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The absence of immunoglobulin D B cell receptor-mediated signals promotes the production of autoantibodies and exacerbates glomerulonephritis in murine lupus

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Summary

Immunoglobulin (Ig)D is the major antigen receptor isotype co-expressed with IgM on the surface of most peripheral B cells in mice and humans. However, the biological role of IgD as B cell receptor (BCR) has remained unclear. Previous studies have indicated that IgD may play a role in B cell tolerance. To understand the role of IgD in B cell tolerance and autoimmunity, we have examined the development of autoimmune syndrome in lpr mice deficient for IgD. The present study showed that IgD deficiency did not alter lymphoproliferation and lymphocyte activation in lpr mice. The survival and proliferation of B cells were not affected by the absence of IgD, indicating that IgD BCR-mediated signals do not have an important role in negative selection of autoreactive B cell clones. Interestingly, compared to IgD-competent littermates, lpr mice with IgD deficiency had elevated autoantibody production, increased deposition of immune complex in the kidney and more severe nephritis. Accumulation of abnormal $CD4^{-}CD8^{-}\alpha\beta^{+}$ T cells was accelerated in $IgD^{-/-}$ lpr mice compared to lpr mice. These results suggest that IgD BCR-mediated signals may be involved in the differentiation of autoreactive B cells into plasma cells and abnormal T cell expansion.

Keywords: autoantibodies, autoimmunity, IgD deficiency, lupus

Introduction

The expression of membrane immunoglobulin M (mIgM) and IgD (mIgD) is regulated strictly during B cell development. IgM begins to be expressed on B cells at the immature stage in the bone marrow (BM), whereas IgD expression is first up-regulated in transitional B cells in the spleen [1]. The vast majority of naive mature B cells in peripheral lymphoid tissues co-express IgM and IgD. To date, however, the biological function of the dual expression of IgM and IgD by most B cells and the differential expression of IgM and IgD during B cell development and differentiation are not understood fully.

As B cell receptors (BCRs), surface IgM and IgD are anchored in the plasma membrane and linked non-covalently to heterodimers of Igα (CD79a) and Igβ (CD79b) chains [2]. Due to different carbohydrate contents, IgM- and IgD- associated Igα and Igβ chains have different molecular masses [3,4]. IgD-BCR is more stable than the IgM-BCR [5]. B cell antigen receptor of IgD class induces a stronger and more prolonged protein tyrosine phosphorylation than that of IgM class [6]. Interestingly, IgD can be expressed on the surface in two alternative ways. In the canonical pathway, IgD is associated with Igα and Igβ; in the alternative pathway, IgD can be linked to membrane lipids via a glycosyl-phosphatidylinositol (GPI) linkage [7]. The GPI-linked isoform of mIgD activates the cAMP-dependent signalling pathway selectively, which promotes signalling synergistically from the canonical pathway [8]. Recently, it has been reported that IgM-to-IgD class-switching occurs in B cells of the human upper respiratory mucosa, and IgD may orchestrate an ancestral surveillance system at the interface between immunity and inflammation [9,10].

An interesting phenomenon, with respect to expression of IgM and IgD, was observed in mice transgenic for rearranged H and L chain genes encoding a self-reactive antibody [11]. Autospecific B cells, found to be anergic, had down-regulated surface expression of IgM, whereas the level of IgD expression remained high. These data led to the suggestion that IgD may play a role in maintaining B cell tolerance. An alternative explanation is that maintenance of IgD expression may rescue the cells undergoing programmed cell death. However, the role of IgM and IgD in tolerance induction has remained a matter of controversy [12–17].

Mice deficient for IgD show a reduced B cell compartment, with 30–50% fewer B cells in the spleen and lymph nodes, indicating that IgD may be important in homeostasis of B cells [18]. Although IgD-deficient mice respond well to T cell-independent and -dependent antigens, they exhibited delayed affinity maturation in the primary response [19]. To understand IgD function and its potential role in self-tolerance, we investigated the development of autoimmune syndrome in lpr mice deficient for IgD. The results showed that the absence of IgD BCR-mediated signals did not alleviate the autoimmune disease. In contrast, lpr mice with IgD deficiency exhibited elevated autoantibodies and more severe nephritis.

