

Deficiency in activation-induced cytidine deaminase promotes systemic autoimmunity in *lpr* mice on a C57BL/6 background

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Introduction

High levels of autoantibodies are associated with many autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) [1–3]. SLE is considered a systemic autoimmune disease in which immune complex deposition or direct autoantibody deposition leads to complement fixation, Fc receptor (FcR) activation and subsequent tissue inflammation [2]. The majority of autoantibodies in human and murine lupus are hypermutated high-affinity immunoglobulin (Ig)G antibodies, which have usually been considered pathogenic [4–10]. The role of IgM autoantibodies in autoimmune diseases is controversial. The relative contribution of IgG and IgM antibodies to lupus-like pathogenesis remains to be defined [11–15]. Importantly, it has not been determined whether high-affinity antibodies that result from Ig gene somatic hypermutation (SHM) and subsequent clonal selection of activated B cells are critical in eliciting organ damage and systemic autoimmune syndrome in lupus.

Activation-induced cytidine deaminase (AID), a member of the cytidine deaminase family that acts as an editor of

Summary

Activation-induced deaminase (AID) is a prerequisite for immunoglobulin (Ig) class-switch recombination and somatic hypermutation, which is critical for antibody affinity maturation. IgM and IgG autoantibodies are characteristic of the systemic autoimmune disorders such as lupus. However, the relative contributions of hypermutated high-affinity IgG antibodies and germline-encoded IgM antibodies to systemic autoimmunity are not defined fully. The role of AID in autoimmunity is unclear. The current study used AID-deficient mice to investigate the role of AID in the development and pathogenesis of murine lupus. C57BL/6 mice deficient in both Fas and AID were generated. Compared to their AID-competent littermates, AID^{-/-} lymphoproliferative (*lpr*) mice produced significantly elevated levels of IgM autoreactive antibodies with enhanced germinal centre (GC) response, developed more advanced splenomegaly and exhibited more severe glomerulonephritis. Thus, AID may play an important role in the negative regulation of systemic autoimmune manifestations in murine lupus. The results also indicate that hypermutated high-affinity IgG antibodies are not necessary for the development of autoimmune syndrome in *lpr* mice on a C57BL/6 background.

Keywords: AID, autoantibodies, autoimmunity, lupus

DNA and RNA, is required for Ig gene SHM and isotype class-switch recombination (CSR) [16–18]. To study the role of AID in the pathogenesis of murine lupus, we introduced the AID deficiency into the lupus-prone lymphoproliferative (*lpr*) mice of C57BL/6 background (B6). Our results showed that, compared to their AID-competent littermates, AID-deficient B6-*lpr* mice produced significantly elevated levels of IgM autoreactive antibodies, developed more severe splenomegaly and suffered from more severe glomerulonephritis. Thus, our findings indicate that AID deficiency promotes systemic autoimmune manifestations.

Materials and methods

Mice

AID-deficient mice were kindly provided by Dr T. Honjo (Kyoto University, Kyoto, Japan) and Dr David Schatz (Yale University). C57BL/6-*lpr* (B6-*lpr*) mice were purchased from the Jackson Laboratory. AID^{-/-} mice were back-crossed to B6-*lpr* mice for six generations. All groups of mice (B6,

AID^{-/-}, B6-lpr and AID^{-/-}lpr) used in the experiments were littermates. Mice of both sexes were kept in specific pathogen-free facilities. All animals were housed in autoclaved microisolators, provided with sterile bedding, food and water, and maintained on a 12-h day/night cycle. Animal experimentation was performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Baylor College of Medicine.

Flow cytometry

Single-cell suspensions of spleens and lymph nodes from mice of various genotypes were depleted of erythrocytes and first incubated with purified anti-mouse CD32/CD16 (PharMingen, San Diego, CA, USA) to block antibody binding to Fcγ receptors. The cells were then stained with different antibodies to cell surface markers. The samples were acquired by a fluorescence activated cell sorter (FACS) Calibur flow cytometer (BD Bioscience, San Jose, CA, USA) and analysed using Flowjo software (TreeStar, Inc., San Carlos, CA, USA).

