

**Increased serum IgA in Fc $\alpha$ / $\mu$ R-deficient mice  
on the (129 x C57BL/6) F1 genetic background.**

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Keywords

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## Abstract

Fc $\alpha$ / $\mu$ R (CD351) is an Fc receptor for both IgA and IgM, which is abundantly expressed in the small intestine. However, the role of Fc $\alpha$ / $\mu$ R in the intestinal tissue is largely unknown. Here, we found that Fc $\alpha$ / $\mu$ R is highly expressed on follicular dendritic cells (FDCs) in Peyer's patches (PP) in the small intestine. Fc $\alpha$ / $\mu$ R-deficient mice on the (129 x C57BL/6) F1 background showed increased serum, but not fecal, IgA level in response to gut-oriented antigens. IgA<sup>+</sup> B cells were increased in PP, but not in the lamina propria, of Fc $\alpha$ / $\mu$ R-deficient mice, which was attenuated after reduction of commensal microbiota by oral treatment with antibiotics. Analyses of bone marrow chimeric mice, in which either FDCs or blood cells or both lack the expression of Fc $\alpha$ / $\mu$ R, suggested that FDCs, but not blood cells, were responsible for the increased serum IgA concentration in Fc $\alpha$ / $\mu$ R-deficient mice. Moreover, Fc $\alpha$ / $\mu$ R-deficient mice showed enhanced germinal center formation against commensal microbiota in PP. Thus, serum IgA production against gut-oriented antigens is negatively regulated by Fc $\alpha$ / $\mu$ R on FDCs in the F1 mice.

## 1. Introduction

IgA is an antibody produced most abundantly among the classes of immunoglobulins and mainly secreted to the mucosal lumen (Kerr et al, 1990; Macpherson et al, 2012). Secreted IgA prevents penetration of pathogens into the mucosal lumen, neutralizes toxins and pathogens (Monteiro & Van De Winkel, 2003), and anchors the pathogenic bacteria to the mucus, leading to down-modulation of their pro-inflammatory epitopes (Fernandez et al, 2003). In Peyer's patches (PP), antigen-stimulated B cells form germinal centers (GC) with T cell help, leading to class switch recombination to IgA (IgA-CSR) in the presence of IgA-CSR-inducing factors, such as TGF- $\beta$  and retinoic acid (Cazac & Roes, 2000). IgA<sup>+</sup> B cells then circulate through the bloodstream and home to the intestinal lamina propria via expressions of  $\alpha$ 4 $\beta$ 7 integrin and CCR9 (Mora & von Andrian, 2009). In addition, IgA<sup>+</sup> B cells are also generated in the intestinal lamina propria (Fagarasan & Honjo, 2004). These in situ IgA-CSR does not require T cells, but does DC (Litinskiy et al, 2002) or epithelial cells (Xu et al, 2007), which secrete IgA-CSR-inducing factors (He et al, 2007). IgA

produced in the lamina propria is transported and secreted into the mucosal lumen via binding to polymeric Ig receptor (pIgR) expressed on the mucosal epithelial cells (Kaetzel, 2005). Although the molecular mechanisms for IgA secretion into the mucosal lumen have well been studied, little is known about the maintenance of IgA in the sera.

Fc receptors are expressed on immune cells and play pivotal roles in immune responses. Several Fc receptors for IgG [Fc $\gamma$ RI (CD64), Fc $\gamma$ RII (CD32), Fc $\gamma$ RIII (CD16) and Fc $\gamma$ RIV] and IgE [Fc $\epsilon$ RI and Fc $\epsilon$ RII (CD23)] have been identified and shown to be pivotal for host immunity, allergy and autoimmune diseases (Ravetch, 1997), (Takai, 2002). We have previously identified an Fc receptor for both IgA and IgM (Fc $\alpha$ / $\mu$ R) (Shibuya et al, 2000). *Fcamr* that encodes Fc $\alpha$ / $\mu$ R was mapped on chromosome 1, in which other Fc receptor genes are closely located each other (Shibuya et al, 2000; Shimizu et al, 2001). Although Fc $\alpha$ / $\mu$ R is abundantly expressed in the small intestine (Sakamoto et al, 2001), its role in the mucosal tissue is largely unknown. Here, we found that Fc $\alpha$ / $\mu$ R was preferentially expressed on FDCs in PP and negatively regulate the serum IgA level in response to gut-oriented antigens.