Materials and methods

Mice

C57BL/6-lpr (B6-lpr) mice were purchased from The Jackson Laboratory (Bar Harbour, ME, USA). $IgD^{-/-}$ mice [18] were back-crossed to C57BL/6 background more than 10 generations. $IgD^{-/-}$ mice were then bred with B6-lpr mice to generate $IgD^{-/-}$lpr mice. Four groups of mice including B6, $IgD^{-/-}$, B6-lpr and $IgD^{-/-}$lpr mice used in the experiments were littermates. Mice of both sexes were kept in specific pathogen-free facilities. Animal experimentation was performed in accordance with protocols approved by 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 stained with different antibodies to cell surface markers. Antibodies to CD3, CD4, CD8, CD19, CD44, CD62L, T cell receptor (TCR)-αβ, interferon (IFN)-γ, interleukin (IL)-10, IL-17, B220, Igκ and Igλ were purchased from BD Pharmingen (San Diego, CA, USA). Anti-forkhead box P3 (FoxP3) antibody was purchased from eBioscience (San Diego, CA, USA). Cell death was determined by annexin V staining. The samples were acquired by a fluorescence activated cell sorter (FACS) FACScalibur flow cytometer (BD Bioscience, San Jose, CA, USA) and analysed using FlowJo software (Tree Star, Inc., San Carlos, CA, USA).

Cell proliferation and cell death

For bromodeoxyuridine (BrdU) labelling, 2–3-month-old mice were injected intraperitoneally (i.p.) with 1 mg BrdU daily for 4 days. The mice were killed next day after the last injection and spleen cells were stained with anti-BrdU antibody and analysed by flow cytometry. For cell death, spleen cells from 2–3-month-old mice were stained with annexin V-FITC (BD PharMingen) *ex vivo* and the cell death of individual B cell subsets within the lymphocyte gate were analysed by flow cytometry.

Serum autoantibody assays

Serum levels of anti-dsDNA antibodies were analysed by enzyme-linked immunosorbent assay (ELISA), as described previously [20]. Microplates were coated with dsDNA (Invitrogen, Carlsbad, CA, USA). Serum samples were incubated for 1 h at 37°C. Horseradish peroxidise (HRP)-conjugated goat antibodies to IgM and IgG1, IgG2a, IgG2b and IgG3 (Southern Biotechnology Associates, Birmingham, AL, USA) were used as secondary detection antibodies. Ginsenoside RF (RF) activity was measured as described previously [21]. Briefly, plates were coated with NP [(4-hydroxy-5-iodo-3-nitrophenyl)acetyl]-bovine serum albumin (BSA) (20 μg/ml). An NP-specific monoclonal antibody (mAb) (IgG1/λ) was added and bound anti-NP mAb served as ligands for RF binding. RFs bound to plates are detected by HRP-conjugated anti-mouse IgM, IgG2a, IgG2b and IgG3 for each isotype of RFs. Total levels of serum antibodies were determined by sandwich ELISA.

Histology

The procedures for freezing tissues, sectioning and immunohistochemical staining were conducted as described previously [22]. Briefly, kidneys were frozen in optimal cutting temperature (OCT) compound and serial 6-μm-thick frozen sections were cut in a cryostat microtome. To examine immune complex deposition in glomeruli, kidney sections were incubated with fluorescein-conjugated goat anti-mouse C3 antibody (ICN Biomedicals, Inc., Aliso Viejo, 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 was calculated. The pathology scores of renal tissue of H&E-stained sections were graded on a 0–4 scale as described previously [23]: 0, normal; 1, a small increase in numbers of cells within the mesangium of glomerulus; 2, prominent increase in the number of mesangial cells and a perivascular lymphocytic infiltration; 3, lobular formation of the glomerulus, thickening of basement membrane and large numbers of mononuclear cell surrounding vessels; 4, glomerular crescent formation, some sclerotic glomeruli, tubular atrophy, tubular cast and occasional vasculitis.

Statistical analysis

Data were analysed using unpaired Student's *t*-test, two-tailed (GraphPad Prism).

Results

IgD-deficiency does not alter lymphoproliferation in lpr mice

To study whether IgD deficiency affects the development of systemic autoimmunity in lpr mice, we generated IgD$^{-/-}$lpr mice by crossing IgD$^{-/-}$ mice to B6-lpr mice. Wild-type B6 and IgD$^{-/-}$ mice were littermate controls. All the mice were on a B6 background. Because 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.

The results showed that both lpr and IgD$^{-/-}$lpr mice had significantly enlarged spleens compared to wild-type controls (Fig. 1a). The total numbers of T cells in the spleen of both lpr and IgD$^{-/-}$lpr mice were increased significantly (Fig. 1b). The percentages of B cells in the spleen of lpr mice were reduced (data not shown), but total B cell numbers in the spleen of lpr and IgD$^{-/-}$lpr mice were similar to wild-type controls (Fig. 1c). Interestingly, unlike the enlarged spleen, the inguinal lymph nodes (iLN) in lpr and IgD$^{-/-}$lpr mice were similar to that of wild-type B6 mice (Fig. 1d). The percentages and total T cell numbers in iLN of lpr and IgD$^{-/-}$lpr mice were also similar to wild-type counterparts (Fig. 1e), but the total numbers of B cells in both lpr and IgD$^{-/-}$lpr mice were decreased significantly compared to that of wild-type mice (Fig. 1f). This is consistent with previous findings that B cell numbers were reduced under some autoimmune syndromes [24,25]. However, no differences have been found in the sizes of the spleen and iLN, as well as the total cell numbers of T and B lymphocytes between lpr mice and IgD$^{-/-}$lpr mice.

