



Allergy inhibitory receptor-1 inhibits autoantibody production via upregulation of apoptotic debris clearance by macrophages

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Abstract

Aim: Allergy inhibitory receptor-1 (Allergin-1) is a newly identified immune regulatory molecule thought to influence autoantibody production. Autoantibody production, like that observed in Allergin-1-deficient mice, is crucial in the pathogenesis of several autoimmune diseases such as systemic lupus erythematosus. The purpose of this study is to clarify the regulatory role of Allergin-1-mediated autoantibody production using a murine model of thymocytic anaphylaxis.

Methods: C57BL/6 (WT) and Allergin-1-deficient mice were treated with apoptotic cells from naive thymocytes stimulated by dexamethasone. Antibody titers of total or immunoglobulin G (IgG) subclass of anti-double-stranded DNA (anti-dsDNA) and anti-histone antibody from serum were measured using an enzyme-linked immunosorbent assay. Macrophages from wild-type (WT) or Allergin-1-deficient mice were co-cultured with fluorescence-labeled apoptotic thymocytes or fluorogenic reagent and resultant phagocytic activity was quantified by with flow cytometry.

Results: After apoptotic cells injection, antibody titers of total and IgG3 anti-dsDNA and total anti-histone from serum were significantly increased in Allergin-1-deficient versus WT mice. Phagocytic activity was significantly lower in macrophages from Allergin-1-deficient mice versus WT mice.

Conclusion: Allergin-1 might play an inhibitory role in autoantibody production via upregulation of macrophage phagocytosis.

KEYWORDS

Allergin-1, autoantibody, lupus nephritis, phagocytosis, systemic lupus erythematosus

1 | INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by high levels of autoantibody production, accumulation of immune complexes and multiple organ system involvement.¹⁻³

However, the etiology and pathogenic mechanisms of SLE have yet to be clearly elucidated.

Myeloid-derived cells such as macrophages, dendritic cells and neutrophils were found to be involved in the pathogenesis of SLE.⁴ As working phagocytes, macrophages are important for homeostatic

maintenance by removal of apoptotic debris.⁵ On the other hand, phagocytic dysfunction in macrophages results in SLE-like phenotypes including autoantibody production and accumulation of immune complexes.^{6,7} This idea is bolstered by a study in which autoantibody production was observed in the serum of wild-type (WT) mice injected with a large number of apoptotic cells.⁸ In SLE patients, a similar accumulation of apoptotic cells and resulting inflammation can also be detected in sites such as the kidneys.^{9,10} Thus, the role of macrophage dysfunction in the failure to clear immunogenic apoptotic debris might be an important factor in the relentless pathogenesis of SLE phenotypes.

Allergy inhibitory receptor-1 (Allergin-1) is a newly identified immune regulatory molecule found in cells of myeloid lineage (mast cells, macrophages, dendritic cells and neutrophils) that contains an immunoreceptor tyrosine-based inhibitory motif (ITIM)-like domain.¹¹ It suppresses immunoglobulin E (IgE)-mediated degradation in mast cells and inhibits systemic and passive cutaneous anaphylaxis in vivo.¹¹ Another report showed that it also suppresses mast cell Toll-like receptor (TLR2) signaling in a TLR2-mediated dermatitis mouse model.¹² Although Allergin-1 was shown to suppress several immune reactions, such as inflammatory cytokine production in mast cells, the function of Allergin-1 in macrophages remains unknown.

In this study, we show that serum anti-double-stranded DNA (anti-dsDNA) and anti-histone antibodies are significantly increased in Allergin-1-deficient mice compared with WT mice after injection of apoptotic cells. Macrophages from Allergin-1-deficient mice had lower phagocytic activity compared to those from WT mice. These results suggest that Allergin-1 is an inhibitory molecule for autoantibody production via promotion of apoptotic cell clearance by macrophages.

2 | MATERIALS AND METHODS

2.1 | Mice

Female C57BL/6 (WT) mice, 8-12 weeks old, were obtained from Charles River Laboratory (Wilmington, MA, USA). Allergin-1-deficient mice were kindly provided by ONO Pharmaceutical Company, Ltd. (Osaka, Japan). Allergin-1-deficient mice are from a C57BL/6 background. The Committee on Institutional Animal Care and Use of the University of Tsukuba approved all experimental protocols.

2.2 | Apoptotic cells injection model

Thymocytes were isolated from WT mice and apoptosis was induced via 10 nmol/L dexamethasone treatment (Wako, Osaka, Japan) for 20 hours in 6 cm dishes (Techno Plastic Products, Trasadingen, Switzerland) containing RPMI medium with 5% fetal bovine serum (FBS: Sigma-Aldrich, St Louis, MO, USA). Apoptotic cells (1×10^7) in phosphate-buffered saline (PBS) were injected into WT or Allergin-1-deficient mice via the tail vein. Apoptotic cells were injected once every 7 days for 28 days (4 weeks) then once every 14 days (2 weeks) until sampling time points.