## **2.Methods**

### **2.1 Mice**

Two lines of Fc $\alpha$ / $\mu$ R-deficient mice were generated, as described previously (Honda et al, 2009). One line was E14 ES origin and backcrossed to one generation to C57BL/6J mice [Fc $\alpha$ / $\mu$ R-deficient mice on (129 x C57BL/6) F1 genetic background]. Another line was BALB/c ES origin and then backcrossed to C57BL/6J mice for more than twelve generations [Fc $\alpha$ / $\mu$ R-deficient mice on BALB/c and C57BL/6 genetic backgrounds, respectively]. BALB/c and C57BL/6J mice were purchased from Clea Japan, inc. (Tokyo, Japan). These mice were maintained under the specific pathogen free (SPF) condition. For antibiotics treatment, mice were fed with drinking water containing ampicillin 1 g/L, neomycin 1 g/L, vancomycin 0.5 g/L and metronidazole 0.5 g/L for four weeks (Rakoff-Nahoum et al, 2004). All experiments were performed in accordance with the guidelines of the animal ethics committee of the University of Tsukuba Animal Research Center.

### **2.2 Antibodies**

Fc $\alpha$ / $\mu$ R-specific monoclonal antibody (mAb) (TX61) was established previously (Cho et al, 2006). TNP-specific mouse IgA (MOPC315) was purchased from Cappel (Solon, OH, USA). mAbs against B220 (RA3-6B2), CD21/35 (7G6), CD45 (30-F11), CD138 (281-2), IgA (C10-3), and T- and B- cell activating antigen (GL-7) were purchased from Pharmingen (San Diego, CA, USA).

### **2.3 Immunization**

Eight- to twelve-week-old Fc $\alpha$ / $\mu$ R-deficient mice of (129 x C57BL/6) F1 background and wild-type littermate were immunized p.o. via gastric tubes with 1 mg of ovalubumin (OVA, Sigma, St. Louis, MO, USA) mixed with 10  $\mu$ g of cholera toxin (List Biological Laboratories, Inc., Campbell, CA, USA) once a week for three consecutive weeks, or with 50  $\mu$ g of NP-LPS (Biosearch Technologies, Inc., Novato, CA, USA) once a week for five consecutive weeks. One week after the final immunization, sera and fecal contents were obtained, as described (Shi et al, 1999).

### **2.4 ELISA**

Serum and fecal antibody concentrations were measured by ELISA by using HRP-conjugated anti-mouse Ig isotype Abs (Southern Biotechnologies, Birmingham, AL, USA) and ABTS substrate (BioFX laboratories, Owings Mills, MD, USA). For measurement of anti-OVA Abs, plates were coated with OVA and serially diluted sera or fecal extract were added, and developed with HRP-conjugated anti-mouse IgA Ab. Ab titers were determined by the dilution factor which showed OD<sub>405</sub> twice as high as that of the background. For measurement of anti-NP Abs, plates were coated with NP-conjugated bovine serum albumin, and were developed with HRP-conjugated anti-mouse IgA Ab. For measurement of TNP-specific mouse IgA mAb concentration, plates were coated with TNP-conjugated OVA and were developed with HRP-conjugated anti-mouse IgA Ab.

## **2.5 Analyses for IgA clearance**

After injection of 50 µg TNP-specific mouse IgA (MOPC315) via tail vein, sera were collected at indicated time point, and measured for TNP-specific mouse IgA concentration by ELISA, as described above.