IgD deficiency also did not affect lymphocyte activation and cytokine production in lpr mice (data not shown). The percentages of activated T cells and effector memory cells were similar between lpr mice and IgD$^{-/-}$lpr mice. We also examined the development of regulatory T cells. The percentages of FoxP3$^+$ regulatory T cells of the total CD4$^+$ T cells were similar in lpr and IgD$^{-/-}$lpr mice (not shown).

CD4$^-$CD8$^-$ double-negative (DN) T cells are accumulated in IgD$^{-/-}$lpr mice

It is known that a population of CD4$^-$CD8$^-$B220$^+$TCR$\alpha\beta^+$ 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 [26,27]. Interestingly, this DN T cell population was increased greatly in the spleen and lymph nodes of IgD$^{-/-}$lpr

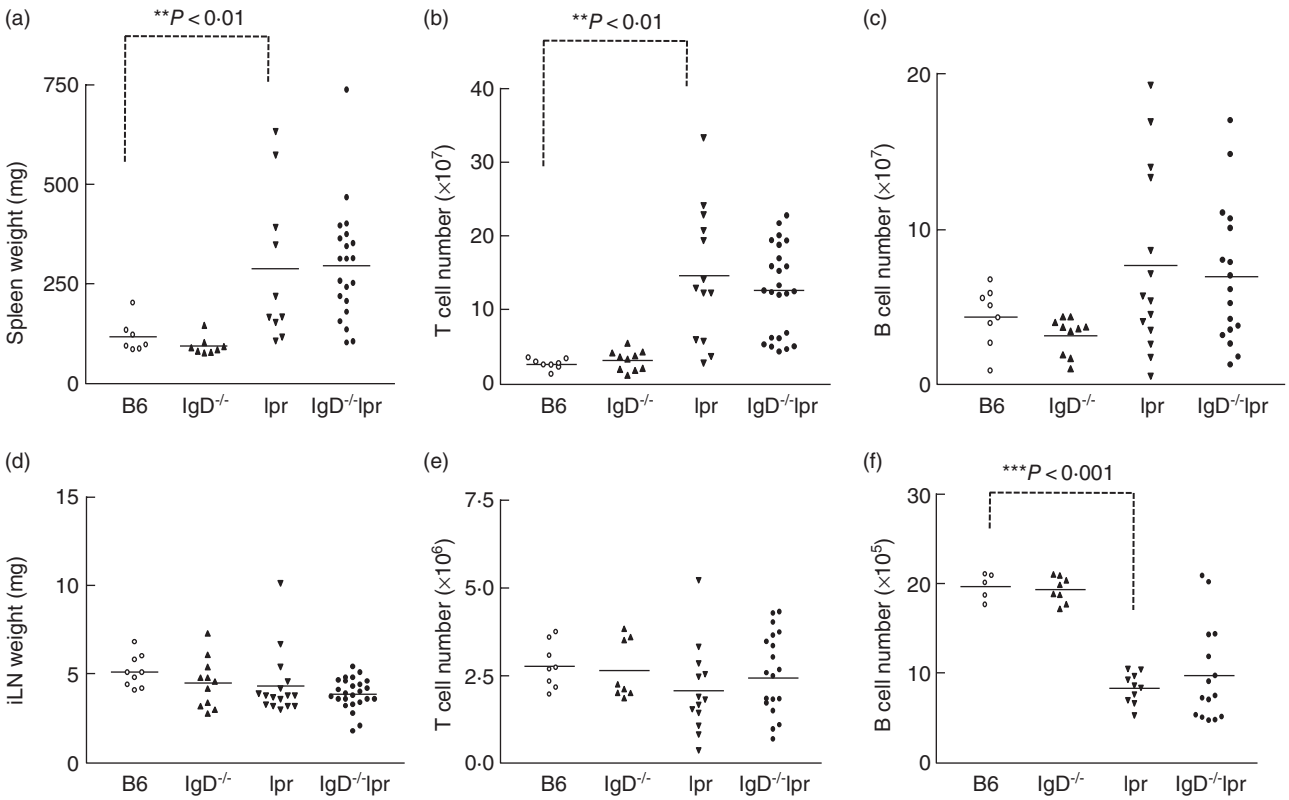

Fig. 1. Immunoglobulin (Ig)D deficiency does not alter lymphoproliferation in lpr mice. (a) The spleen weight of B6, IgD$^{-/-}$, lpr and IgD$^{-/-}$lpr mice at 12 months old. (b) Total numbers of T cells (gated on CD3$^+$) in the spleen. (c) Total numbers of B cells (gated on CD19$^+$) in the spleen. (d) The weight of inguinal lymph nodes (iLNs). (e) Total T cell numbers in iLNs. (f) Total numbers of B cells in iLNs. Each symbol represents one mouse.