2.3 | Enzyme-linked immunosorbent assay (ELISA)

Anti-dsDNA and anti-histone antibody titers in serum were measured every 2 weeks for 12 weeks after injection of apoptotic cells by ELISA. For the measurement of anti-dsDNA antibodies, 96-well plates were first coated with 10 µg/mL protamine sulfate (Wako) diluted in carbonate buffer for 1 hour at room temperature (RT). Next, a second coating was done with 5 µg/mL ds-DNA (Sigma-Aldrich) diluted in carbonate buffer for 1 hour at RT. Plates were blocked in PBS containing 5% FBS (Sigma-Aldrich) for 1 hour at RT. Serum diluent (100 × dilution in PBS 1 × 5% FBS) from experimental mice was applied to the coated plates for 2 hours at RT. Anti-mouse IgG-horseradish peroxidase (HRP: Dako, Carpinteria, CA, USA), anti-mouse IgG1-HRP (Bethyl, Montgomery, TX, USA), anti-mouse IgG2a-HRP (Bethyl), anti-mouse IgG2b-HRP (Bethyl) and anti-mouse IgG3-HRP (Bethyl) detection antibodies were used as secondary antibodies. These antibodies were applied for 2 hours at RT then SureBlue™ TMB 1-Component Microwell Peroxidase Substrate (SeraCare Life Sciences, Milford, MA, USA) was added and incubated at RT for 15 minutes and 1 mol/L sulfuric acid (Wako) was used as a stop solution. For anti-histone antibody assays, 10 µg/mL histone (Roche Diagnostics, Basel, Switzerland) diluted in carbonate buffer, was used to coat plates for 3 hours at RT. After 1-hour blocking in PBS 1 × 5% FBS, the serum diluent (100× dilution in PBS 1 × 5% FBS) derived from experimental mice was applied to the coated

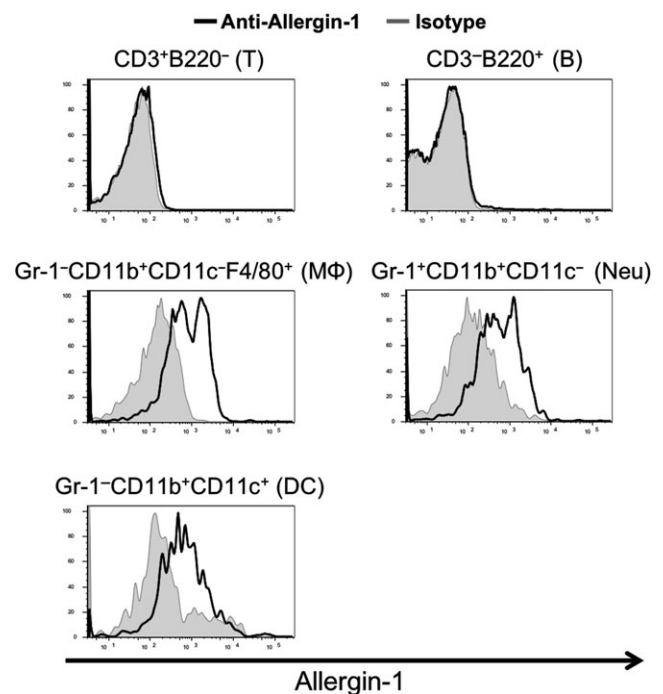


FIGURE 1 The expression of Allergin-1 on splenocytes. Splenocytes were harvested from naïve wild-type (WT) mice. T cells (T), B cells (B), macrophages (MΦ), neutrophils (Neu) and dendritic cells (DC) were stained with the following antibodies: anti-CD3 mAb, anti-B220 mAb, anti-CD11b mAb, anti-Gr-1 mAb, anti-CD11c mAb, anti-Allergin-1 (TX83), and isotype controls. Data are representative of 3 independent experiments

plates for 2 hours at RT. Anti-mouse IgG-HRP (Dako), anti-mouse IgG1-HRP (Bethyl), anti-mouse IgG2a-HRP (Bethyl), anti-mouse IgG2b-HRP (Bethyl) and anti-mouse IgG3-HRP (Bethyl) detection antibodies were used as secondary antibodies. These antibodies were applied for 2 hours at RT then SureBlue™ TMB 1-Component Microwell Peroxidase Substrate (SeraCare Life Sciences) was added and incubated at RT for 15 minutes. 1M sulfuric acid (Wako) was used as a stop solution. The absorbance (optical density [OD] at 450 nm) in each of the samples was measured with an ARVO MX™ plate reader (PerkinElmer, Waltham, MA, USA). The serum of MRL/MpJ-Fas^{lpr/lpr} mice over 20 weeks of age (Japan SLC, Shizuoka, Japan) was used as a positive control.

