

Interleukin-5 Plays a Key Role in Mouse Strain-Dependent Susceptibility to Contact Hypersensitivity through Its Effects on Initiator B Cells

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Key Words

Contact hypersensitivity · Interleukin-5 · B-1 cells

Abstract

Background: Elicitation of contact hypersensitivity requires antigen-specific immunoglobulin M (IgM) antibodies that trigger recruitment of effector T cells to the local tissue. These antigen-specific IgM antibodies are produced by B-1-like 'initiator B cells'. In this study, we compared susceptibility to hapten-induced contact hypersensitivity between BALB/c and C57BL/6 mice. **Methods:** BALB/c and C57BL/6 mice were sensitized by painting oxazolone onto the skin and were challenged on the ears with the same hapten on day 4. Ear thickness and serum hapten-specific IgM levels were measured at 24 h post-challenge. Peritoneal cells were harvested and the numbers of B cell subpopulations were counted. Interleukin (IL)-5 was intraperitoneally injected into BALB/c and C57BL/6 mice, and the change in numbers of B cell subpopulations and serum IgM levels was monitored. **Results:** Oxazolone induced stronger ear swelling and specific IgM responses in BALB/c mice than in C57BL/6 mice. BALB/c mice had higher numbers of peritoneal B-1 cells than C57BL/6 mice at steady state. IL-5 injection increased the number of peritoneal B-1 cells and serum IgM levels more

significantly in BALB/c mice than in C57BL/6 mice. **Conclusions:** BALB/c mice exhibit higher susceptibility to hapten-induced contact hypersensitivity than C57BL/6 mice, most likely because they have a higher number of B-1 cells, leading to massive production of hapten-specific IgM antibodies upon contact sensitization. The differences in the number of B-1 cells and IgM responses between the two strains of mice may be attributed to the difference in responsiveness of B-1 cells to IL-5.

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Introduction

Contact hypersensitivity (CHS) is a classic example of delayed-type hypersensitivity, which is thought to be mediated by Th1 cells [1, 2]. Upon primary contact sensitization with antigen, skin-resident Langerhans cells and dermal dendritic cells capture antigens and migrate to the draining lymph nodes, where they present antigens to antigen-specific naïve T cells. Naïve T cells are then activated and differentiate into Th1 cells within 3–4 days. Following secondary contact with the same antigen, the antigen-specific Th1 cells are recruited to local tissues; this triggers infiltration of antigen-nonspecific inflam-

matory cells such as macrophages, neutrophils and eosinophils [3]. We have demonstrated that antigen-specific immunoglobulin M (IgM) antibodies are essential for the recruitment of antigen-specific Th1 cells to local tissues [4]. The B cell population that produces these IgM antibodies was originally identified as CD19⁺CD5⁺IgM^{high}IgD^{low} B-1 cells [4]. However, a recent study by Kerfoot et al. [5] revealed that a CD19⁺CD5⁺IgM^{high}IgD^{high} B-1-like cell population called 'initiator B cells' is responsible for the production of hapten-specific IgM antibodies. Initiator B cells are activated in the peritoneal cavity 1 h after skin sensitization and migrate to lymphoid tissues, where they produce antigen-specific IgM antibodies [6]. Hapten-specific IgM antibodies produced by initiator B cells are also required for the elicitation of non-atopic asthma [7].

Several investigators have reported mouse strain-associated differences in susceptibility to hapten-induced CHS [8–10]. The differences among mouse strains in terms of severity of skin inflammation are often related to the degree of expansion of different helper T cell types (Th1 or Th2) that mediate the CHS response. Takeshita et al. [8] reported that fluorescein isothiocyanate (FITC) induced stronger CHS in BALB/c mice than in C57BL/6 mice. Because FITC-induced CHS is mediated by Th2 cytokines such as interleukin (IL)-4 and IL-5, they concluded that Th2-dominant BALB/c mice exhibit a stronger response to FITC than Th1-dominant C57BL/6 mice. However, no significant difference was observed between BALB/c mice and C57BL/6 mice in dinitrofluorobenzene (DNFB)-induced CHS, which is mediated by Th1 cells. Interestingly, however, Nagai et al. [9] reported that BALB/c mice exhibit stronger CHS responses than C57BL/6 mice when DNFB is applied repeatedly, which is conceivable as repeated application of DNFB induces a shift from a Th1-mediated response to a Th2-mediated response. Although CHS is mediated not only by T cells but also by various immunocompetent cells such as initiator B cells, mast cells and polymorphonuclear cells, their contribution to strain-associated differences in susceptibility to hapten-induced CHS has not yet been clarified.

IL-5 is known to promote the survival and proliferation of B-1 cells and to stimulate B-1 cells to produce IgM antibodies through IL-5 receptor (IL-5R), consisting of IL-5R α and IL-5R β c chains [11, 12]. IL-5 also affects eosinophils and controls their differentiation, survival and function [13]. We found that CHS to oxazolone is impaired in IL-5R α -deficient mice, most likely because the numbers of CD19⁺CD5⁺ peritoneal B-1 cells (including

initiator B cells) are diminished in these mice [14, 15], resulting in limited production of antigen-specific IgM antibodies upon contact sensitization. Moreover, infiltration of eosinophils to antigen-challenged sites is impaired in these mice. Other studies have also proposed a crucial role of IL-5 in the elicitation of delayed-type hypersensitivity [8, 16].