Serum autoantibody assays

Serum levels of anti-dsDNA antibodies were analysed by enzyme-linked immunosorbent assay (ELISA) as described previously [19]. Microplates were coated with dsDNA (Invitrogen, Carlsbad, CA, USA) and blocked with 10% fetal calf serum (FCS). Samples were added and incubated for 1 h at 37°C and washed. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgM (Southern Biotechnology Associates, Birmingham, AL, USA) was used as secondary detection antibody. HRP activity was visualized using a tetramethylbenzidine (TMB) peroxidase substrate kit (Bio-Rad Laboratories, Hercules, CA, USA), and optical densities were determined at 450 nm using an automated reader.

Urine analysis

Urine from all groups of mice was collected and levels of urine protein, leucocytes and red blood cells were determined semi-quantitatively with Multistix 8 SG (Bayer Corporation, Elkhart, IN, USA).

Histology

The procedures for freezing tissues, sectioning and immunohistochemical staining were conducted as described previously [20]. Briefly, kidneys and spleens were frozen in optical cutting temperature (OCT) compound and serial 6-μm-thick frozen sections were cut in a cryostat microtome, thaw-mounted onto poly-L-lysine-coated slides, air-dried, fixed in ice-cold acetone for 10 min and stored at -80°C. To examine immune complex deposition in glomeruli, kidney sections were incubated with fluorescein-

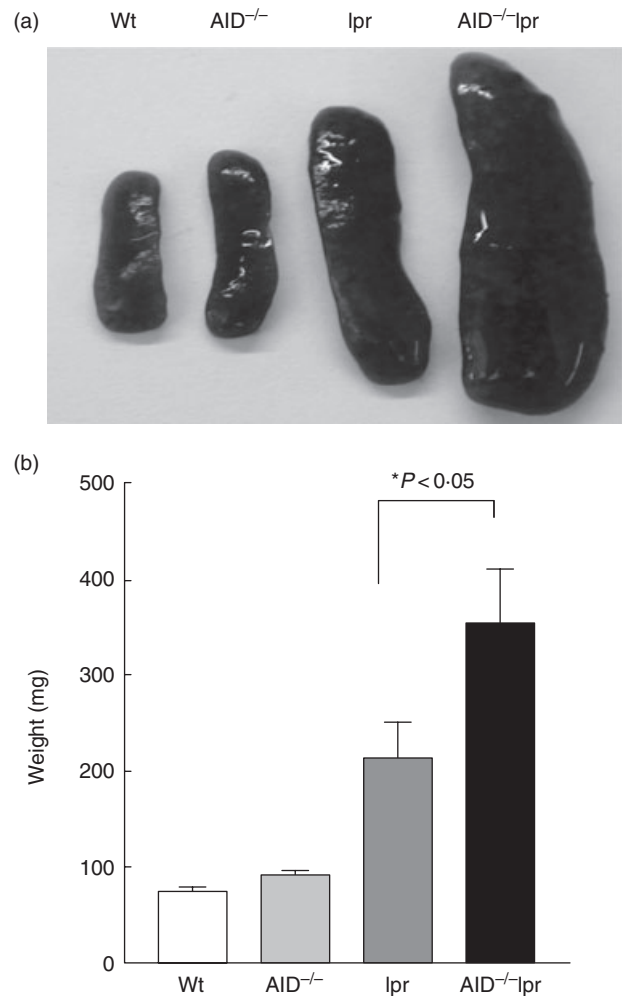


Fig. 1. Enhanced splenomegaly in activation-induced deaminase (AID)^{-/-} lymphoproliferative (lpr) mice. (a) Representative photographs of spleens from 12-month-old wild-type B6, AID^{-/-}, B6-lpr and AID^{-/-}lpr mice. (b) Weight of spleens from wild-type B6, AID^{-/-}, B6-lpr and AID^{-/-}lpr mice ($n = 19, 26, 23, 22$, respectively).

conjugated goat anti-mouse C3 antibody (ICN Biomedicals, Inc., Irvine, CA, USA) and goat F(ab')₂ anti-mouse Ig antibody (Dako, Glostrup, Denmark) and examined under a fluorescent microscope. The numbers of positive glomeruli were counted. Then the same sections were restained with haematoxylin and eosin (H&E) to obtain total numbers of glomeruli. The percentage of glomeruli with immune complex deposition were calculated. The pathology scores of renal tissue of H&E-stained sections were graded on 0–4 scale as described [21].