2.4 | Expression of Allergen-1 on splenocytes and macrophages

The spleens, inguinal lymph nodes, livers and kidneys of naïve WT mice were harvested and incubated in PBS containing 2 mg/mL collagenase D (Roche Diagnostics) for 30 minutes at 37°C. Peritoneal macrophages were collected from WT mice via intraperitoneal

administration (i.p.) of 2 mL of 3% thioglycollate medium (Becton Dickinson, Franklin Lakes, NJ, USA). After 3 days, peritoneal cavity cells (PECs) were collected and stained with the following monoclonal antibodies: anti-mouse CD16/32, anti-F4/80-FITC (fluorescein isothiocyanate), anti-CD11b-FITC, anti-Gr-1-PE, anti-B220-PE/Cy7, anti-CD3-PerCP/Cy5.5, anti-CD11b-APC/Cy7, anti-CD11c-APC/Cy7 and streptavidin-APC (all from Biolegend, San Diego, CA, USA). Anti-Allergen-1 antibodies (TX83; mouse IgG1 [provided by the Department of Immunology, Faculty of Medicine, University of Tsukuba]) were biotinylated with a Biotinylation Kit (Dojindo, Kumamoto, Japan). Analysis was performed using fluorescence-assisted flow cytometry (FACS) Verse flow cytometer (Becton Dickinson) and FlowJo software (FlowJo, Ashland, OR, USA).

2.5 | Apoptotic cell clearance in vitro

Thioglycollate-elicited PECs (1×10^7 cells) from WT and Allergen-1-deficient mice were adhered overnight on 6 cm dishes (Techno Plastic Products) in RPMI-1640 medium containing 10% FBS (Sigma-Aldrich). The remaining adherent macrophages were then collected

