robo4 can be an effective marker for tumor vascular targeting. (*Blood.* 2013;121 (14):2804-2813)

Introduction

Antibody drug conjugates (ADCs), ie, monoclonal antibodies (mAbs) labeled with certain anticancer agents, are currently the focus of antibody-based drug discovery. ADCs have mAb-derived specificity and allow for targeted delivery of cytotoxic drugs to a tumor, which is expected to significantly enhance the antitumor activity of mAbs.[1] Trastuzumab ematansine (T-DM1)[2] for human epidermal growth factor receptor 2 (Her-2)–positive breast cancer and brentuximab vedotin (SGN-35)[3] for relapsed or refractory CD30-positive lymphoproliferative disorders are now in phase 3 clinical trials as effective ADCs.[4] ADCs will have an important role in overcoming some types of refractory cancers and will contribute to the field of tumor vascular targeting.[5]

An essential property of ADCs is that the mAb should be efficiently internalized into the cell where the cytotoxic effects of anticancer drugs occur.[1] The isolation of mAbs with high cell-internalizing activity (cell-internalizing mAbs) is a limiting factor in the development of ADCs. The discovery of potent cell-internalizing mAbs, however, requires labor-intensive screening of a massive number of candidates, and therefore the development of phage display–based methods to identify these candidates is highly desirable.[6,7] In the phage display–based method, a phage antibody library is added to the desired cells and then phages bound to the cell surface are removed. Only internalized phages are rescued from the intracellular compartment. Even with this method, however, the internalizing activities of individual antibody candidates must be assessed, because the concentrated phage pool comprises a "polyclonal" population. To address this issue, we used high-throughput screening methods to estimate "monoclonal" cell-internalization activities using a protein synthesis inhibitory factor (PSIF),[8] which provided a breakthrough in reducing the time-consuming screening of the cell-internalizing activity.

PSIF is a fragment of a bacterial exotoxin derived from *Pseudomonas aeruginosa*.[9] PSIF lacks its cell binding domain, and its cytotoxic portion is used in a recombinant immunotoxin.[10] Upon entry into the cell, PSIF has strong cytotoxicity by inducing ADP-ribosylation of elongation factor-2, which is essential for protein synthesis.[11] Our group previously accelerated the identification of cell-internalizing novel protein transduction domains (PTDs) by expressing PTD-PSIF fusion proteins in the supernatant of *Escherichia coli*.[8] Using this system, we successfully discovered superior HIV-Tat PTD mutants by simultaneously estimating

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M.Y. and Y.M. contributed equally to this study.

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the cell-internalizing activities of several hundred monoclonal PTD-PSIF fusions.[8] Therefore, we expect this method to contribute to the identification of mAbs with high cell-internalizing activity (cell-internalizing mAbs) by expressing single-chain antibody Fv (scFv)-PSIF fusion proteins to estimate the cell-internalizing activities of a very large number of antibodies.

Roundabout homolog 4 (Robo4) is a potential tumor angiogenesis marker.[12] Robo4 expression is restricted to areas of in vivo angiogenesis[13,14] and the subpopulation of hematopoietic stem cells localized in the bone marrow.[15] At angiogenic sites, Robo4 is present in the endothelial lining of blood vessels in the developing embryo,[16] placenta,[14] and tumors.[17] We previously confirmed the endothelial cell–specific expression of Robo4 using transgenic mouse lines.[18,19] Robo4 acts as a receptor that modulates vascular endothelial growth factor A (VEGF)–VEGF receptor (VEGFR) signaling.[20-23] Therefore, Robo4 is a potential marker for tumor vascular targeting because angiogenesis is only activated in tumors in the adult,[24] with the exception of some pathological states.[25,26] Another potential tumor angiogenesis marker is VEGFR2, a well-established tumor endothelial marker.[27] The VEGF-VEGFR2 signaling pathway plays a crucial role in angiogenesis, and anti-VEGF mAbs and small molecule inhibitors against VEGFR are approved for various types of cancers.[28] Anti-VEGFR2 mAbs are also used for tumor vascular targeting.[29] Although VEGFR2 is strongly expressed in active angiogenic sites, its expression is also observed in normal tissues.[30] Hypertension and proteinuria are common side effects of anti-VEGF therapy because VEGF-VEGFR signaling is also inhibited in normal tissue, including the kidney, heart, and resistance vessels.[31-33]

Here we applied the PSIF system to search for novel cell-internalizing mAbs from an immune phage antibody library. Application of this method to Robo4 and VEGFR2 led to the successful discovery of anti-Robo4 and anti-VEGFR2 cell-internalizing mAbs, as well as mAbs with low cell-internalizing activity (low-internalizing mAbs) to be used as controls. Comparing mAbs with different cell-internalizing activities revealed that higher cell-internalizing activity enhanced the tumor targeting potency of mAbs. Furthermore, comparative studies with anti-Robo4 and anti-VEGFR2 cell-internalizing mAbs in vivo indicated that Robo4 was superior to VEGFR2 in terms of the therapeutic window. This is the first report demonstrating the benefits of cell-internalizing mAbs in tumor vascular targeting. Further, these findings demonstrate the potential of Robo4 as a target for further development of novel ADCs against tumor blood vasculature.