B-1 cells are found in the peritoneal and pleural cavities at high frequency [17]. However, the IL-5-producing cells that maintain B-1 cell homeostasis in these cavities have not been identified. We recently identified innate IL-5-producing non-T lymphoid cells that localize to the peritoneal cavity as well as the lung and intestine using an IL-5 reporter mouse whose genetic background is C57BL/6 [18]. They are lineage negative and express Sca-1, c-kit, Thy 1.2, CD25, CD27, IL-7R α and T1/ST2, a subunit of IL-33R. However, it has not been elucidated whether the innate IL-5-producing non-T lymphoid cells also localize to the peritoneal cavity of other strains of mice.

In this study, we compared the susceptibility to oxazolone-induced CHS which is thought to be mediated by Th1 cells between Th2-dominant BALB/c mice and Th1-dominant C57BL/6 mice. We found that oxazolone induced stronger CHS responses in BALB/c mice than in C57BL/6 mice. To clarify the mechanisms of strain-dependent susceptibility to oxazolone-induced CHS, we examined production of hapten-specific IgM antibodies following contact sensitization, and evaluated the responsiveness of both strains of mice to IL-5 stimulation. Our findings suggest an important role of IL-5 in strain-dependent susceptibility to hapten-induced CHS.

Materials and Methods

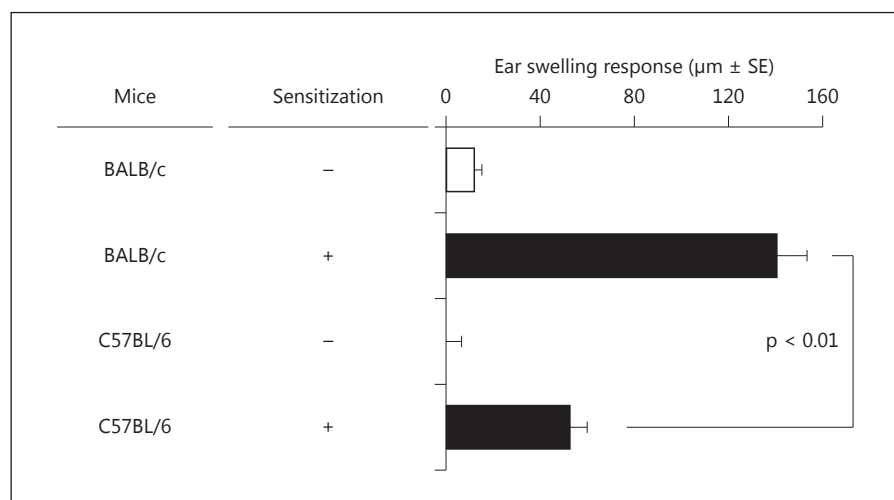
Mice

Female BALB/c and C57BL/6 mice were purchased from CLEA Japan (Tokyo, Japan), and were rested for at least 1 week before use. IL-5/Venus knockin (KI) mice were generated as described previously [18], and were backcrossed to C57BL/6 or BALB/c mice at least 10 generations. All mice were housed at the animal facility of the Iwaki Meisei University under specific pathogen-free conditions, and were used at the age of 7–10 weeks. All experiments were performed according to the guidelines of Iwaki Meisei University.

Elicitation of CHS

Mice were sensitized by painting 150 μ l of 3% oxazolone (Sigma, St. Louis, Mo., USA) in ethanol:acetone = 4:1 on the shaved abdomen, chest and footpads. Four days later, mice were challenged by topical application of 10 μ l of 0.4% oxazolone in acetone:olive oil = 1:1 on each side of the ears, and ear thickness was measured using a micrometer (Mitutoyo, Japan) at 24 h after

Fig. 1. Ear swelling responses to oxazolone. BALB/c and C57BL/6 mice were sensitized and were challenged on the ears 4 days later. Ear thickness was measured at 24 h after challenge, and the net increase in ear thickness was calculated by subtracting ear thickness measured before challenge. Data are expressed as mean $\pm$ SE of 5 mice.



challenge. The net increase in ear thickness was calculated by subtracting ear thickness measured before challenge and expressed as mean $\pm$ SE of 4–6 mice, unless otherwise stated.

Measurement of Serum IgM Antibodies

Appropriately diluted serum was applied to 96-well plates coated with anti-mouse IgM antibodies or oxazolone-bovine serum albumin prepared as described previously [14], and the plates were incubated for 1 h at room temperature. After washing 3 times with PBS containing 0.05% Tween-20, the plates were incubated with horseradish peroxidase-conjugated anti-mouse IgM antibodies (Southern Biotech, Birmingham, Ala., USA) for 1 h at room temperature. The plates were then washed and color was developed with *o*-phenylenediamine. The reaction was stopped by adding 4N H₂SO₄, and the absorbance was measured at 490 nm.