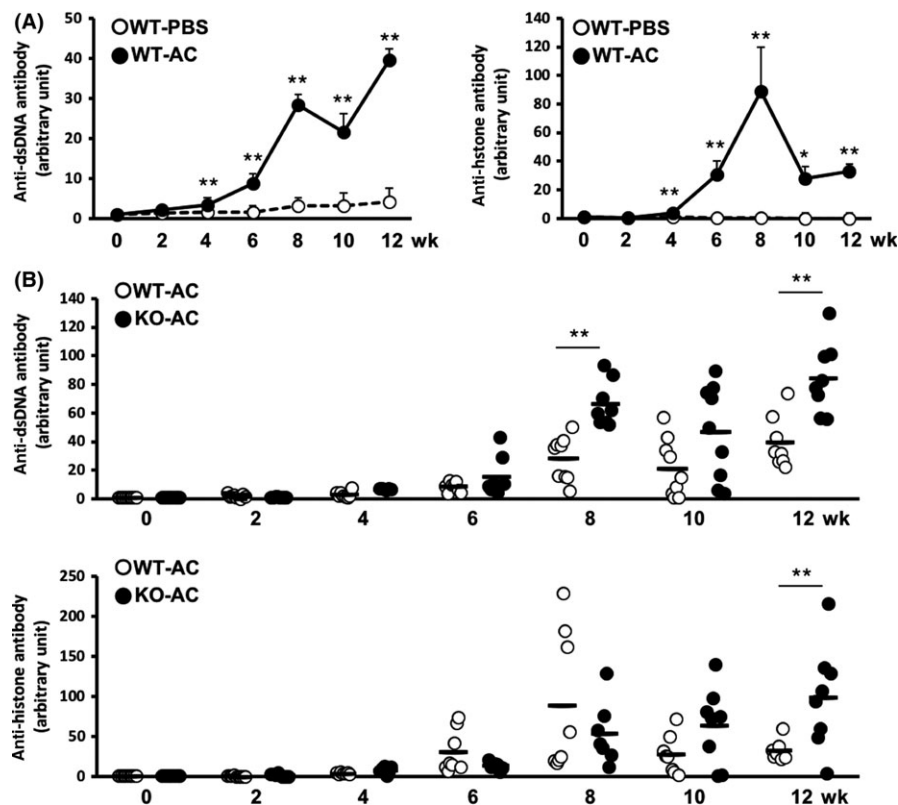


FIGURE 2 The titers of autoantibodies after apoptotic cells injection. A, At 0, 2, 4, 6, 8, 10 and 12 weeks after injection with either phosphate-buffered saline (PBS: white circle, $n = 7$) or apoptotic cells (AC: black circle, $n = 7$), the titers of total immunoglobulin G (IgG) anti-double-stranded DNA (anti-dsDNA) antibodies (left panel) and anti-histone antibodies (right panel) were measured using enzyme-linked immunosorbent assay (ELISA). B, Wild-type (WT) mice (white circle, $n = 7$) or Allergen-1-deficient mice (black circle, $n = 8$) were treated with AC. After 0, 2, 4, 6, 8, 10 and 12 weeks, the titers of total IgG anti-dsDNA antibodies (upper panel) and anti-histone antibodies (lower panel) were measured using ELISA. WT, C57BL/6 mice; KO, Allergen-1-deficient mice; arbitrary unit, data are shown as a ratio of anti-dsDNA and anti-histone antibodies compared with those in 0 weeks. The value of 0 weeks mice is indicated as 1.0. Data were pooled with 2 independent experiments. Data are means $\pm$ SEM; * $P < 0.05$, ** $P < 0.01$

and stained with anti-F4/80-FITC and anti-CD11b-APC/Cy7 (all from Biolegend) antibodies. CD11b⁺F4/80⁺ macrophages were sorted using a MoFlo XDP cell sorter (Beckman Coulter, Brea, CA, USA), and these cells were seeded to adhere overnight on 48-well plates (2×10^5 cells, Thermo Fisher Scientific) in RPMI-1640 medium containing 10% FBS (Sigma-Aldrich). The remaining adherent cells were then washed with PBS and 200 μ L of pHrodo[®] Green Zymosan Bioparticles[®] Conjugate for Phagocytosis (Thermo Fisher Scientific) was applied. After 0, 30 and 60 minutes, fluorescent CD11b⁺F4/80⁺ macrophages were analyzed using a FACSVerse flow cytometer (Becton Dickinson) and data were processed using FlowJo software. To collect apoptotic cells, thymocytes from WT mice were treated with 10 nmol/L dexamethasone (Wako) for 14 hours and 10 μ mol/L Cell Tracker-FITC (Life Technologies, Carlsbad, CA, USA) in RPMI-1640 medium containing 5% FBS (Sigma-Aldrich). After 6 hours, apoptotic cell fractions (Annexin V⁺PI⁻) were sorted using a MoFlo XDP cell sorter (Beckman Coulter) according to the manufacturer's instructions. CD11b⁺F4/80⁺ peritoneal macrophages were prepared as described earlier and these cells were adhered in 24-well plates (2×10^5 cells, Thermo Fisher Scientific) in RPMI-1640 medium containing 10% FBS (Sigma-Aldrich) and left overnight. The remaining adherent cells were washed with PBS and co-cultured with CellTracker[™] Green CMFDA Dye⁺-apoptotic cells (1×10^6 cells, Thermo

Fisher Scientific) in RPMI-1640 medium containing 10% FBS (Sigma-Aldrich). Inguinal lymph nodes, livers and kidneys from naïve WT and Allergin-1-deficient mice were incubated in PBS containing 2 mg/mL collagenase D (Roche Diagnostics) for 30 minutes at 37°C. Isolated cells were stained with anti-F4/80-FITC and anti-CD11b-APC/Cy7 antibodies (all from Biolegend). These cells (1×10^5 cells) were applied to 48-well plates (1×10^5 cells, Thermo Fisher Scientific) and co-cultured with CellTracker[™] Green CMFDA Dye⁺-apoptotic cells (5×10^5 cells, Thermo Fisher Scientific) in RPMI-1640 medium containing 10% FBS (Sigma-Aldrich). After 0, 30 and 60 minutes, CellTracker[™] Green CMFDA Dye⁺-apoptotic cells within CD11b⁺F4/80⁺ cells were quantified using a BD FACS Verse flow cytometer (Becton Dickinson) and data were processed using FlowJo software.

2.6 | Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics version 22 (IBM, Armonk, NY, USA). Statistical differences between the 2 groups were calculated with Student's *t* test. For statistical analyses, outliers were excluded using exploratory data analysis (EDA). *P* values of less than 0.05 were considered statistically significant. Data are presented as the mean $\pm$ SD where appropriate.