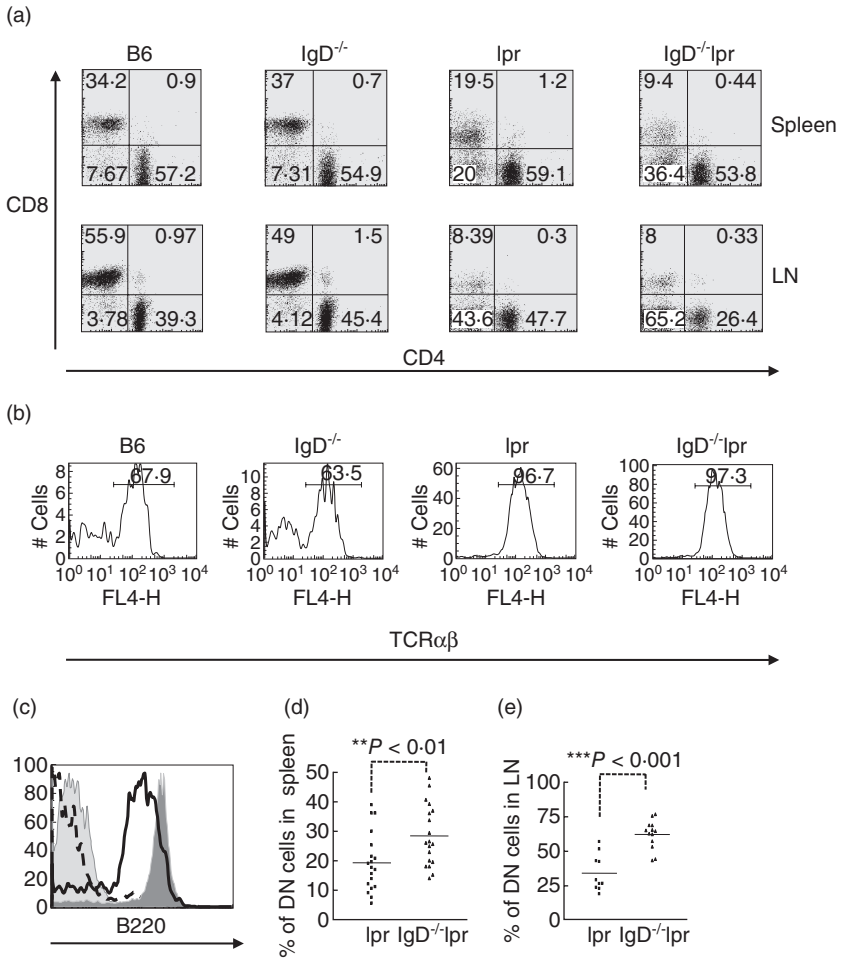

Fig. 2. Accumulation of double-negative (DN) T cells in immunoglobulin (Ig)D$^{-/-}$lpr mice. (a) CD3$^+$ T cells in the spleen and lymph nodes of different mice were gated and analysed further for CD4 and CD8 expression. (b) T cell receptor (TCR) expression on CD4$^-$CD8$^-$ DN T cells. CD4$^-$CD8$^-$ DN T cells were gated and analysed for TCR-αβ expression. Data in (a) and (b) were representative of 10–20 mice in each group. (c) B220 expression on DN T cells in lpr mice. CD3$^+$CD4$^+$ T cells (light grey area), CD3$^+$CD8$^+$ T cells (dotted line), CD3$^+$CD4$^-$CD8$^-$ DN T cells (solid line) and CD19$^+$ B cells (dark grey area) were gated and analysed for B220 expression, respectively. (d) Percentage of DN T cells out of total CD3$^+$ T cells in the spleen of lpr and IgD$^{-/-}$lpr mice. (e) Percentage of DN T cells of total CD3$^+$ T cells in the lymph nodes of lpr and IgD$^{-/-}$lpr mice. Each dot represents one mouse (d,e). The data are representative of three independent experiments.

mice compared to that in B6-lpr mice (Fig. 2a–c). Additionally, almost all these DN T cells were TCR-αβ$^+$ (Fig. 2d) and B220$^+$ (Fig. 2c).

The absence of IgD results in elevated autoantibodies in lpr mice

We analysed the serum autoantibodies to dsDNA by ELISA. By 12 months of age, the levels of IgM antibodies against dsDNA and IgM RF were significantly higher in IgD$^{-/-}$lpr mice than that in lpr mice (Fig. 3). The levels of most of IgG isotypes tested were also increased significantly in the absence of IgD. These results demonstrate that IgD deficiency promotes the production of autoantibodies.