Enzyme-Linked Immunospot Assay for Specific IgM-Producing Cells

Single cell suspensions were prepared from spleens 4 days after contact sensitization, and were subjected to enzyme-linked immunospot (ELISPOT) assay as described previously [14]. Briefly, cells were seeded into MultiScreen-IP plates coated with oxazolone-bovine serum albumin and incubated at 37°C overnight. After washing, plates were incubated with biotin-conjugated anti-mouse IgM antibodies (BD Biosciences, San Jose, Calif., USA) for 1 h followed by incubation with streptavidin-peroxidase (Life Technologies, Carlsbad, Calif., USA) for 1 h at room temperature. Spots were developed using a 3-amino-9-ethylcarbazole Chromogen Kit (Sigma) and reactions were stopped by washing with H₂O. After drying in the dark, the membranes were removed and the spots were counted under a phase-contrast microscope by a single observer unaware of the groups.

Flow Cytometric Analysis

Flow cytometric analysis was performed with FACSCanto II (BD Biosciences) after staining with combinations of antibodies specific for c-kit (2B8), CD3 ϵ (145-2C11), CD4 (RM4-5), CD5 (53-7.3), CD19 (1D3), IL-5 (TRFK5), IFN- γ (XMGI.2), Mac1 (M1/70) and Sca-1 (E13-161.7) obtained from BD Biosciences and eBiosci-

ences (San Diego, Calif., USA). In some experiments, peritoneal cells were harvested from mice injected intraperitoneally with recombinant IL-5 (rIL-5, 100 U) or PBS 4 times weekly. For intracellular staining of IL-5 and IFN- γ , cells were stained using a fixation/permeabilization kit (eBiosciences) after in vitro stimulation with PMA (10 ng/ml) and ionomycin (1 μ M) for 6 h in the presence of GolgiStop (BD Biosciences).

Cell Proliferation Assay

CD19⁺CD5⁺ B-1 cells were purified from peritoneal exudate cells with FACSaria (BD Biosciences), and stained with 1 μ M of CFSE at 37°C for 10 min. Stained cells were then incubated with 1 ng/ml of rIL-5 (R&D Systems, Minneapolis, Minn., USA) and 1 μ g/ml of anti-CD40 antibodies (clone 1C10; eBiosciences) for 4 days, and subjected to flow cytometric analysis with FACSCanto II.

Statistics

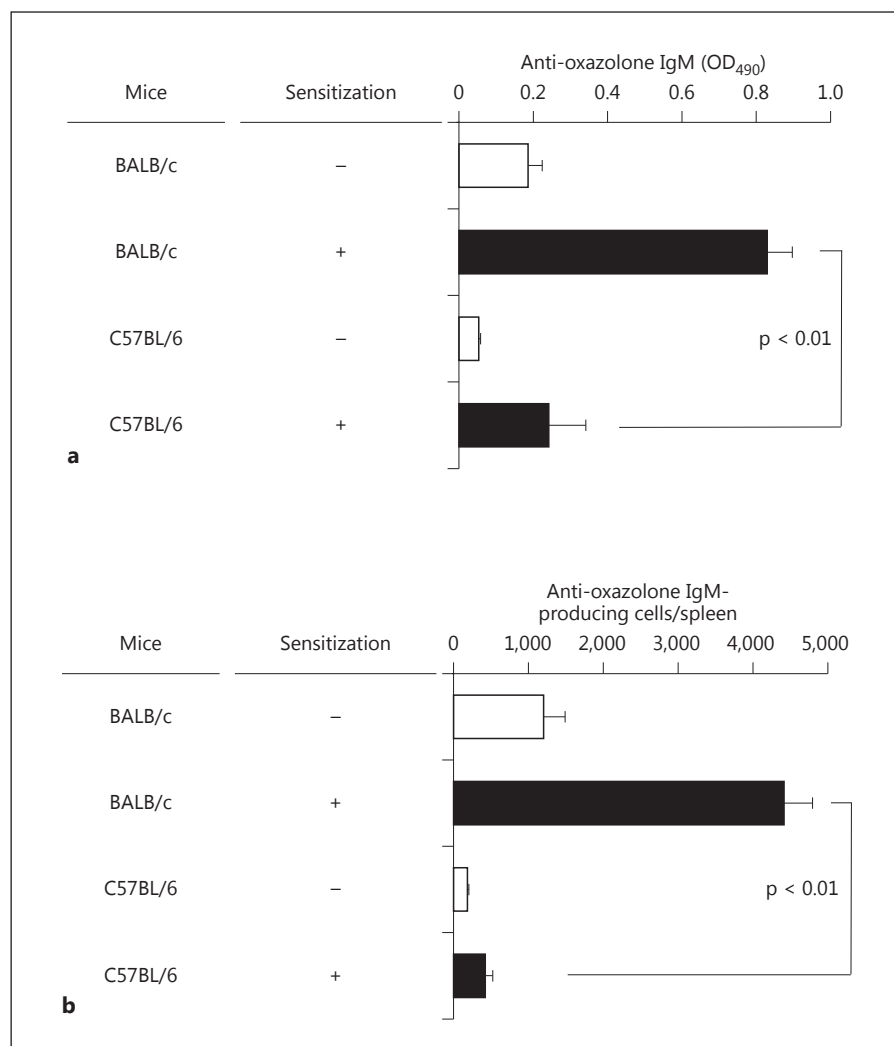
Statistics were performed using the paired two-tailed Student *t* test, and *p* < 0.05 was taken as the level of significance.