## **2.6 Cell preparation**

Cells were harvested from the bone marrow (BM), spleen, mesenteric lymph node (MLN), Peyer's patch (PP), peritoneal exudate cell (PEC) and lamina propria (LP) one week after intragastric injection of 100 µg of LPS. LP cells were prepared, as previously described with minor modification (Hurst et al, 1999). Briefly, after PPs were excised, intestines were cut into small pieces and digested with dithiothreitol (DTT), followed by collagenase type VIII (Sigma, St. Louis, MO, USA). Cells were collected from the digested tissues by using centrifugation with 100 %-40 % Percoll.

## **2.7 Flow cytometry analyses**

The cells were stained with cychrome-conjugated anti-B220 and PE-conjugated anti-CD138 mAbs, followed by FITC-conjugated anti-mouse IgA mAb after permeabilization with Fix & Perm kit (Invitrogen, Carlsbad, CA, USA). LP cells were stained with biotin-conjugated anti-CD45 mAb, followed by APC-conjugated streptavidin with cychrome-conjugated anti-B220 mAb. Cells were then permeabilized

and stained with FITC-conjugated anti-mouse IgA mAb. For analyses of germinal center (GC) B cells, cells were stained with FITC-conjugated GL7 mAb, PE-conjugated anti-B2200 mAb and biotin-conjugated-PNA, followed by APC-conjugated streptavidin. Cells were analyzed on a FACS Calibur (BD, Franklin Lakes, NJ, USA).

## **2.8 Immunohistochemistry**

For staining GC, frozen sections were stained with biotin-conjugated PNA, followed by Alexa594-conjugated streptavidin with FITC-conjugated anti-CD21/35 mAb. For staining Fc $\alpha$ / $\mu$ R, frozen sections were stained with biotin-conjugated anti-Fc $\alpha$ / $\mu$ R mAb (TX61), followed by HRP-conjugated streptavidin with TSA amplification reagent (PerkinElmer, Wellesley, MA, USA) in combination with APC-conjugated anti-CD21/35 mAb.

## **2.9 Bone marrow chimeric mice**

Bone marrow (BM) cells ( $1 \times 10^7$  cells) from Fc $\alpha$ / $\mu$ R-deficient mice or wild-type littermate mice were injected intravenously via tail vein into lethally

irradiated (2 x 0.55 Gy) Fc $\alpha$ / $\mu$ R-deficient or control mice. Eight weeks after BM cells transfer, the blood lymphocytes were analyzed to confirm the engraftment of donor BM cells and used for analyses.

## **2.10 Statistics**

Statistical analyses were performed by using unpaired Student's *t*-test. The Smirnov-Grubbs test was used to exclude outliers.

### 3. Results

#### 3.1 Increased serum IgA in Fc $\alpha$ / $\mu$ R-deficient mice on the (129 x C57BL/6) F1 genetic background.

Previous reported demonstrated that *Fcamr* transcript was abundantly expressed in the small intestine (Sakamoto et al, 2001). By using immunohistochemical analyses, we observed that Fc $\alpha$ / $\mu$ R was strongly expressed on FDCs in Peyer's patches (PP) of the small intestine (Fig. 1A). Since FDCs are involved in mucosal IgA production (Suzuki et al, 2010), IgA concentration in the serum and feces was analyzed. We found that serum IgA was significantly increased in Fc $\alpha$ / $\mu$ R-deficient mice on the (129 x C57BL/6) F1 genetic background compared with that of littermate control mice (Fig. 1B). In contrast, IgA in feces and other immunoglobulin isotypes in serum of Fc $\alpha$ / $\mu$ R-deficient mice were comparable to those of littermate control mice (Fig. 1C and data not shown). However, both Fc $\alpha$ / $\mu$ R-deficient mice on the BALB/c and C57BL/6 genetic backgrounds showed comparable serum IgA levels to respective control mice (Fig. 1B). These results suggested that serum IgA level of Fc $\alpha$ / $\mu$ R-deficient mice seems to be influenced by the genetic background of mice. For further analyses, we used