Statistical analysis

Data were analysed using either a one-way analysis of variance (ANOVA) and Newman–Keuls *post-hoc* test or an unpaired Student's *t*-test with Welch's correction (GraphPad Prism, Inc., La Jolla, CA, USA).

Table 1. Cell numbers in the spleen ($\times 10^6$).

Spleen	B6	AID ^{-/-}	lpr	AID ^{-/-} lpr
Total	53.7 \pm 13.0	67.1 \pm 6.2	96.3 \pm 26.6	122.8 \pm 46.9
CD19 ⁺ B cells	28.5 \pm 4.5	32.1 \pm 7.5	42.1 \pm 3.5	61.2 \pm 4.5
CD3 ⁺ T cells	19.4 \pm 3.7	22.6 \pm 2.3	35.8 \pm 5.3	42.5 \pm 3.8
CD4 ⁺ T cells	12.1 \pm 2.0	13.2 \pm 2.2	24.3 \pm 3.7	25.2 \pm 4.4
CD8 ⁺ T cells	5.9 \pm 1.3	6.8 \pm 1.5	5.4 \pm 2.8	6.6 \pm 1.1
DN T cells*	1.2 \pm 0.5	2.2 \pm 0.3	5.7 \pm 1.7	9.5 \pm 1.4

*Double negative (DN) T cells represent CD3⁺CD4⁻CD8⁻ T cells. Splensens were removed from 12-month old wild-type B6, AID^{-/-}, lymphoproliferative (lpr) and activation-induced deaminase (AID)^{-/-} mice, respectively, and numbers of total cells and lymphocytes were enumerated; $n = 5-8$.

Results

AID deficiency promotes splenomegaly in B6-lpr mice

To study whether AID deficiency affects the development of systemic autoimmunity in lpr mice, we generated AID^{-/-}lpr mice by back-crossing AID^{-/-} mice to B6-lpr mice. Control animals including both wild-type B6 and AID^{-/-} mice were their littermates. All the mice were on a B6 background. As the onset of the autoimmune syndrome in lpr

mice with a B6 background is relatively delayed compared to MRL/lpr mice, all the mice were analysed at 12–14 months of age.

Compared to B6-lpr mice, AID^{-/-}lpr mice exhibited more pronounced splenomegaly (Fig. 1). At 12 months of age, the sizes of spleens from AID^{-/-}lpr mice were significantly larger than those of lpr mice. In addition, the total numbers of cells from the spleen of AID^{-/-}lpr mice were significantly higher than those in lpr mice (Table 1). The total numbers of B cells in AID^{-/-}lpr mice were increased significantly compared to