Results

Ear Swelling and Antigen-Specific IgM Responses to Contact Sensitization

To compare the response to contact sensitization between mouse strains, BALB/c and C57BL/6 mice were contact-sensitized with oxazolone and challenged on the ears 4 days later. Sensitized BALB/c mice developed massive ear swelling 24 h after the challenge compared to unsensitized BALB/c mice (fig. 1). In contrast, ear swelling in sensitized C57BL/6 mice was significantly lower than that in sensitized BALB/c mice (fig. 1). As antigen-specific IgM antibodies are essential for the recruitment of effector T cells to local tissues, we then measured oxazo-

Fig. 2. Antigen-specific IgM production in sensitized BALB/c and C57BL/6 mice. Serum and spleens were collected 4 days after the contact sensitization. **a** Serum oxazolone-specific IgM antibodies were measured by ELISA. **b** The number of oxazolone-specific IgM-producing cells in spleens was measured by ELISPOT assay. Data are expressed as mean $\pm$ SE of 5 mice.



lone-specific IgM antibodies in the serum. As shown in figure 2a, serum levels of oxazolone-specific IgM antibodies in sensitized BALB/c mice were 3.5-fold higher than those in sensitized C57BL/6 mice, although contact sensitization with oxazolone increased specific IgM antibodies in both strains of mice. In addition, ELISPOT assay revealed that the number of cells producing oxazolone-specific IgM antibodies in the spleen increased 3.7-fold after contact sensitization in BALB/c mice, whereas the increase in sensitized C57BL/6 mice was significantly less (2.3-fold; fig. 2b).

B Cell Subpopulations in the Peritoneal Cavity

Hapten-specific IgM antibodies are produced in the spleen by initiator B cells that are primarily activated in the peritoneal cavity and migrate to the lymphoid tissues [6]. To clarify the factors that account for the difference

in hapten-specific IgM responses between BALB/c and C57BL/6 mice, we next measured the numbers of B-1a, B-1b and B-2 cells in the peritoneal cavity (fig. 3). Initiator B cells express CD19 and CD5, and are thus identified as either B-1a (CD19⁺CD5⁺Mac1⁺) or B-1b (CD19⁺CD5⁺Mac1⁻) cells [5]. BALB/c mice had about 3-fold higher numbers of B-1a cells than C57BL/6 mice. The numbers of B-1b cells and CD19⁺CD5⁻ B-2 cells also appeared to be higher in BALB/c mice than in C57BL/6 mice, although the difference was not statistically significant.

BALB/c B-1 Cells Are More Sensitive to IL-5 Stimulation than C57BL/6 B-1 Cells

IL-5 promotes the development and proliferation of B-1 cells and maintains their survival [11, 15]. Gene-targeted mice with a defect in IL-5 signaling have decreased numbers of peritoneal B-1 cells due to impaired prolif-

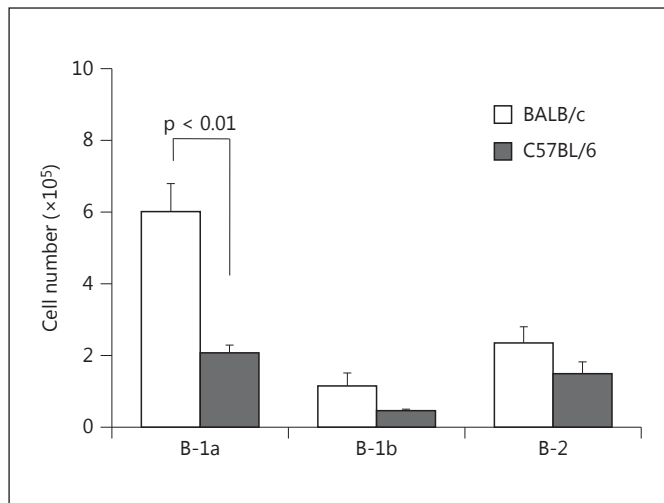


Fig. 3. Numbers of B cell populations in the peritoneal cavity. Peritoneal cells were harvested from unsensitized mice, and total cell numbers were counted. Cells were then stained with fluorescence-labeled antibodies, and were analyzed by flow cytometry. Data are expressed as mean $\pm$ SE of 5 mice.

eration and survival of B-1 cells [11, 15]. We thus hypothesized that the difference in numbers of peritoneal B-1a cells between BALB/c and C57BL/6 mice may be attributable to the different responsiveness of B-1 cells to IL-5. To compare the proliferative ability of B-1 cells in response to IL-5, B-1 cells purified from peritoneal exudate cells were labeled with CFSE and cultured in vitro with rIL-5 and anti-CD40 antibodies for 4 days. As shown in figure 4 (right panel), the intensity of CFSE was lower and more widely distributed in BALB/c B-1 cells, indicating that these cells had divided rapidly. In contrast, the intensity of CFSE in C57BL/6 B-1 cells was higher and distributed over a limited range. These results indicate that IL-5 induces stronger proliferation of BALB/c B-1 cells than C57BL/6 B-1 cells. Neither BALB/c nor C57BL/6 B-1 cells showed significant proliferative responses when cultured with anti-CD40 antibody alone (fig. 4, left panel). To evaluate the responsiveness to IL-5 in vivo, we injected rIL-5 intraperitoneally 4 times weekly, and examined the change in numbers of peritoneal B cells. As shown in figure 5a, the number of B-1a cells in rIL-5-injected BALB/c mice was 2.9-fold higher than in untreated BALB/c mice, whereas rIL-5-induced expansion of C57BL/6 B-1a cells was more limited (1.7-fold). B-1b cells proliferated in both strains of mice in response to rIL-5 injection (BALB/c, 5.3-fold; C57BL/6, 4.4-fold), although the number of B-1b cells in rIL-5-injected BALB/c mice was sig-