Fc $\alpha$ / $\mu$ R-deficient mice on the (129 x C57BL/6) F1 background and their littermates as a control. We speculated that serum IgA level was increased in response to gut-oriented antigens in Fc $\alpha$ / $\mu$ R-deficient mice. To test this possibility, IgA production was analyzed after oral immunization of OVA and NP-LPS, T cell-dependent and independent type antigens, respectively. In response to both OVA and NP-LPS, antigen-specific IgA titer in the sera was significantly increased in Fc $\alpha$ / $\mu$ R-deficient mice compared with that of control mice (Fig. 1D). In contrast, antigen-specific IgA titer in feces was comparable between Fc $\alpha$ / $\mu$ R-deficient and control mice (Fig. 1E). These results indicated that serum, but not fecal, IgA titers in response to gut-oriented antigens were increased in Fc $\alpha$ / $\mu$ R-deficient mice on the (129 x C57BL/6) F1 background. In the current study, we therefore focused on this genotype of mice to clarify how serum IgA is increased.

### **3.2 Normal IgA clearance in Fc $\alpha$ / $\mu$ R-deficient mice.**

In mice lacking pIgR or J chain, serum IgA is increased as a result of impaired IgA transfer from the blood to the bile (Johansen et al, 1999). We speculated that increased serum IgA level in Fc $\alpha$ / $\mu$ R-deficient mice might be a result of impaired IgA

clearance. To address this question, anti-TNP mouse IgA was intravenously injected into Fc $\alpha$ / $\mu$ R-deficient and control mice, and then measured for its concentration in the sera. Anti-TNP mouse IgA concentration was declined with time, and the half life in the sera was comparable between Fc $\alpha$ / $\mu$ R-deficient and control mice (Fc $\alpha$ / $\mu$ R-deficient mice, 14.7 hours and control mice, 13.3 hours, respectively) (Fig. 1F). Thus, the increased serum IgA in Fc $\alpha$ / $\mu$ R-deficient mice did not result from impaired IgA clearance from the serum. Rather, the production of IgA might be increased in Fc $\alpha$ / $\mu$ R-deficient mice.

### **3.3 Increased IgA<sup>+</sup> B cell populations in PP of Fc $\alpha$ / $\mu$ R-deficient mice.**

To determine whether IgA production in response to gut-oriented antigens is increased in Fc $\alpha$ / $\mu$ R-deficient mice, IgA<sup>+</sup> B cell population of lymphoid organs were analyzed by flow cytometry. We observed that IgA<sup>+</sup> B cell population (B220<sup>+</sup>, IgA<sup>+</sup>) was significantly larger in PPs in Fc $\alpha$ / $\mu$ R-deficient mice than those in control mice (Fig. 2A). In contrast, IgA producing plasma cell population (B220<sup>-</sup>, IgA<sup>+</sup>) in lamina propria was comparable between Fc $\alpha$ / $\mu$ R-deficient and control mice (Fig. 2A). We assumed that IgA<sup>+</sup> B cell generation might be increased in PP in response to commensal

microbiota in the gut of  $Fc\alpha/\mu R$ -deficient mice. To address this issue, commensal microbiota was reduced by oral treatment with antibiotics (Rakoff-Nahoum et al, 2004). As expected, serum IgA levels were down-regulated after antibiotics treatment in  $Fc\alpha/\mu R$ -deficient mice (Fig. 2B). Moreover,  $IgA^+$  B cell population in PPs of  $Fc\alpha/\mu R$ -deficient mice was comparable to those of control mice after antibiotics treatment (Fig. 2C). These results demonstrated that the increased  $IgA^+$  B cell population in PP of  $Fc\alpha/\mu R$ -deficient mice was dependent on gut commensal microbiota.