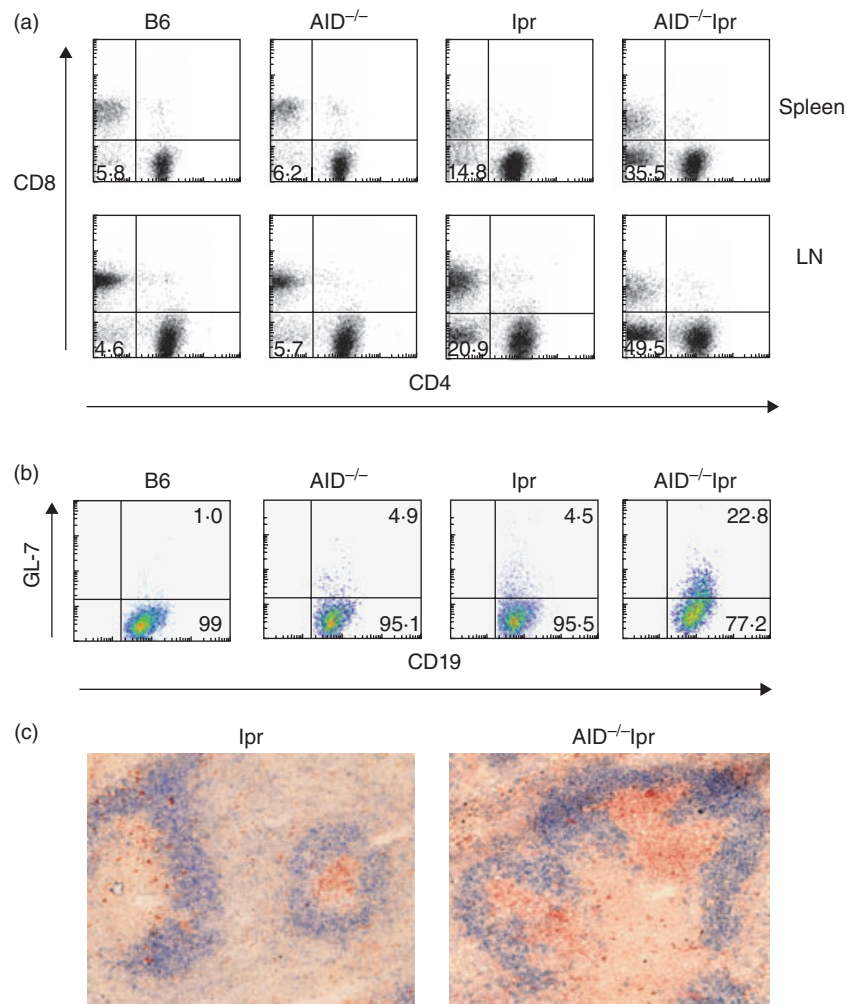


Fig. 2. Increased double negative (DN) T cells and enhanced germinal centre (GC) response in activation-induced deaminase (AID)^{-/-} lymphoproliferative (lpr) mice. (a) CD3⁺ T cells were gated and analysed further for CD4⁻CD8⁻ DN T cells. The numbers shown represent the percentages of DN T cells within the T cell gate. (b) Splenic cells were gated on CD19⁺ B cells and analysed further for the percentages of GC B cells within the CD19⁺ B cell gate. The data were representative of results from eight to 10 mice (12–14 months old) in each group. (c) Representative splenic sections stained with peanut agglutinin (PNA) (red) and anti-IgD antibody (blue) to visualize GCs in the spleen of B6-lpr and AID^{-/-}lpr mice.

those in B6-*lpr* mice, indicating more B cell expansion in the absence of AID. There were no significant differences in the sizes of mesenteric lymph nodes from *lpr* and *AID*^{-/-}*lpr* mice (data not shown).

Increased double negative (DN) T cells and enhanced germinal centre (GC) response in *AID*^{-/-}*lpr* mice

It is known that a population of CD4⁻CD8⁻B220⁺TCRαβ⁺ DN T cells is greatly accumulated in both lupus patients and *lpr* mice, which is caused in part by *fas* defects, leading to abnormal lymphocyte survival with subsequent autoimmunity [22,23]. Interestingly, we found that this population was increased greatly in *AID*^{-/-}*lpr* mice compared to B6-*lpr* mice (Fig. 2a and Table 1). These DN T cells were B220⁺ and the vast majority of them were T cell receptor (TCR)αβ⁺ (data not shown). The data indicate profound abnormal expansion of T cells in the absence of AID.

Enhanced development of spontaneous GC response is a hallmark of systemic autoimmunity in lupus-prone mice. To determine whether AID deficiency promotes GC response, we analysed GC formation in AID-deficient mice and their AID-competent counterparts. At 12 months old, wild-type B6 mice had few GL-7⁺ GC B cells in the spleen (Fig. 2b). However, the GL-7⁺ B cell population was increased significantly in *AID*^{-/-}*lpr* mice compared to that in *lpr* mice. The increase in GC formation in *AID*^{-/-}*lpr* mice was confirmed by immunohistology. GCs are significantly larger in size in the spleens of *AID*^{-/-}*lpr* mice compared to those of B6-*lpr* mice (Fig. 2c). The data demonstrated that activated B cells were expanded in *AID*^{-/-}*lpr* mice.

Elevated levels of IgM autoantibodies in *AID*^{-/-}*lpr* mice

Because AID deficiency leads to impaired Ig somatic hypermutation and isotype class-switch, antibodies other than IgM isotype are not produced in AID-deficient mice [16–18]. We analysed the serum IgM autoantibodies to dsDNA by ELISA. By 12 months of age, the levels of IgM antibodies against dsDNA were significantly higher in *AID*^{-/-}*lpr* mice than those in *lpr* mice (Fig. 3a). We further determined total serum IgM levels in different groups of mice. *AID*^{-/-} mice at 12 months of age had significantly elevated serum IgM levels compared to wild-type controls (Fig. 3b). The total IgM levels in *AID*^{-/-}*lpr* mice were also high, but with no statistical significance if compared to total IgM levels in B6-*lpr* mice.