nificantly higher than that in rIL-5-injected C57BL/6 mice. In contrast, we observed a comparable increase in the numbers of B-2 cells in rIL-5-injected BALB/c and C57BL/6 mice. Since B-1 cells produce natural antibodies, we monitored serum levels of IgM after rIL-5 injection (fig. 5b). Although serum levels of IgM antibodies increased in both BALB/c and C57BL/6 mice 3 weeks from the first rIL-5 injection, the absolute value of IgM antibodies in BALB/c mice remained higher than those in C57BL/6 mice throughout the experimental period. At 4 weeks from the first injection, the serum IgM levels in rIL-5-injected BALB/c mice further increased 1.5-fold relative to PBS-injected mice, whereas they started to decrease in C57BL/6 mice. The serum levels of IgM antibodies in PBS-injected BALB/c mice remained 1.5 times higher than those in PBS-injected C57BL/6 mice.

Expression of IL-5R α on B Cells

To identify factors accountable for the difference in responsiveness of the two strains of mice to IL-5, we next examined the expression levels of IL-5R α on B cells (fig. 6). Flow cytometric analysis revealed that the expression of IL-5R α on BALB/c B-1a cells was slightly higher than that on C57BL/6 B-1a cells, although the difference was not significant. We did not observe any difference between BALB/c and C57BL/6 mice in terms of IL-5R α expression on B-1b cells and B-2 cells.

IL-5-Producing Cells in the Peritoneal Cavity

To identify the source of IL-5 that maintains the survival and proliferation of B-1 cells in the peritoneal cavity, we generated IL-5/Venus KI mice with a C57BL/6 and BALB/c background. We have already demonstrated that Venus⁺ cells in IL-5/Venus KI mice produce IL-5 and are identifiable as innate IL-5-producing non-T cells [18]. As shown in figure 7a, most of the Venus⁺ cells among the peritoneal lymphoid cells derived from the IL-5/Venus KI mouse with C57BL/6 background were c-kit⁺CD3 ϵ ⁻, which is consistent with our previous report [18]. In contrast, the percentage of Venus⁺c-kit⁺CD3 ϵ ⁻ cells in the IL-5/Venus KI mouse with BALB/c background was much lower. Instead, Venus⁺c-kit⁻CD3 ϵ ⁺ cells were most abundant in the IL-5/Venus KI mouse with a BALB/c background. It is notable that the total number of Venus⁺ cells in BALB/c mice was twice that in C57BL/6 mice (BALB/c, $11.3 \pm 5.2 \times 10^2$; C57BL/6, $5.6 \pm 2.4 \times 10^2$).

To confirm the difference in IL-5-producing cells between the two strains of mice, peritoneal cells from BALB/c and C57BL/6 mice were stimulated with PMA and ionomycin, and were analyzed by flow cytometry

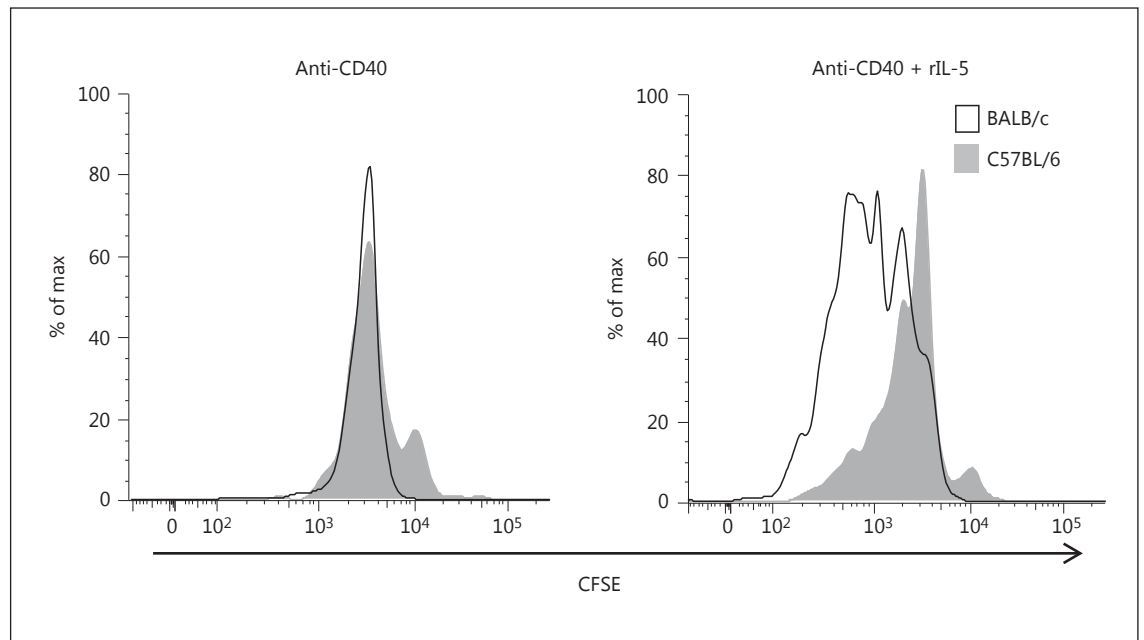


Fig. 4. Proliferation of B-1 cells stimulated with IL-5 and anti-CD40 antibodies. Purified CD19⁺CD5⁺ B-1 cells were labeled with CFSE, and were incubated with anti-CD40 antibody or anti-CD40 antibody + rIL-5. Four days later, cells were collected and were subjected to flow cytometric analysis. Data are representative of 3 independent experiments.