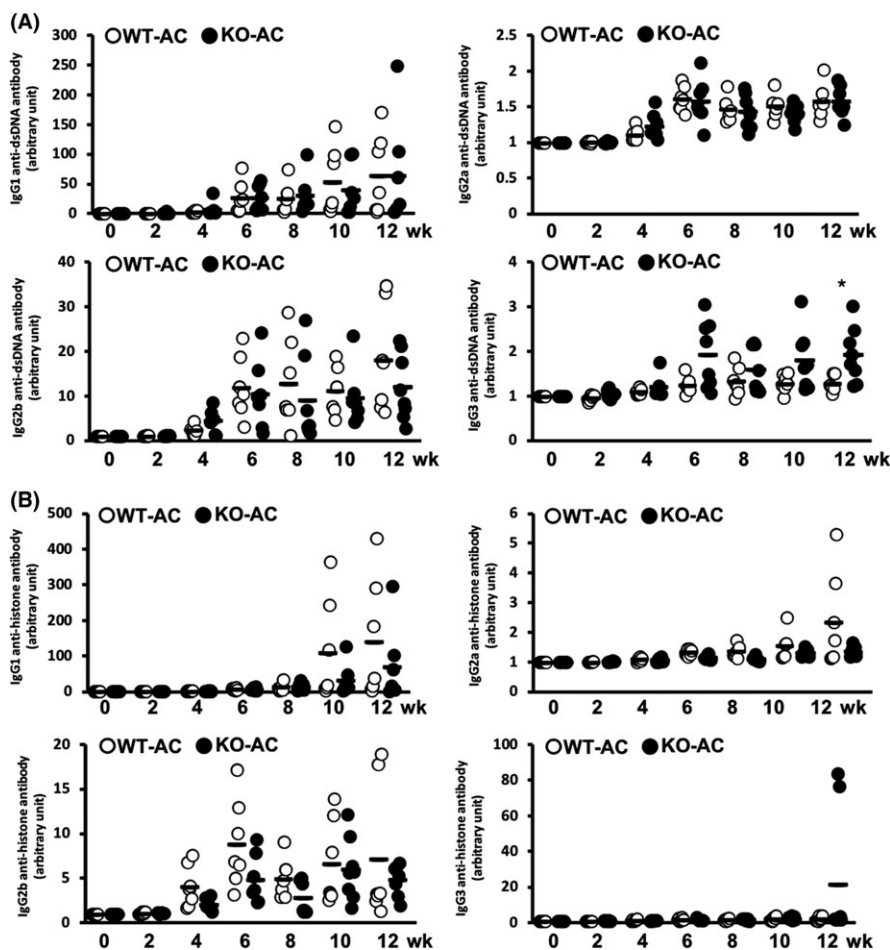


FIGURE 3 The titers of immunoglobulin G (IgG) subclass after apoptotic cells injection. A, Wild type (WT; white circle, *n* = 7) and Allergin-1-deficient mice (black circle, *n* = 8) were treated with apoptotic cells (AC). After 0, 2, 4, 6, 8, 10 and 12 weeks, the titers of IgG1 (upper left panel), IgG2a (upper right panel), IgG2b (lower left panel) and IgG3 (lower right panel) anti-double-stranded DNA (anti-dsDNA) antibodies were measured using enzyme-linked immunosorbent assay (ELISA). B, WT (white circle, *n* = 7) and Allergin-1-deficient mice (black circle, *n* = 8) were treated with AC. After 0, 2, 4, 6, 8, 10 and 12 weeks, the titers of IgG1 (upper left panel), IgG2a (upper right panel), IgG2b (lower left panel) and IgG3 (lower right panel) anti-histone antibodies were measured using ELISA. WT, C57BL/6 mice; KO, Allergin-1-deficient mice; arbitrary unit, data are shown as a ratio of anti-dsDNA and anti-histone antibodies compared with those in 0 weeks. The value of 0 weeks mice is indicated as 1.0. Data were pooled with two independent experiments. Data are means $\pm$ SEM; **P* < 0.05

3 | RESULTS

3.1 | Allergin-1 was expressed on myeloid cells

Splenocytes were analyzed with FACS to clarify which cell types expressed Allergin-1. Allergin-1 was found to be expressed on macrophages, dendritic cells, and neutrophils (Figure 1); however, it was not expressed on T cells or B cells.

3.2 | Allergin-1-deficient mice serum showed high autoantibody titer

To examine whether Allergin-1 affects autoantibody production after apoptotic cell injection, the antibody titers of anti-dsDNA and anti-histone from serum were measured using ELISA. In WT mice, total IgG anti-dsDNA and anti-histone antibodies increased at 4, 6, 8, 10 and 12 weeks after injection (Figure 2A). In Allergin-1 deficient mice, total anti-dsDNA antibodies were significantly increased at 8 and 12 weeks and total anti-histone antibodies were significantly increased at 12 weeks compared with WT mice (Figure 2B). Furthermore, we examined the subtypes of IgG in these autoantibodies. As shown in Figure 3A, serum IgG3 anti-dsDNA antibodies were significantly increased at 12 weeks in Allergin-1-deficient mice compared with WT mice, whereas IgG1, IgG2a, and IgG2b anti-dsDNA antibodies were not. However, there was no significant difference in IgG1, IgG2a, IgG2b, and IgG3 anti-histone antibodies between Allergin-1-deficient and WT mice (Figure 3B).