AID^{-/-}*lpr* mice develop more severe glomerulonephritis

To determine the impact of AID deficiency on glomerulonephritis, we examined the occurrence and severity of glomerulonephritis in mice of various genotypes. The results showed that compared to B6-*lpr* mice, *AID*^{-/-}*lpr* mice devel-

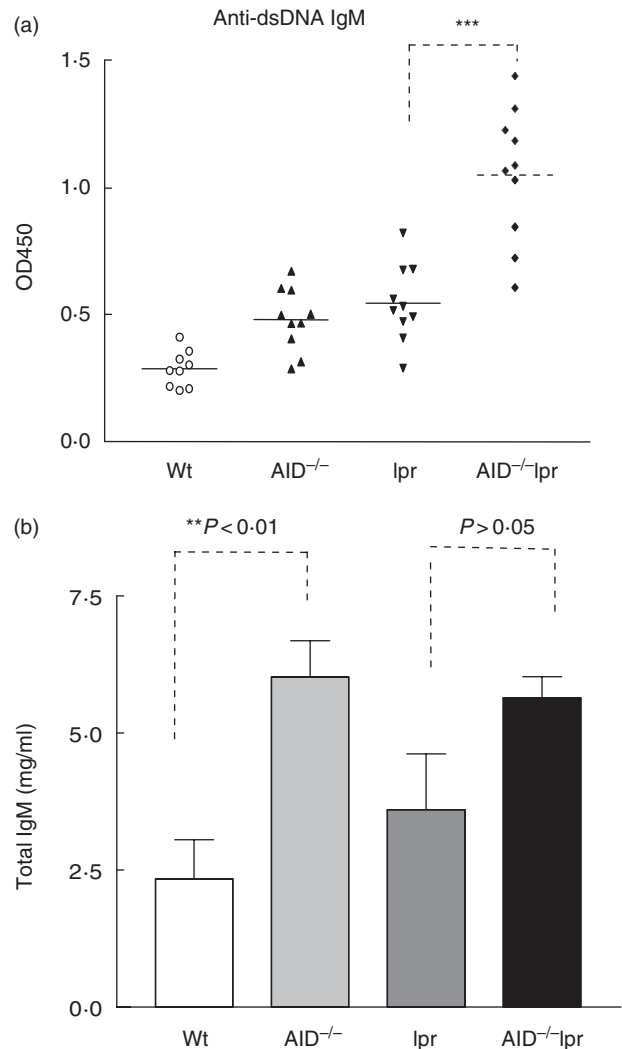


Fig. 3. Elevated production of immunoglobulin (IgM) autoantibodies in activation-induced deaminase (*AID*^{-/-} lymphoproliferative (*lpr*) mice. (a) Anti-dsDNA autoantibodies in the sera from wild-type B6, *AID*^{-/-}, *lpr* and *AID*^{-/-}*lpr* mice at 12 months old were measured by enzyme-linked immunosorbent assay. (b) Total serum IgM levels in 12-month-old mice were measured; 10 mice each group. ***P* < 0.01; ****P* < 0.001 by Student's *t*-test.

oped more severe proliferative glomerulonephritis, demonstrated by hypercellularity, enlarged Bowman's capsules and more severe destruction of glomerular structure (Fig. 4a). The renal pathology score was significantly higher in *AID*^{-/-}*lpr* mice compared to that in *lpr* mice (Fig. 4d). However, the percentage of glomeruli positive for immune complex deposition in *AID*^{-/-}*lpr* mice was increased slightly compared to that in *lpr* mice, but was not statistically significant (Fig. 4b, c, e and f).