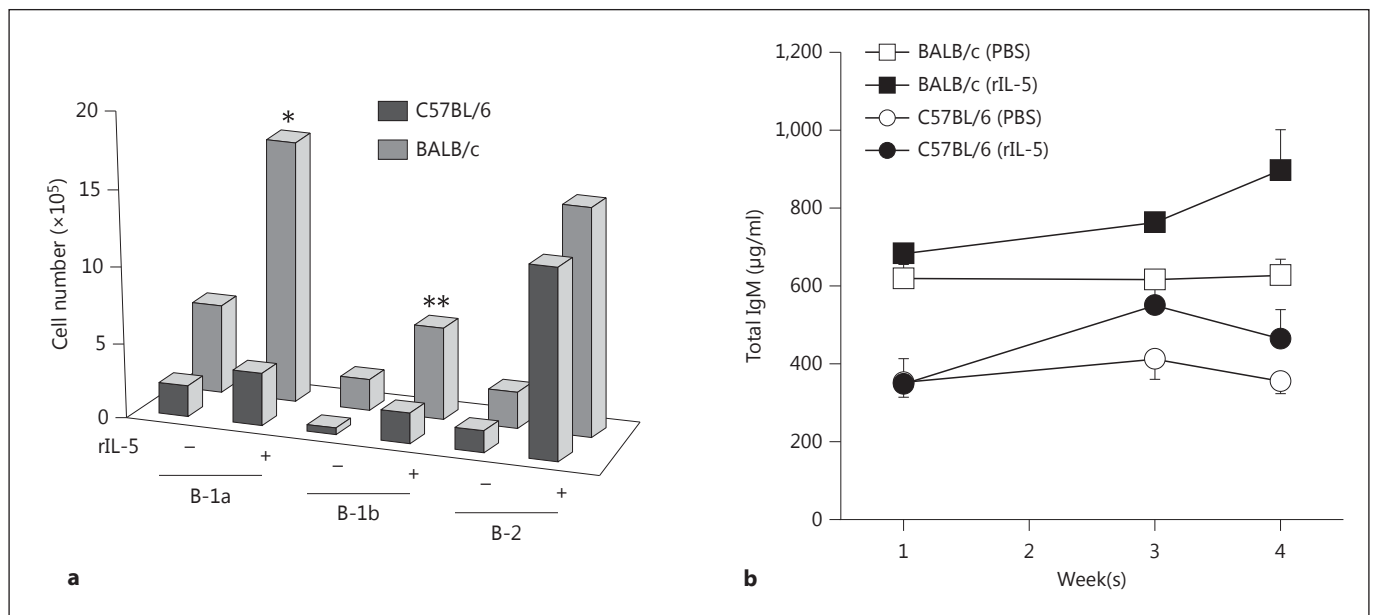


Fig. 5. In vivo analysis of B cell expansion and IgM production in response to rIL-5 injection. BALB/c and C57BL/6 mice were injected with rIL-5 4 times weekly. **a** Peritoneal cells were collected 1 week after the last injection and were subjected to flow cytometric analysis. Data are expressed as mean of 5 mice. * $p < 0.01$ and ** $p < 0.05$ (vs. rIL-5-injected C57BL/6 mice). **b** Serum levels of IgM were measured by ELISA. Data are expressed as mean $\pm$ SE of 5 mice.