3.3 | Allergin-1-deficient macrophages had decreased phagocytic activity

To examine the effect of Allergin-1 in phagocytic activities in vitro, macrophages from the peritoneal cavity, inguinal lymph nodes, liver, and kidney were co-cultured with either fluorescence labeled-apoptotic cells or reagents that fluoresced in phagosomes. We confirmed that Allergin-1 was expressed on macrophages from the peritoneal cavity, inguinal lymph nodes, liver and kidney (Figure 4). As shown in Figure 5A,B, the phagocytic activity was significantly lower in macrophages from Allergin-1-deficient mice than those in WT mice at 30 and 60 minutes.

4 | DISCUSSION

In this study we examined the role of Allergin-1 in apoptosis-mediated autoantibody production. Allergin-1 was expressed on macrophages, dendritic cells and neutrophils. After injection of apoptotic cells, the antibody titers of total IgG, anti-dsDNA and anti-histone in serum were significantly increased in Allergin-1-deficient mice compared with WT mice. Additionally, macrophages from Allergin-1-deficient mice had low phagocytic activity compared with WT mice. Collectively, these results indicate that Allergin-1 might play an inhibitory role in autoantibody production through the upregulation

of phagocytosis in the macrophages responsible for removing apoptotic cell debris.

Autoantibody production is crucial in the pathogenesis of autoimmune diseases such as SLE. Autoantibodies such as anti-dsDNA, anti-histone and anti-nuclear antibodies are increased in the sera of SLE patients.¹³ To corroborate this, we show here that an injection of a large number of apoptotic cells induced anti-dsDNA and anti-histone antibodies in WT mice. Furthermore, anti-dsDNA and anti-histone antibody titers were significantly increased in Allergin-1-deficient mice compared with WT mice. These results indicated that Allergin-1 suppressed autoantibody production through upregulation of macrophage phagocytosis of apoptotic debris. In addition to this result, the titers of anti-dsDNA and anti-histone antibodies

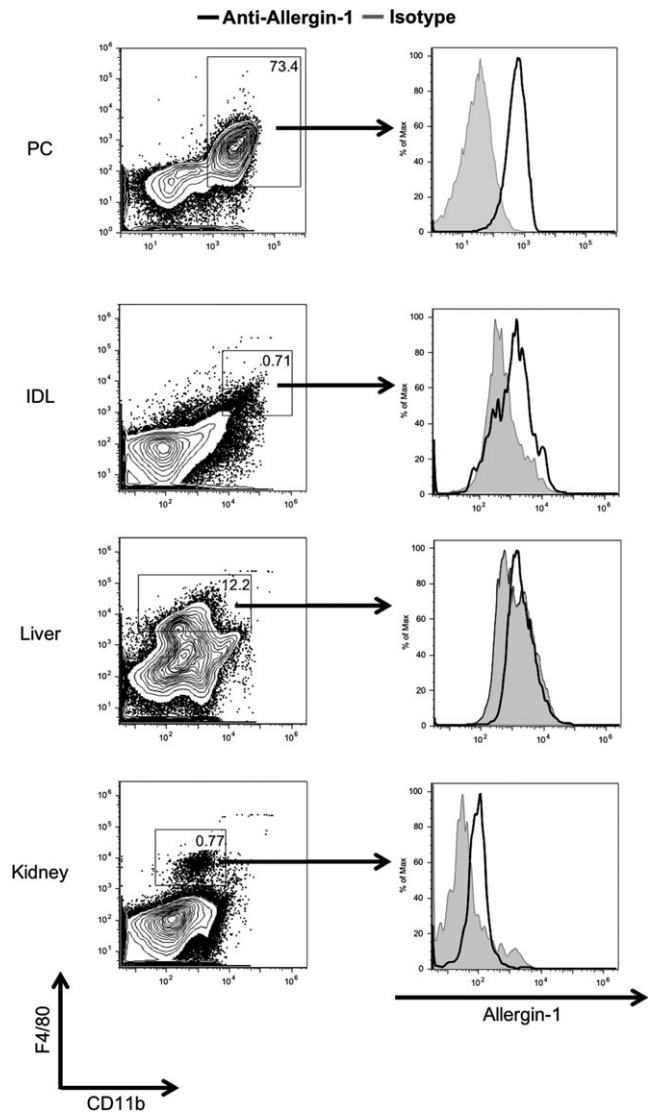


FIGURE 4 The expression of Allergin-1 on macrophages. Macrophages from peritoneal cavity (PC), inguinal lymph nodes (IDL), liver and kidney were harvested from wild-type (WT) mice. Macrophages were stained with the following antibodies: anti-CD11b mAb, anti-F4/80 mAb, anti-Allergin-1 (TX83), and isotype controls. Data are representative of two independent experiments