We further examined total levels of serum antibodies. Interestingly, total IgM levels were similar in lpr mice with or without IgD expression (Fig. 4). Total levels of IgG1, IgG2b and IgG3 antibodies were also similar between lpr mice and IgD$^{-/-}$lpr mice. However, the levels of IgG2a were increased dramatically in IgD$^{-/-}$lpr mice compared to that in lpr mice. This increase was not caused by Fas deficiency, because IgD$^{-/-}$ mice also had significantly elevated IgG2a (Fig. 4).

IgD$^{-/-}$lpr mice develop more severe glomerulonephritis

To determine whether the increased levels of autoantibodies in IgD$^{-/-}$lpr mice leads to more deposition of immune complex in kidney, we examined the kidney sections by immunohistochemistry. The results showed that compared to B6-lpr mice, IgD$^{-/-}$lpr mice developed more severe proliferative glomerulonephritis, demonstrated by hypercellularity and more severe destruction of glomerular structure (Fig. 5a). The parietal epithelium of Bowman's capsule proliferated significantly and grew into a crescent shape to replace the filtration space (Fig. 5a). In addition, there were more focal and diffused infiltrating inflammatory cells in the interstitial tissue of IgD$^{-/-}$lpr kidney (not shown). The renal pathology score was significantly higher in IgD$^{-/-}$lpr mice compared to that in lpr mice (Fig. 5e).

There was no significant difference in the percentages of Ig deposition in the glomeruli of the kidney of IgD$^{-/-}$lpr mice and lpr mice (Fig. 5b and d). However, the deposition of C3 was increased significantly in IgD$^{-/-}$lpr mice compared to that in lpr mice (Fig. 5c and d). Thus, our data

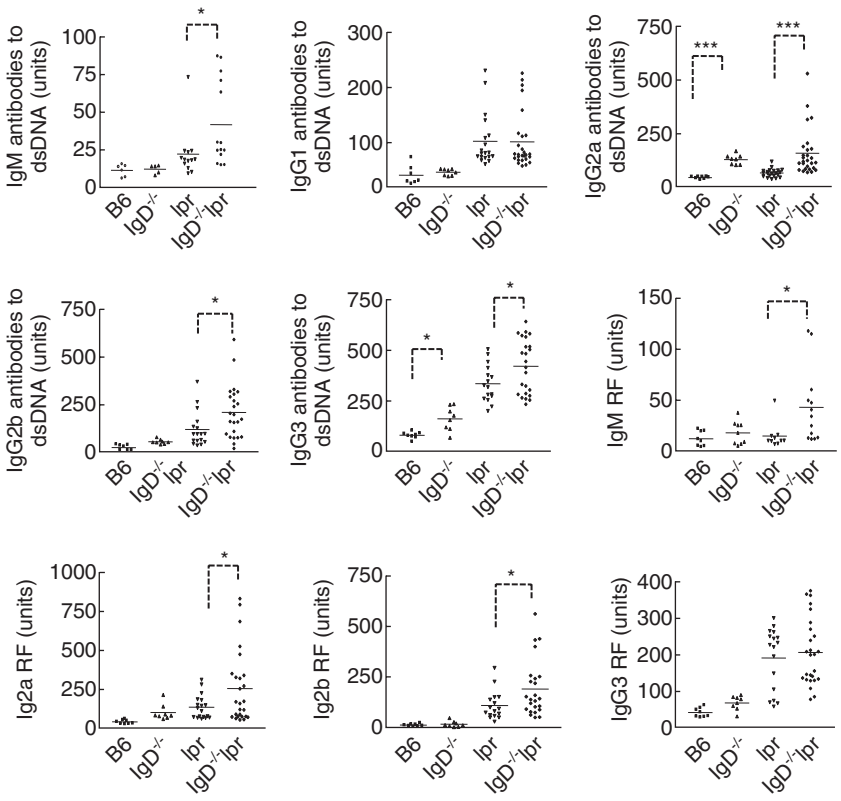

Fig. 3. Elevated autoantibody production in immunoglobulin (Ig)D$^{-/-}$lpr mice. Different isotypes of autoantibodies to dsDNA or ginsenoside RF in the sera of mice were determined by enzyme-linked immunosorbent assay. Each symbol represents one mouse. $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

indicated that IgD-deficiency in B6-lpr mice leads to more severe glomerulonephritis.

IgD deficiency does not affect B cell survival and proliferation

We analysed whether IgD BCR plays a role in cell survival, particularly in the survival of transitional B cells by annexin V staining. As CD21 is down-regulated significantly in B cells of lpr mice at a late stage, we analysed 2–3-month-old young adult mice. If IgD-BCR mediates survival signals for B cells, then IgD deficiency could lead to an increase in cell death. However, the results showed even slightly reduced cell death in transitional B cells and mature B cells in the spleen of IgD$^{-/-}$lpr mice compared to that of B cells in lpr mice, although statistically not significant (Fig. 6a). This result indicated that IgD does not have anti-apoptotic effects for B cells in lpr mice.