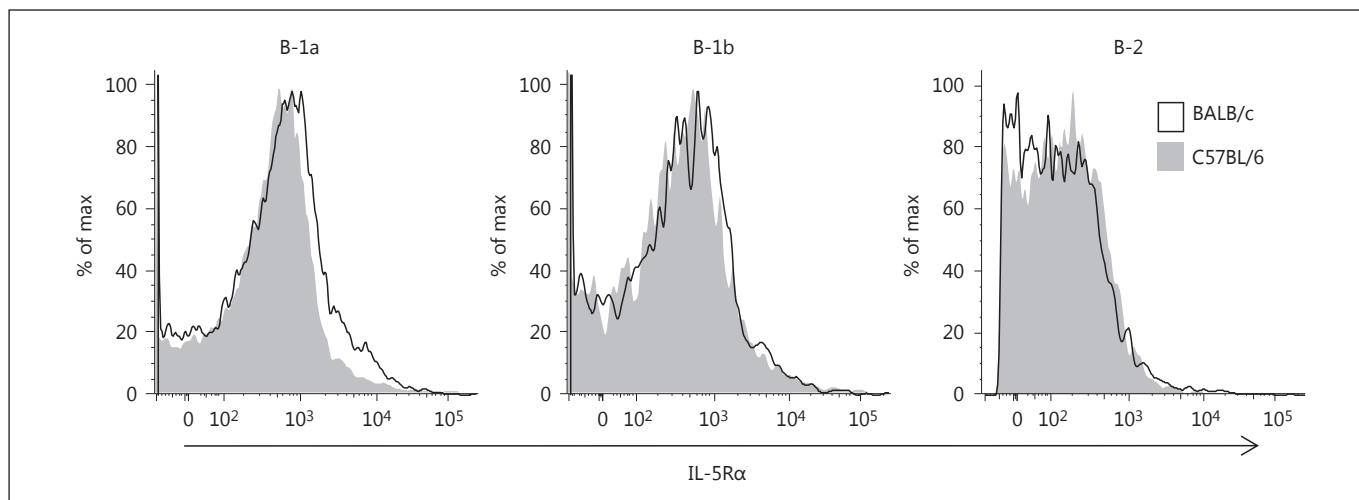


Fig. 6. Expression of IL-5R α on B cells. Peritoneal cells were stained with fluorescence-labeled antibodies against CD19, Mac-1, CD5 and IL-5R α (clone H7), and were analyzed by flow cytometry. Histograms are representative of 3 independent experiments.

(fig. 7b). Again, we found c-kit⁺Sca-1⁺ cells and CD3⁺CD4⁺ cells were the major IL-5-producing cells in the peritoneal cavity of C57BL/6 and BALB/c mice, respectively.

Discussion

In this study, we showed that oxazolone induced stronger CHS responses in BALB/c mice than in C57BL/6 mice. Because BALB/c is a Th2-dominant mouse strain, it is intriguing that BALB/c mice are more susceptible to oxazolone-induced CHS, which is thought to be mediated by Th1 cells [1, 2]. It has been reported that FITC and DNFB, when applied to the ears repeatedly, induce stronger CHS responses in BALB/c mice than in C57BL/6 mice [8, 9]. These findings are more in line with expectations, because Th2 cytokines such as IL-4 and IL-5 play a central role in CHS induced by FITC or repeated application of DNFB. Nevertheless, in contrast to these past reports, we here propose a new mechanism that accounts for the difference in susceptibility to oxazolone-induced CHS between the two strains of mice.

To clarify the mechanism of strain-dependent susceptibility to oxazolone-induced CHS, we first focused on the production of hapten-specific IgM antibodies, which are necessary for the recruitment of effector T cells to local sites. We found that BALB/c mice exhibited higher hapten-specific IgM responses than C57BL/6 mice upon contact sensitization, which enabled effector T cells to mi-

grate to antigen-challenged sites more efficiently. Serum levels of hapten-specific IgM antibodies and the number of hapten-specific IgM-producing cells in the spleen correlated well with the number of peritoneal B-1a cells. The hapten-specific IgM antibodies are produced by B-1-like initiator B cells 1 day after contact sensitization [5, 6]. Because both B-1a cells and initiator B cells are identified as CD19⁺CD5⁺ cells, the fact that BALB/c mice have higher numbers of B-1a cells strongly suggests that the number of initiator B cells is also higher in BALB/c mice than in C57BL/6 mice. As initiator B cells were only identified in the spleens of sensitized mice, and their frequency was relatively low, we compared the number of B-1 cells in the peritoneal cavity between BALB/c and C57BL/6 mice. However, further studies are needed to compare the number of oxazolone-specific initiator B cells in the spleen of sensitized BALB/c and C57BL/6 mice.

To investigate the role of IL-5 in causing differences in the number of B-1a cells and serum levels of hapten-specific IgM antibodies, we compared the responsiveness of BALB/c and C57BL/6 mice to IL-5. In vitro and in vivo studies revealed that IL-5 induced proliferation more strongly in BALB/c B-1 cells than in C57BL/6 B-1 cells. Moreover, intraperitoneal injection of rIL-5 induced a stronger IgM response in BALB/c mice than in C57BL/6 mice, indicating that BALB/c B-1 cells produce higher amounts of IgM antibodies than C57BL/6 B-1 cells in response to IL-5, since serum natural IgM antibodies are mostly derived from B-1 cells [17]. These results suggest

