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Th1 polarization in murine IgA nephropathy directed by bone marrow-derived cells

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IgA nephropathy is the most common form of progressive glomerulonephritis although the pathophysiology of this nephropathy is unclear. The ddY mouse is a spontaneous animal model with variable incidence and extent of glomerular injury mimicking human IgA nephropathy. Here, we transplanted bone marrow cells from 20-week-old ddY mice with beginning or quiescent IgA nephropathy into irradiated similar ddY mice, C57Bl/6 (Th1 prone) mice, or BALB/c (Th2 prone) mice. Serum IgA/IgG complex and Th1/Th2 polarization of spleen cells was determined by enzyme-linked immunosorbent assay and confirmed by fluorescent cytometric analysis. The ddY mice with commencing IgA nephropathy demonstrated strong polarization toward Th1, while those with quiescent disease were Th2 polarized. Serum levels of IgA/IgG2a immune complex significantly correlated with the severity of the glomerular lesions. Bone marrow taken from mice with commencing IgA nephropathy conferred IgA nephropathy with Th1 polarization in recipient-quiescent mice, while transplantation from the quiescent mice ablated glomerular injury and mesangial IgA/IgG deposition in those commencing IgA disease. However, adoptive transfer of $CD4^+$ T cells from those whose disease began failed to induce any IgA deposition or renal injury. Our study suggests that bone marrow cells, presuming IgA producing cells, may initiate this disease. Th1 cells may be involved in the pathophysiology of the disease after glomerular IgA deposition.

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KEYWORDS: IgA nephropathy; immune complexes; IgA deposition; lymphocytes; stem cell

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IgA nephropathy (IgAN) is the commonest form of progressive primary glomerulonephritis, exhibiting mesangial IgA and IgG co-deposition.[1] Although the pathogenesis of IgAN remains unclear, many studies convincingly suggest the involvement of dysregulation of the immune system.[2,3] Fundamental pathogenic factors may underlie outside of the kidney, because half of IgAN patients show recurrences after renal transplantation.[4]

A previous report demonstrated that in a patient with IgAN and chronic myeloblastic leukemia, bone marrow transplantation (BMT) resulted not only in remission of leukemia but also in IgAN.[5] In addition, Imasawa *et al.*[6] reported that BMT from normal mice attenuated glomerular lesion in a murine model of IgAN, HIGA (high serum IgA ddY) mice, while the glomerular lesion associated with IgA deposition was reconstituted in normal recipient mice after BMT from HIGA mice. The above background indicates that IgAN may involve disorders of stem cells.

Other studies discussed that functional abnormalities of B- and/or T cells may underlie in the pathogenesis of IgAN. Emerging evidence indicates the involvement of aberrantly glycosylated IgA1 in the pathogenesis.[7,8] Furthermore, Allen *et al.*[9] reported B-cell-restricted reduction of β 1,3 galactosyltransferase activity (responsible for the aberrant *O*-glycosylated serum IgA1). Of note, recent study reported that Th2 cytokines induced altered IgA glycosylation, presumably via reduction of β 1,3 galactosyltransferase enzymatic activity.[10] Moreover, in experimental IgAN, Th2 cytokines may alter the glycosylation of IgA produced by B cells,[11] although the molecular structure of murine IgA is different from that of human IgA1. Indeed, Th1/Th2 imbalance is expected to have an important role in this disease.[12] Many studies have found that the principal cytokines produced by circulating T cells in IgAN are Th2 type.[13,14] However, some other reports indicated Th1 predominance in IgAN,[15,16] emphasizing that the pathogenic importance of Th1/Th2 polarization in this disease remains unclear.

The ddY mice are known as a spontaneous murine IgAN model, but the incidence of IgAN is highly variable. Indeed, although HIGA mice show high serum levels of IgA, serum IgA levels are not associated with the severity of glomerular injury and incidence of the disease. We recently found that the ddY mice could be classified into three groups, the early

onset (~20 weeks), late onset (~40 weeks), and quiescent groups by serial biopsies that confirm the glomerular lesions and IgA deposition.[17] A genome-wide association study of the early onset and the quiescent mice revealed that the susceptibility to murine IgAN is partly regulated by specific loci syntenic to the *IGAN1* gene[18] known as a candidate gene of human familial IgAN.[17] Those results suggested suitability of the grouped ddY mice model for examining the pathogenesis of IgAN. Although administration of Th1 cytokines could induce crescentic glomerular lesions in HIGA mice,[19] it remains unclear whether Th-dependent immune regulation is specifically involved in the onset of IgAN.