To examine cellular proliferation, mice were injected with BrdU daily for 4 days. There were no differences in cell

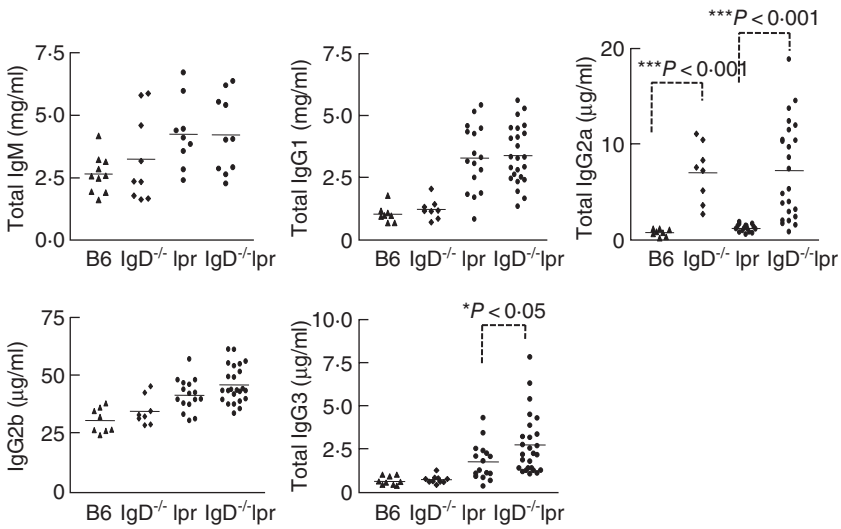

Fig. 4. Total levels of immunoglobulin (Ig) production in IgD$^{-/-}$lpr mice. Different isotypes of antibodies in the sera of the mice were determined by enzyme-linked immunosorbent assay. Each symbol represents one mouse. Data were analysed by Student's *t*-test.