Materials and methods

Cell culture

MS1 immortalized murine endothelial cells were cultured in Dulbecco's Modified Eagle Medium containing 5% fetal bovine serum 1% antibiotic-antimycotic mixed solution. B16BL6 murine melanoma cells were cultured in minimum essential medium containing 10% fetal bovine serum and 1% antibiotic-antimycotic mixed solution at 37°C. These cells were maintained at 37°C under a humidified 5% CO_2 atmosphere.

B16BL6 tumor-bearing mice

B16BL6 cells (1×10^6 cells/100 μL) were inoculated intracutaneously into 6-week-old female C57BL6 mice (Japan SLC Inc., Shizuoka, Japan) (day 0). Biodistribution was analyzed on the day that the tumor width reached 10 mm. The therapy experiment was started on day 3. As a validation of the model, we confirmed the expressions of VEGFR2 and Robo4 on the tumor endothelium, based on the immunofluorescence against B16BL6 tumor sections.

Antigens

Human VEGFR2 (hVEGFR2) and mouse VEGFR2 (mVEGFR2) were commercial recombinant proteins (Merck Chemicals, Inc., Darmstadt, Germany, or R&D Systems, Inc., Minneapolis, MN). Human Robo4 (hRobo4) and mouse Robo4 (mRobo4) were produced as described previously.[34]

Immune phage antibody libraries

Phage antibody libraries were constructed from the spleen and bone marrow cells of immunized mice as previously described.[35,36] Our phage antibody library comprised single-chain Fv fragment (scFv) fused with pIII phage coat protein. Four rounds of affinity panning were performed against hVEGFR2 and mVEGFR2 for the anti-VEGFR2 immune library, and against hRobo4 and mRobo4 for the anti-Robo4 immune library. Anti-FLAG panning was followed by each panning to concentrate the scFv-displaying phages, as described previously.[36]

ELISA and cytotoxicity assay using TG1 supernatant

Plasmids were extracted from TG1 cells after the fourth panning against mVEGFR2 or mRobo4. These "enriched" scFv gene libraries were cloned into a PSIF-fusion expression vector derived from pCANTAB5E.[8] Monoclonal scFv-PSIF protein was induced in the TG1 supernatant, as previously described.[8] mVEGFR2 or mRobo4 was immobilized on an immunoassay plate and blocked with 4% skim milk in phosphate-buffered saline (PBS) (4% MPBS) at 37°C for 2 hours. TG1 supernatant containing 2% MPBS was reacted with antigens at room temperature for 1 hour. Bound scFv-PSIFs were detected by anti–FLAG-horseradish peroxidase (M2, Sigma-Aldrich Corporation, St. Louis, MO). For the cytotoxicity assay, MS1 cells were seeded on a 96-well plate at 1.0×10^4 cells/well. After incubation at 37°C for 24 hours, TG1 supernatant was diluted in MS1 culture medium, and then added to the MS1 cells. After incubation at 37°C for 24 hours, cell viability was assessed using a WST-8 assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). The viability of nontreated MS1 and completely killed MS1 with 1 mM cycloheximide were defined as 100% and 0%, respectively.

Expression and purification of scFv, dscFv, and scFv-PSIF recombinant protein

The isolated scFv gene with 15 amino acids linker (VL-GGGGSGGG GSGGGGS-VH) was cloned into modified pET15b vector, resulting in the scFv fused by FLAG-tag and His×6 tag at the C-terminus. A scFv gene with a 5–amino acid linker (VL-GGGGS-VH) was also cloned into modified pET15b, resulting in a noncovalent scFv dimer (dscFv) fused by FLAG-tag and His×6 tag at the C-terminus. An anti-His scFv gene was also cloned but only a FLAG-tag was fused at the C-terminus. A scFv gene with a 15–amino acid linker was cloned into pYas-PSIF vectors.[37] ScFvs, dscFvs, and scFv-PSIFs were purified from inclusion bodies in *E coli* according to the previously described methods.[37] The binding affinity of each recombinant protein was assessed by surface plasmon resonance using BIAcore3000 (GE Healthcare UK Ltd., Chalfont, United Kingdom).

Expression and purification of IgG recombinant protein

IgG recombinant proteins were expressed using an OptiCHO antibody expression kit (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's instructions. IgGs were purified from cell culture supernatant with protein G column (GE Healthcare). Eluted fractions were further purified with Superdex 200 column (GE Healthcare). Anti-FLAG[IgG] (anti-FLAG M2 antibody) was purchased from Sigma-Aldrich.

Preparation of IgG-NCS

NCS was kindly provided by Kayaku Co, Ltd., Tokyo, Japan. NCS was thiolated by incubating it with 10 molar excess 2-iminothiolane (Thermo Fisher Scientific Inc., Waltham, MA) for 1 hour at room temperature. IgG recombinant proteins were reacted with 10 molar excess of SPDP