This study with the grouped ddY mouse model aimed to examine the role of bone marrow-derived cells (BMC) in the induction and Th polarity of this disease and approach the potential mechanisms.

RESULTS

The murine IgAN is linked to Th1 polarity

We first examined the production of interferon (IFN)-γ and interleukin (IL)-4, which are representative cytokines of Th1 and Th2, respectively, in spleen cells of C57Bl/6 (B6) and BALB/C (BALB) mice (each $n=5$). Spleen cells of B6 and BALB mainly produced IFN-γ and IL-4, respectively. These findings are compatible with previous reports,[20] suggesting that major cells producing these cytokines may be $CD4^+$ T cells. Next, we investigated these cytokines production in the IgAN-onset and the quiescent ddY mice (each $n=5$). Although the levels of IFN-γ and IL-4 produced by cultured splenic cells were stable between 20 and 40 weeks of age in the quiescent group, the levels of IFN-γ were higher and those of IL-4 were lower in the onset mice than those of the quiescent mice at 20 and 40 weeks of age ($P<0.01$, each, Figure 1a). Furthermore, in the onset group, the level of IFN-γ at 40

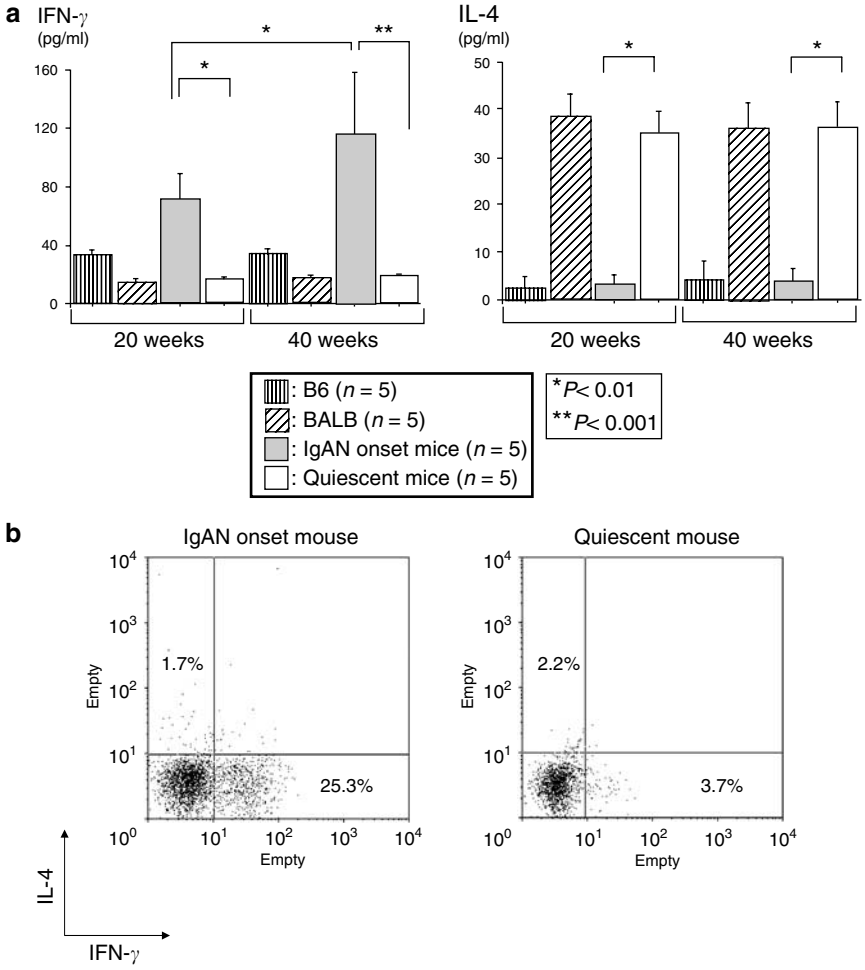

Figure 1 | Production of Th1/Th2 cytokines by splenic T cells. (**a**) Th1- and Th2-prone mice, B6 and BALB, show Th1- and Th2-dominant immune regulation, respectively. Although the levels of IFN-γ and IL-4 remained stable in the quiescent mice from 20 to 40 weeks of age, the onset mice showed significantly higher levels of IFN-γ and lower levels of IL-4 than those of the quiescent mice at 20 and 40 weeks of age ($P<0.01$). Furthermore, the levels of IFN-γ in the onset mice increased from 20 to 40 weeks of age ($P<0.01$). (**b**) To further confirm Th1 polarization in the onset mice, we checked intracellular IFN-γ production in $CD4^+$ T cells by FACS. $CD4^+$ T cells from the onset mice showed higher amounts of intracellular IFN-γ, suggesting strong Th1 polarization. Meanwhile, Th1 polarization is not shown in the quiescent mice; data are mean $\pm$ s.d.