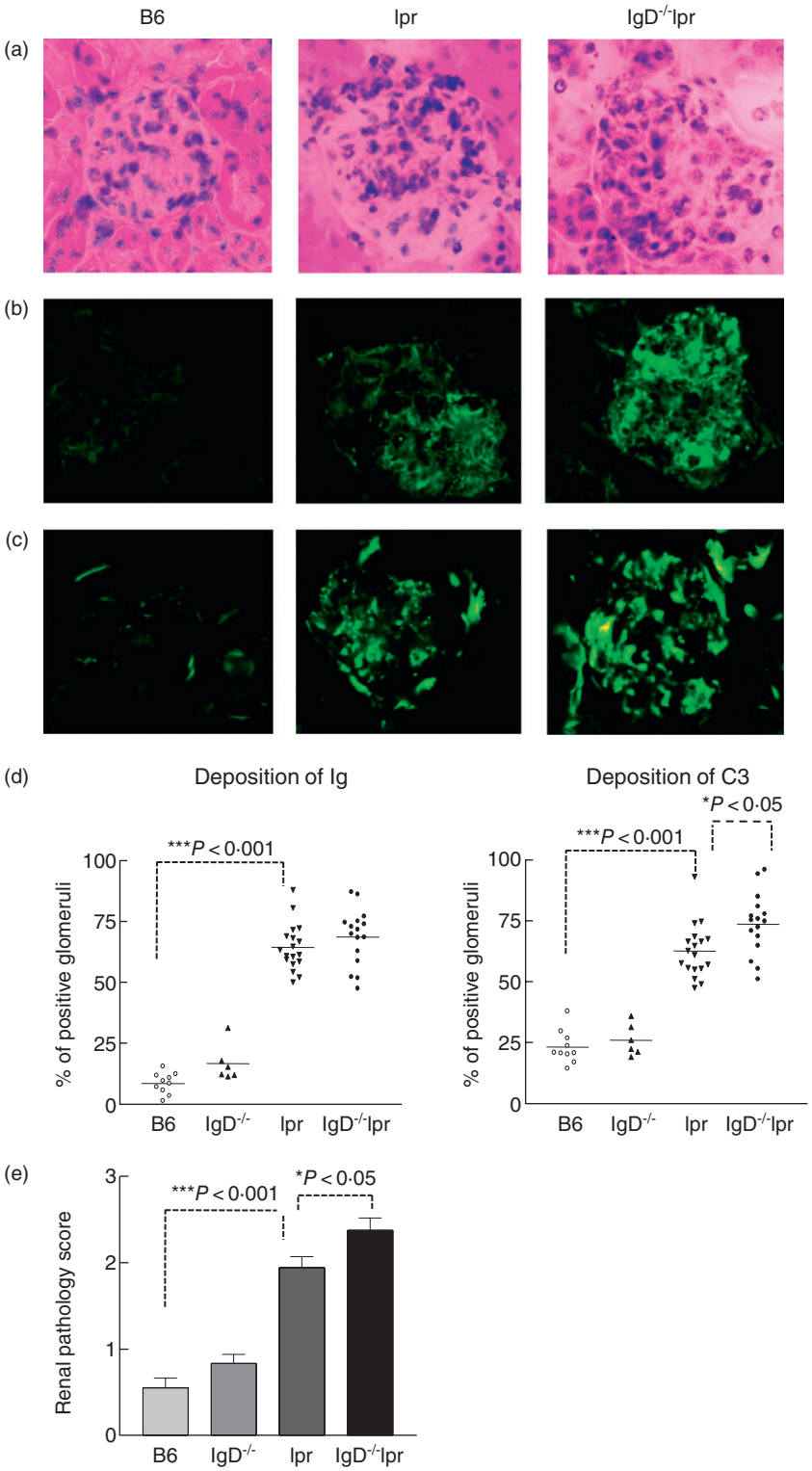

Fig. 5. Immunoglobulin (Ig)D deficiency leads to more severe glomerulonephritis in lpr mice. (a) Haematoxylin and eosin staining showed glomerulonephritis. (b,c) Immunofluorescent staining showed Ig deposition (b) and C3 deposition (c) in the kidney. (d) The numbers of glomeruli positive for Ig deposition and C3 deposition were counted and calculated as percentage of total glomeruli. Kidney sections from eight to 10 mice in each group were analysed. (e) The pathology scores of renal tissue were determined.

proliferation of transitional T1 and T2 cells among all the groups of mice analysed (Fig. 6b). However, mature B cells from lpr and IgD$^{-/-}$lpr mice proliferated more vigorously than wild-type controls (Fig. 6b), but there were no differences in cell proliferation between mature B cells from lpr and IgD$^{-/-}$lpr mice. These data suggested that IgD deficiency does not affect B cell proliferation.

We also found that the percentage of Igλ^+ B cells out of total B cells was not affected by the absence of IgD (Fig. 6c), indicating that IgD deficiency does not affect receptor editing.

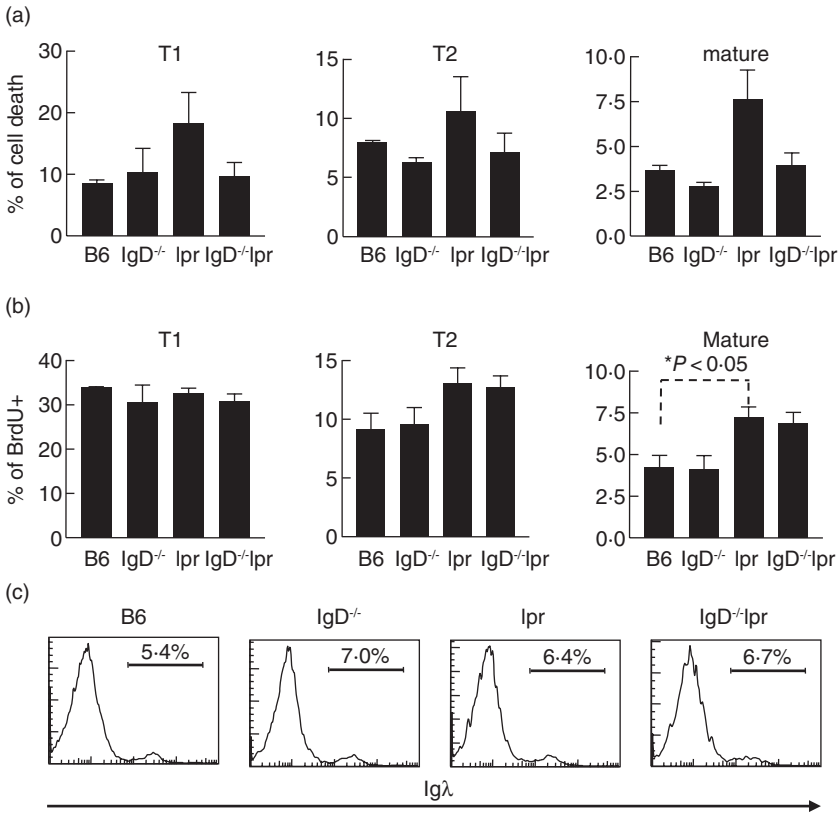

Fig. 6. Immunoglobulin (Ig)D deficiency did not alter B cell survival and proliferation. (a) B cell survival. Different B cell subsets including T1, T2 and mature B cells in the spleen of 2-month-old mice were gated and analysed for cell death by annexin V staining. The percentages of cell death from of each subset are shown; three mice in each group. (b) B cell proliferation by bromodeoxyuridine (BrdU) labelling. After 4 days of BrdU injection, the spleen B cells were analysed for BrdU-positive cells in each B cell subset; three mice in each group. (c) The spleen B cells were determined for Igλ expression. The percentages of B cells expressing λ of total B cells in the spleen are shown.