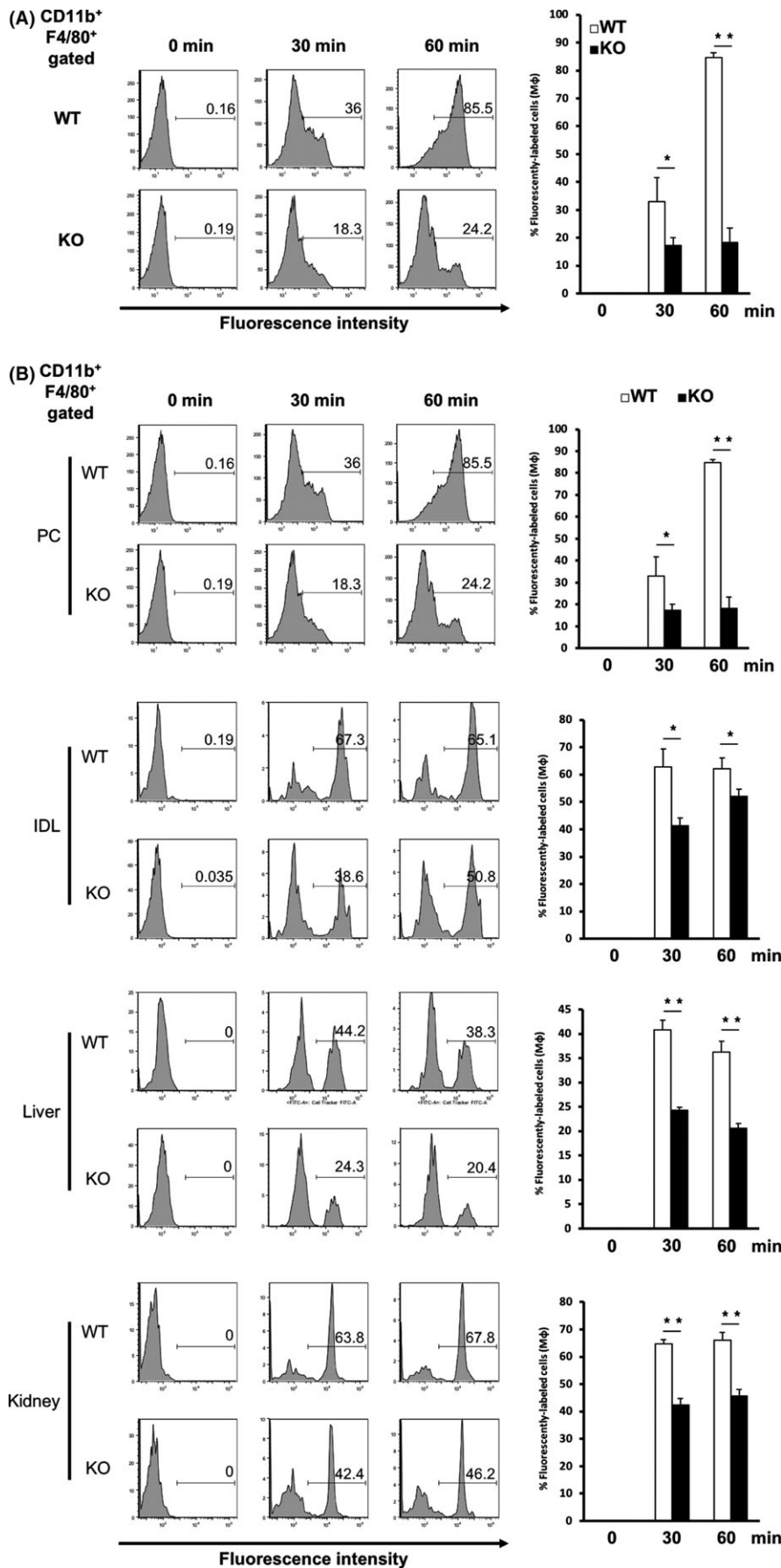


FIGURE 5 Phagocytic activities in macrophages. A, Wild-type (WT) and Allergin-1-deficient peritoneal macrophages were co-cultured with pHrodo bioparticle. At 0, 30, and 60 min, the percentage of fluorescently labeled cells in macrophage (CD11b⁺ F4/80⁺) was detected by flow cytometry (FCM). B, WT and Allergin-1-deficient macrophages from peritoneal cavity (PC), inguinal lymph nodes (IDL), liver and kidney were co-cultured with apoptotic cells stained with fluorescein isothiocyanate (FITC) fluorescent dye. At 0, 30, and 60 min, the percentages of FITC fluorescently labeled cells in macrophages (CD11b⁺ F4/80⁺) were detected by FCM. WT, C57BL/6 mice; KO, Allergin-1-deficient mice. Data are representative of two independent experiments. Data are means $\pm$ SEM; * P < 0.05, ** P < 0.01



tended to be increased in the serum from 24-month-old Allergin-1-deficient mice compared with WT mice (data not shown). These results might also be caused by a mild decrease of phagocytosis for apoptotic debris due to deficiency of Allergin-1.

We next examined the IgG subclass in the serum of apoptotic cell-injected mice. In MRL-lpr/lpr and NZB/WF1 SLE mouse models, IgG autoantibody subtypes were shown to be important to pathogenesis; for an example, the subtypes IgG2a and IgG3 form immune complexes, which facilitate nephritis through deposition in the renal glomerus.^{14,15} In the present study, we showed that the antibody titers of IgG3 anti-dsDNA in serum were significantly increased after injection of apoptotic cells into Allergin-1-deficient mice compared with WT mice. This increase may be due to class switching which induces IgG subclasses. Several cytokines have been reported to specifically induce each IgG subclass.¹⁶ For example, IgG3 is induced by interferon (IFN)- γ .¹⁷ After a large stimulatory event such as apoptotic cell injection, subsequent immune activation, cytokine production and sustained inflammation may induce such IgG class switching.

Several reports have demonstrated that apoptotic cell clearance was important for both the generation of autoantibody production and immune complex accumulation in humans and mice.^{5,18} Milk fat globule-epidermal growth factor 8 protein (MFGE-8) or T-cell immunoglobulin mucin (Tim)-4 are examples of such phagocytic factors.^{19,20} In phagocytic factor-deficient mice, such as MFGE-8 or Tim-4 deficient mice, anti-dsDNA antibody production and glomerulonephritis are often induced by attenuating the apoptotic cell clearance activity of macrophages.