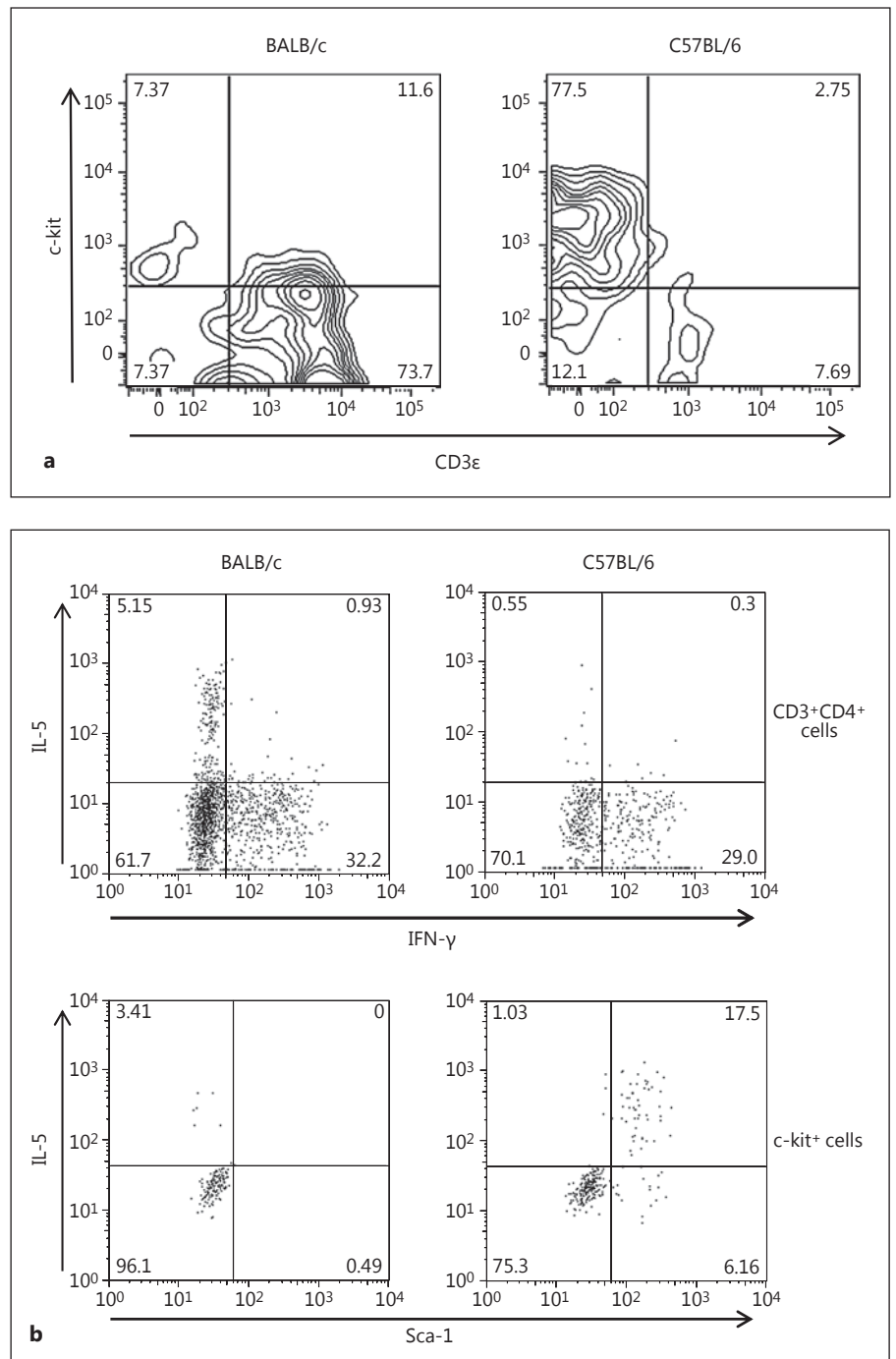


Fig. 7. IL-5-producing cells in the peritoneal cavity. **a** Peritoneal cells were harvested from IL-5/Venus KI mice, and expression of c-kit and CD3ε on Venus⁺ cells was assessed. **b** CD3⁺CD4⁺ cells (upper panels) and c-kit⁺Sca-1⁺ cells (lower panels) were purified from peritoneal cells, and were stimulated in vitro with PMA and ionomycin for 6 h. Expression of intracellular cytokines was assessed. Data are representative of 3 independent experiments.

that BALB/c B-1 cells are more sensitive to IL-5 than C57BL/6 B-1 cells. Because BALB/c and C57BL/6 B-1 cells express roughly comparable levels of IL-5Rα, strain-specific responsiveness to IL-5 might be due to the difference in tyrosine phosphorylation of cellular molecules in IL-5 signal transduction. Thus, we conclude that higher

numbers of B-1a cells and serum levels of hapten-specific IgM antibodies in BALB/c mice may be due to a higher responsiveness of BALB/c B-1 cells to IL-5 stimulation.

With regard to the source of IL-5 that maintains homeostasis of peritoneal B-1 cells, results obtained using IL-5/Venus KI mice and normal mice indicate that IL-5

is produced by distinct cell populations in BALB/c and C57BL/6 mice. We found that IL-5 was mainly produced by c-kit⁺ non-T cells in C57BL/6 mice, which is consistent with our previous findings [18]. In contrast, CD3⁺CD4⁺ cells are the dominant IL-5-producing cells in BALB/c mice. Although characterization of the IL-5-producing CD3⁺CD4⁺ cells has not yet been conducted, we speculate that they are Th2 cells. Further studies are needed to clarify whether c-kit⁺ non-T cells and CD3⁺CD4⁺ cells have the same potential to produce IL-5 and whether the difference in IL-5-producing cells affects susceptibility to CHS between the two strains of mice. Additionally, flow cytometric analysis of IL-5/Venus KI mice revealed that BALB/c mice had twice as many Venus⁺ cells as C57BL/6 mice, suggesting that the difference in IL-5 levels in the peritoneal cavity of BALB/c and C57BL/6 mice also accounts for the difference in numbers of B-1a cells and serum levels of hapten-specific IgM antibodies.

In conclusion, we propose an important role of IL-5 and initiator B cells in mouse strain-dependent susceptibility to hapten-induced CHS. These findings suggest that the IL-5/initiator B cell axis also contributes to the diverse susceptibility to allergic diseases in humans such as delayed-type hypersensitivity and nonatopic asthma.

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Disclosure Statement

The authors declare that no financial or other conflict of interest exists in relation to the content of this article.

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