#### **3.4 $Fc\alpha/\mu R$ expressed on FDCs negatively regulate serum IgA level.**

$Fc\alpha/\mu R$  was expressed most abundantly on FDCs, but also expressed on hematopoietic cells such as B cells and macrophages (Sakamoto et al, 2001). To determine the cell type responsible for the increased serum IgA level in  $Fc\alpha/\mu R$ -deficient mice, we established bone marrow (BM) chimeric mice by transferring BM cells of  $Fc\alpha/\mu R$ -deficient or control mice into lethally irradiated  $Fc\alpha/\mu R$ -deficient or control mice. Since FDCs are radio-resistant cell population, FDCs

are derived from recipient mice, whereas hematopoietic cells of BM chimeric mice are derived from transferred BM cells. We observed that only BM chimeric mice, whose FDCs, but not hematopoietic cells, lack Fc $\alpha$ / $\mu$ R expression, showed increased IgA in the sera (Fig. 3A). Thus, Fc $\alpha$ / $\mu$ R on FDCs is involved in the increased serum IgA in response to gut-oriented antigens.

### **3.5 Enhanced germinal center formation in response to commensal microbiota in Fc $\alpha$ / $\mu$ R-deficient mice.**

Since FDCs contribute the generation of IgA<sup>+</sup> B cell in germinal center (GC) of PP in the presence of commensal microbiota (Suzuki et al, 2010), we analyzed GC formation in Fc $\alpha$ / $\mu$ R-deficient mice. Immunohistochemical analyses showed that CD21/35<sup>+</sup> PNA<sup>+</sup> GC area was enlarged in PPs of Fc $\alpha$ / $\mu$ R-deficient mice compared with control mice (Fig. 3B). These results were confirmed by flow cytometry analyses, showing that GL7<sup>+</sup> PNA<sup>+</sup> GC B cell population was larger in PP of Fc $\alpha$ / $\mu$ R-deficient mice than in control mice (Fig. 3C). In addition, enhanced GC formation in PP of Fc $\alpha$ / $\mu$ R-deficient mice was diminished after reduction of commensal microbiota by

antibiotics treatment (Fig. 3D and 3E). Thus, the increased serum IgA level in  $Fc\alpha/\mu R$ -deficient mice seemed to result from increased germinal center formation in response to gut-oriented antigens.

### 3. Discussion

We observed that naïve  $Fc\alpha/\mu R$ -deficient mice on the (129 x B6) F1 background, but not on the BALB/c or C57BL/6 genetic backgrounds, showed increased serum IgA level. It was previously reported that immune responses, including antibody production, are influenced by genetic background of mice (Dorf et al, 1974) (Lipes et al, 2002). Along with this notion, involvement of  $Fc\alpha/\mu R$  in serum IgA level seemed to be influenced by the genetic background of mice. Although we found that  $Fc\alpha/\mu R$  on FDCs was involved in the increased serum IgA (Fig. 3A), the expression of  $Fc\alpha/\mu R$  on FDCs in PP was comparable among mice on the (129 x B6) F1, BALB/c and C57BL/6 genetic backgrounds (Fig. 1A and data not shown). Thus, differential involvement of  $Fc\alpha/\mu R$  in serum IgA level in each mice strain could not be explained by  $Fc\alpha/\mu R$  expression on FDCs in PP. The molecular basis for this phenomenon has remained unclear.

$Fc\alpha/\mu R$  and  $pIgR$  are receptors for both IgA and IgM, which were expressed in the intestine. In addition, their genes locate very closely on chromosome 1 (Shibuya et al, 2000). However, previous work demonstrated that  $Fc\alpha/\mu R$  and  $pIgR$  are

differentially expressed in mucosal tissue, suggesting the different roles in this site (Wang et al, 2009). Although mice lacking pIgR showed impaired IgA clearance from the blood to the bile (Johansen et al, 1999), our analyses indicated that Fc $\alpha$ / $\mu$ R does not contribute to IgA clearance from the blood. Rather, Fc $\alpha$ / $\mu$ R seems to regulate serum IgA levels in response to gut-oriented antigens.