^{5,21} When apoptotic cells are not removed swiftly, their plasma membranes rupture and release cellular contents in a process called secondary necrosis.²² The contents of necrotic cells are thought to activate macrophages through Fc receptors (FcRs) and TLRs to induce tumor necrosis factor (TNF)- α and interleukin (IL)-8 production.⁶ Thus, this induced phagocytic dysfunction in macrophages and accumulation of apoptotic cells is therefore thought to be one of the factors involved in the pathogenesis of SLE-like phenotypes. In the present study, we showed that phagocytic activity in macrophages from Allergin-1-deficient mice was significantly lower than that from WT mice, suggesting that Allergin-1 modulates macrophage apoptotic cell clearance. Therefore, we propose that, under Allergin-1-deficient conditions, apoptotic cells may not be sufficiently removed. As a result, autoantibodies are induced in the serum, which trigger an immune cascade and lead to inflammation and associated tissue damage consistent with SLE.

During phagocytosis, the downstream molecules of ITIM signaling, including Src homology region 2 domain-containing phosphatase (SHP)-1 and SHP-2, affect the efficiency of the engulfment process.²³⁻²⁵ SHP-1 activates proto-oncogene tyrosine-protein kinase Src (SRC) family kinases Syk, Cbl, phosphatidylinositol-3 (PI-3) kinase, and ras-related C3 botulinum toxin substrate (Rac) while suppressing phagocytosis-related molecules such as Fc γ RIIA.^{24,26} However, SHP-2 inhibits the expression of SHP-1 and thereby enhances phagocytosis.²⁷ Thus, SHP-1 and SHP-2 were thought to control phagocytic activity in an antagonistic fashion. Previous reports

clearly showed that Allergin-1 contains an ITIM-like domain and recruits both phosphatase SHP-1 and SHP-2.¹¹ Therefore, during the process of apoptotic cell clearance, we speculate that Allergin-1 might upregulate phagocytosis through the regulation of SHP-1 and SHP-2 activation.

In conclusion, our results suggest that Allergin-1 might suppress autoantibody production via the promotion of apoptotic cell clearance. These findings should enhance our understanding of the process of autoantibody production as well as suggesting a novel target in Allergin-1 for regulation of apoptotically mediated inflammation in SLE.

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