Discussion

Unlike IgM, secreted IgD is present in very low quantities in human serum and is virtually absent in mouse serum. However, IgD, as one major BCR, is expressed on most of B cells. It is a long-standing question as to why IgD BCRs are co-expressed with IgM BCRs in mature B cells. This study intends to explore the role of IgD in B cell tolerance and in the development of autoimmune disease using the murine lupus model.

Our results showed that IgD$^{-/-}$lpr mice exhibited many similarities to lpr mice. For example, they had a similar extent of lymphoproliferation, lymphocyte activation and production of inflammatory cytokines. In addition, the absence of IgD expression did not affect cell survival and clonal expansion of B cells. These results indicated that IgD does not play an important role in the elimination or expansion of autoreactive B cells, and the absence of IgD-BCRs does not influence B cell activation and cytokine production in the lupus model.

However, surprisingly, IgD deficiency enhanced autoantibody production. IgD$^{-/-}$lpr mice produced significantly higher levels of IgM and IgG autoantibodies compared to lpr mice. The results indicated that IgD may influence the differentiation of autoreactive B cells towards plasma cells by negatively regulated antibody production. The underlying mechanisms remain to be investigated. It has been shown that IgG autoantibodies to dsDNA play a prominent role in the immune complex glomerulonephritis of systemic lupus erythematosus (SLE) [28]. IgG2a and IgG2b are the most pathogenic subclasses *in vivo* due to their unique ability to engage Fcγ receptor IV (FcγRIV), which is expressed mainly on macrophages, monocytes and neutrophils [29,30]. Thus, the significantly increased autoantibodies, particularly the IgG2a and IgG2b isotypes of autoantibodies, in IgD$^{-/-}$lpr mice are likely to contribute to the severity of glomerulonephritis by increased deposition of immune complex (IC) in the glomeruli. In addition, heightened levels of IgM autoantibodies in IgD$^{-/-}$lpr mice may facilitate activation and deposition of complement in the kidney. However, our data show that complement deposition, but not Ig deposition, in the kidney of IgD$^{-/-}$lpr mice is increased significantly compared to that in lpr mice. This finding indicates an antibody-independent means of complement deposition, which may be implicated with activation of complement by the alternative pathway. In human, there is one unique type of glomerular inflammation classified as C3 glomerulopathy with complement C3 deposition within the glomeruli in the absence of substantial Ig deposition [31]. However, in IgD$^{-/-}$lpr mice, although not statistically significant, Ig deposition in kidney is increased slightly in most of the mice compared to that in B6-lpr mice. Therefore, it is possible that the absence of IgD promotes some degree of complement deposition independent of immunoglobulin in the glomeruli in addition to classical immune complex deposition.

This study has also revealed that $IgD^{-/-}$ mice produce high levels of IgG2a. Coincidentally, we have found previously that $IgM^{-/-}$ mice on a BALB/c background have significantly reduced levels of IgG2a [32]. To examine whether the alterations of IgG2a production are due to the genetic background of the mice or associated with the deletion of IgM or IgD, we have determined the levels of IgG2a in $IgM^{-/-}$ mice that have back-crossed to C57BL/6 background for at least 10 generations. Surprisingly, like $IgD^{-/-}$ mice, $IgM^{-/-}$ mice on a B6 background also have significantly higher levels of serum IgG2a than wild-type control mice (data not shown). Thus, the absence of either IgM or IgD promotes the production of IgG2a in mice with a B6 background.

It is known that lack of Fas results in accumulation of abnormal $B220^+CD4^-CD8^-CD3^+$ DN T cells in lpr mice [33]. Interestingly, accumulation of these cells is accelerated in the $IgD^{-/-}$lpr mice. These cells express TCR-$\alpha\beta$. $\alpha\beta$ T cells have been shown to have a central role in the pathogenesis of murine lupus [34]. Therefore, the increase of DN T cells in $IgD^{-/-}$lpr mice may contribute to the severity of glomerulonephritis. Although the mechanisms responsible for the elevation of DN T cells in $IgD^{-/-}$lpr mice are currently unknown, our data suggest a role of IgD in regulating T cell differentiation in the context of autoimmunity. It has been shown that $CD4^+$ T cells in murine and both $CD4^+$ and $CD8^+$ T cells in human can up-regulate IgD-specific receptors (IgD-R) that bind to the heavily glycosylated constant region of the heavy chain of IgD molecules [35–38]. The cognate interactions between IgD-R and IgD mediate signal transduction and may affect T cell activation and differentiation [39], which may ultimately modulate DN T cell generation in lpr mice.

Taken together, our results reveal novel information about the role of IgD in the development of murine lupus and may help in understanding of the overall function of IgD in self-tolerance and autoimmunity.

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Disclosure

The authors have no financial conflict of interest.

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