IgA<sup>+</sup> B cells are generated in the presence of IgA-CSR-inducing factors in PP, and among those factors, retinoic acid induces the expression of  $\alpha$ 4 $\beta$ 7 integrin and CCR9 on B cells surface (Mora & von Andrian, 2009). IgA<sup>+</sup> B cells then circulate through the bloodstream and home to the intestinal lamina propria via  $\alpha$ 4 $\beta$ 7 and CCR9 expression. In Fc $\alpha$ / $\mu$ R-deficient mice, IgA<sup>+</sup> B cells were increased in PP, but not in lamina propria, which is a dominant site for IgA production and secretion into feces. These results indicated that the generation of IgA<sup>+</sup> B cells in PP was increased, whereas the homing of IgA<sup>+</sup> B cells to lamina propria was not affected, in Fc $\alpha$ / $\mu$ R-deficient mice. As a result, Fc $\alpha$ / $\mu$ R-deficient mice showed increased serum IgA with normal fecal IgA in response to intestinal microbiota. Recently, it was reported that FDCs directly secrete IgA-CSR-inducing factors in response to gut microbiota for production of IgA<sup>+</sup> B cells

in PP (Suzuki et al, 2010). We speculate that Fc $\alpha$ / $\mu$ R on FDCs in mice on the (129 x B6) F1 genetic background regulates production of IgA-CSR-inducing factors, except retinoic acid, in response to gut-oriented antigens in PP. In addition to cytokine secretion, FDCs capture and retain antigens for B cells activation (Heesters et al, 2014). We have shown that the retention of systemic administered T-independent antigen on splenic FDCs was prolonged in the Fc $\alpha$ / $\mu$ R-deficient mice (Honda et al, 2009). Therefore, we also speculate that retention of gut-oriented antigens on FDCs in PP is increased in the absence Fc $\alpha$ / $\mu$ R, resulted in increased GC formation and IgA-CSR. The mechanisms how Fc $\alpha$ / $\mu$ R on FDCs regulates production of IgA-CSR-inducing factors, and retention of gut-oriented antigens should be clarified in future.

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## Figure Legends

### Figure 1. Increased serum IgA in Fc $\alpha$ / $\mu$ R-deficient mice.

(A) Immunohistochemical analysis of Peyer's patches of naïve Fc $\alpha$ / $\mu$ R-deficient on the (129 x B6) F1 genetic background (-/-) and its littermate control (+/+). (B and C) IgA concentration of serum (B) and feces (C) of naïve Fc $\alpha$ / $\mu$ R-deficient and littermate control of the F1, BALB/c, or C57BL/6 genetic backgrounds. (D and E) Antigen-specific IgA titers in the serum (D) and feces (E) in -/- and +/+ mice after oral immunization with OVA or NP-LPS, respectively. (F) The serum IgA clearance in -/- and +/+ mice. n.s, not significant.

### Figure 2. Increased IgA<sup>+</sup> B cell population in lymphoid organs of Fc $\alpha$ / $\mu$ R-deficient mice.

(A and C) IgA<sup>+</sup> B cell populations of bone marrow (BM), spleen, mesenteric lymph node (MLN), Peyer's patches (PP), peritoneum (PEC) and lamina propria (LP) in Fc $\alpha$ / $\mu$ R-deficient (-/-) on the F1 genetic background and littermate control mice (+/+) before (A) and after (C) oral antibiotics treatment. Data are shown as percentages of

IgA<sup>+</sup> B cells among total B220<sup>+</sup> B cells. **(B)** IgA concentration in the serum of -/- and +/+ mice after oral antibiotics treatment. n.s, not significant.

**Figure 3. Fcα/μR on FDCs negatively regulates germinal center formation of PP.**

**(A)** Serum IgA concentration of naïve bone marrow chimeric mice, which were generated by transfer of bone marrow cells from Fcα/μR-deficient mice (-/-) on the F1 genetic background or littermate control (+/+) into lethally irradiated -/- or +/+ mice. **(B)** to **(E)** Germinal center (GC) formation. Immunohistochemical analyses of PP of -/- or +/+ mice before **(B)** and after **(D)** oral antibiotics treatment. The white arrowhead indicates GC. The flow cytometry analysis of PP cells obtained from -/- or +/+ mice before **(C)** and after **(E)** oral antibiotics treatment. The percentage of GC B cells (GL7<sup>+</sup>PNA<sup>+</sup>B220<sup>+</sup>) among B cells (B220<sup>+</sup>) was shown. n.s, not significant.