To assess kidney function, urine of different strains of mice was analysed for the levels of protein, leucocytes and red blood cells. Consistent with more severe destruction of glomeruli, the levels of protein, leucocytes and blood in the

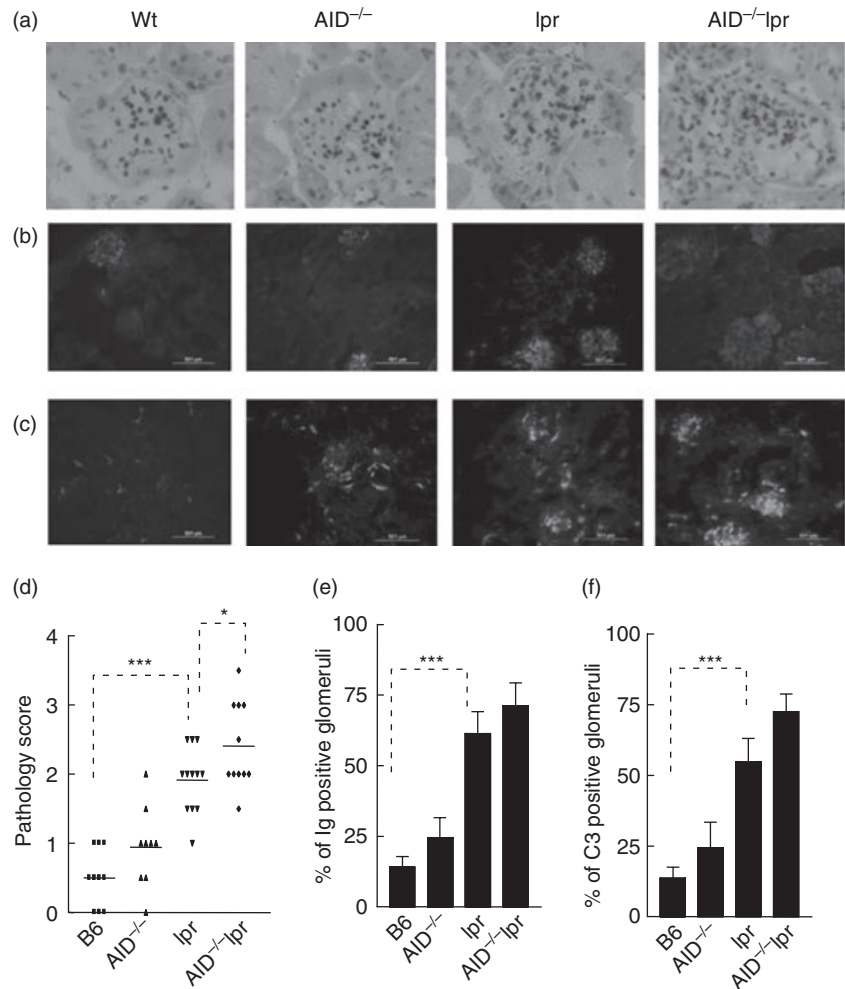


Fig. 4. AID-deficiency leads to more severe glomerulonephritis in lpr mice with B6 background. (a) Haematoxylin and eosin (H&E) staining showed glomerulonephritis. (b,c) Immunofluorescent staining showed immunoglobulin (Ig) deposition (b) and C3 deposition (c) in the kidney. (d) The pathology scores of renal tissue were determined. (e,f) The numbers of glomeruli positive for Ig deposition (e) and C3 deposition (f) were counted and calculated as percentage of total glomeruli. Kidney sections from eight to ten mice in each group were analysed.

urine of AID^{-/-}lpr mice were significantly higher than those in lpr littermates (Fig. 5). Thus, our data indicated that AID deficiency in B6-lpr mice leads to more severe glomerulonephritis and deteriorated kidney function.

Discussion

It is clear that B cells play a critical role in the development of systemic autoimmune disease [24]. B cells can promote autoimmune pathology by several potential mechanisms including antigen presentation, cytokine production and autoantibody production. Although autoantibodies are characteristic of the systemic autoimmune disease, their involvement in the pathogenesis has long been debated. The present study revealed that the absence of AID did not alleviate the autoimmune syndrome in lpr mice on a B6 background. Interestingly, AID^{-/-}lpr mice had more profound B cell expansion and severe disease manifestations than lpr mice. As AID deficiency results in little class-switching and somatic hypermutation, the results indicate that IgG autoantibodies are not required for the disease