Fig. 1

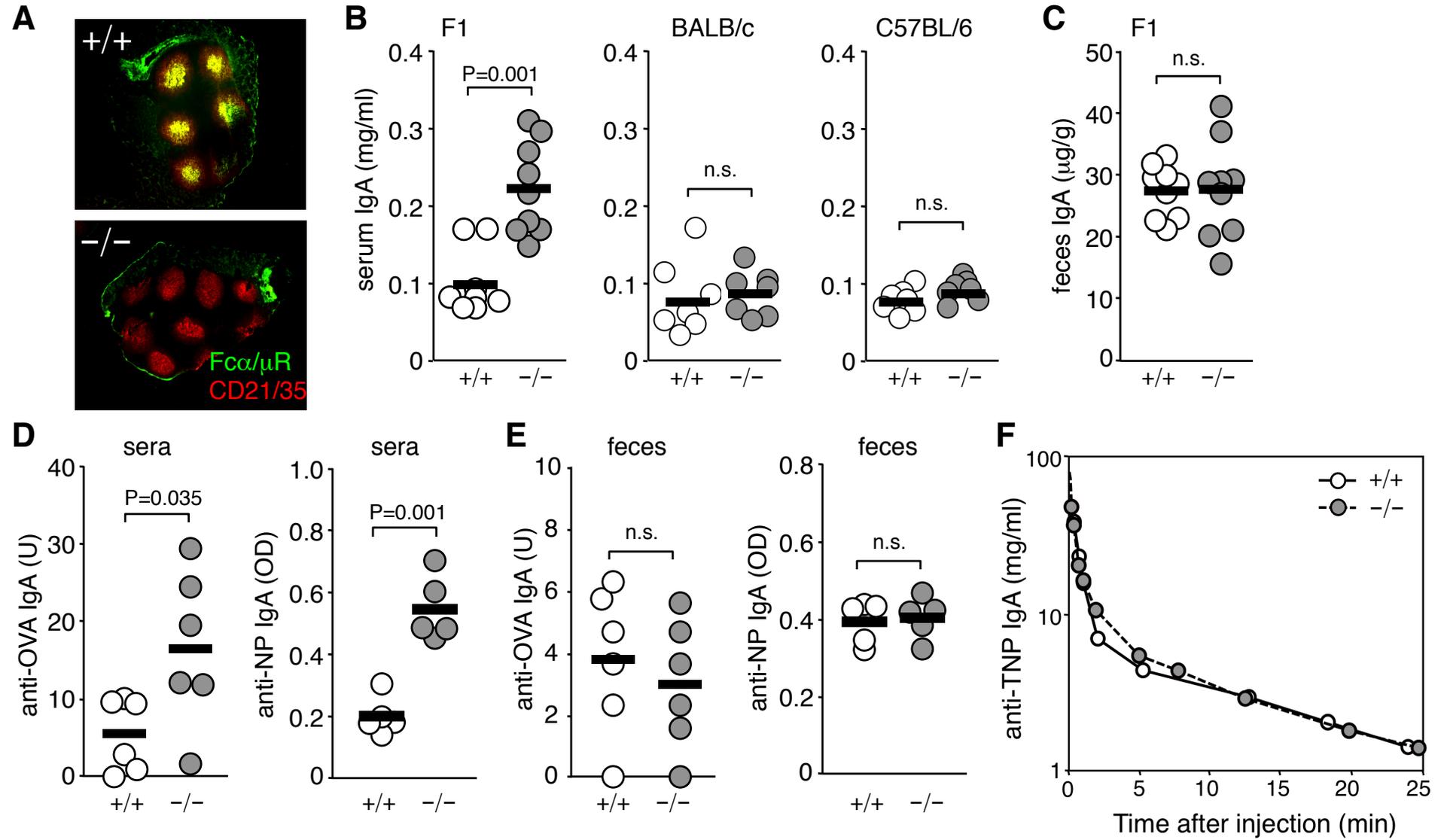


Fig. 2

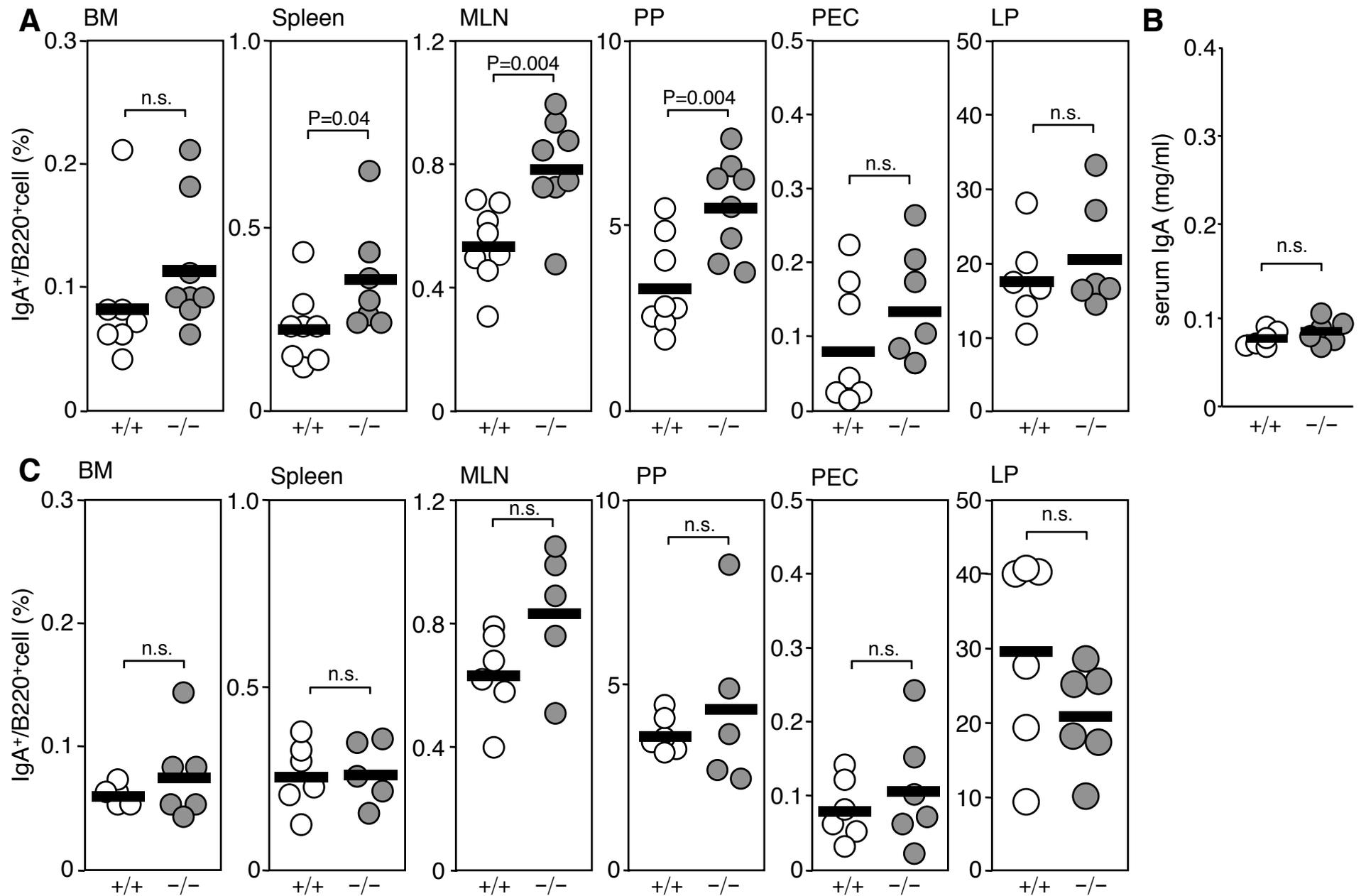


Fig. 3