manifestation. AID deficiency in B6-lpr mice had significantly higher levels of IgM autoantibodies compared to B6-lpr littermates, which may contribute to the increased IC deposition in kidney and more severe kidney pathology in AID^{-/-}lpr mice. In addition, it has been shown that AID deficiency drives B cell autoimmunity [25]. AID^{-/-} mice develop tertiary lymphoid organs spontaneously in non-lymphoid tissues and develop severe gastritis with elevated gastric autoantigen-specific serum IgM levels [25]. Thus, the severe autoimmune syndrome in AID^{-/-}lpr mice may also be contributed by AID deficiency. Indeed, our results also revealed that AID deficiency resulted in significantly enhanced GC formation in AID^{-/-}lpr mice, which is consistent with previous reports showing that there is intense proliferation of GC B cells leading to giant GCs in AID-deficient patients [17,26]. Although the mechanism for the dramatically increased GC B cell population caused by AID deficiency is not clear, this property may promote expansion of autoreactive B cells *in vivo*. In combination with *fas* deficiency, AID deficiency may accelerate the expansion of autoreactive B cells. The increased GC B cell population

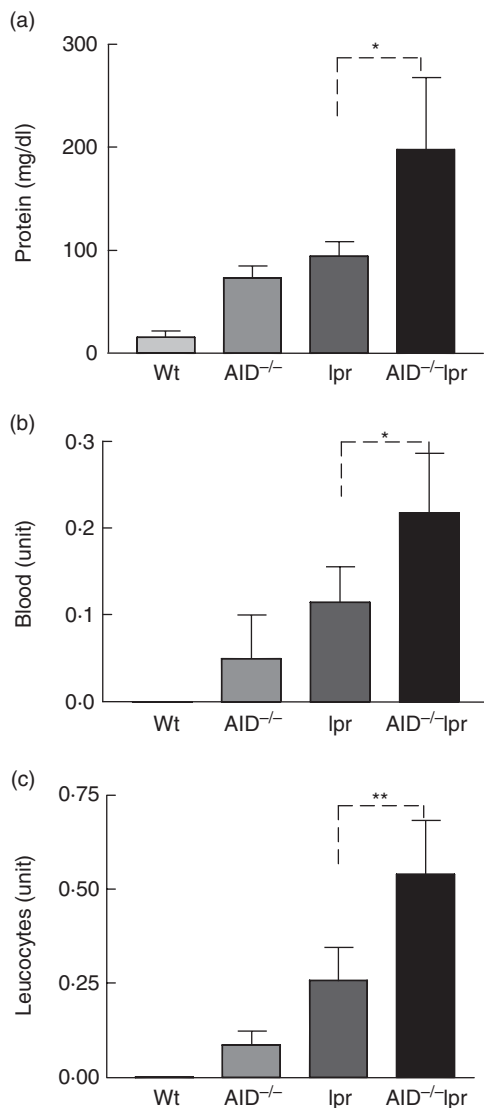


Fig. 5. Urinalysis of protein, leucocytes and blood. Data (mean \pm standard deviation) are shown for the levels of protein (a), blood (b) and leucocytes (c) in the urine of different strains [B6, $n = 19$; activation-induced deaminase (AID)^{-/-}, $n = 30$; lymphoproliferative (lpr), $n = 35$; AID^{-/-}lpr, $n = 35$] of mice at 12–14 months of age. * $P < 0.05$; ** $P < 0.01$.

may contribute to the severity of autoimmunity in AID^{-/-}lpr mice. Thus, AID may play an important role in the containment of autoimmune diseases by negative regulation of autoreactive B cells.

In summary, the current study demonstrated that AID deficiency results in more severe autoimmune syndrome in the relatively less susceptible C57BL/6 background. Our results are supported by previous reports from AID-deficient patients showing that AID-deficient patients are not only prone to infections, but also to lymphoid hyperplasia, autoimmune diseases and inflammatory disorders [26]. Twenty-one per cent of AID-deficient patients developed autoimmune disorders, including polyarthritis, autoimmune

hepatitis and Crohn's disease [26]. Thus, attempts to limit AID function as a therapeutic strategy for the treatment of autoimmune diseases should be cautious.

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Disclosure

The authors declare that they have no conflict of interest related to the publication of this manuscript.

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