weeks of age was higher than that at 20 weeks ($P<0.01$, Figure 1a). To further confirm Th1 polarization in the onset ddY mice, intracellular IFN-γ produced by $CD4^+$ T cells were analyzed using flow cytometric analysis (FACS). The FACS analysis showed that $CD4^+$ T cells strongly expressed IFN-γ in the onset mice, but not in the quiescent mice (Figure 1b).

Serum IgA–IgG2a immune complex levels correlate with severity of glomerular lesions

Immunohistological analyses showed that most of glomerular IgA was colocalized with IgG (Figure 2a), especially IgG2a (Figure 2b and c) in the onset group. Interestingly, IgA formed an immune complex (IC) with IgG2a in the sera of these mice, and the serum levels of this complex were higher in the onset group than the quiescent group ($P=0.0031$, Figure 3a, each $n=30$). Moreover, serum levels of IgA–IgG2a IC correlated with the severity of glomerular injury ($P<0.0001$, Figure 3b).

Murine IgAN is reconstituted in the quiescent mice by BMC from the onset mice

To verify that IgAN is directed by BMC, we conducted three different BMT (the onset mice to the quiescent mice, the onset mice to the onset mice, and the quiescent mice to the onset mice; $n=5$ for each BMT). BMT from the onset mice induced glomerular injury and mesangial IgA/IgG deposition with progression of albuminuria in the quiescent recipients (Figure 4a and d). BMT from the onset mice accelerated the glomerular injury and albuminuria in the recipient-onset mice (Figure 4b and d). Conversely, BMT from the quiescent mice improved not only glomerular injury and mesangial IgA/IgG deposition but also albuminuria in the recipient-onset mice (Figure 4c and d). These results are summarized in Table 1.

BMT from the onset mice resulted in Th1 polarization and a rise in serum IgA–IgG2a IC in recipient-normal mice

Next, we examined Th polarization after BMT. BMT (from the onset mice to the quiescent mice) resulted in increase in IFN-γ ($P<0.01$) and decrease in IL-4 production by spleen cells ($P<0.01$, Figure 5a, $n=5$). FACS analysis for the intracellular cytokine production revealed strong IFN-γ expression in $CD4^+$ T cells in the transplanted normal mice (Figure 5b), further confirming Th1 polarization with glomerular injury. Moreover, BMT from the onset mice further increased the level of IFN-γ in recipient-onset mice ($P<0.001$, Figure 5a, $n=5$). Importantly, serum levels of IgA–IgG2a IC were increased after BMT from the onset mice to both the quiescent ($P<0.01$) and the onset mice ($P<0.05$) (Figure 5c), although total serum levels of IgA, IgG, and IgM did not change significantly (Figure 5d). In contrast, a significant decrease in IFN-γ and increase in IL-4 were found after BMT from the quiescent mice in recipient-onset mice ($P<0.01$, Figure 5a, $n=5$) with improvement of glomerular injury and marked decrease in serum levels of IgA–IgG2a IC ($P<0.05$) (Figure 5c).

Adoptive transfer of $CD4^+$ T cells failed to induce murine IgA nephropathy

To further investigate whether $CD4^+$ T cells directly regulate the production of nephritogenic IgA production and thus induce this disease, $CD4^+$ T cells from the onset mice were adoptively transferred into BALB/cAJcl-*nu*/*nu* (nude) mice with or without serum from the onset mice. However, the $CD4^+$ T cells could not induce any renal injuries or IgA deposition in any cases.

DISCUSSION

In this study, we showed that systemic immune response in ddY mice with IgAN was strongly polarized toward Th1, which could be reconstituted in the quiescent and normal control mice by BMC from the onset mice. IFN-γ production by splenic T cells in the onset mice increased significantly from 20 to 40 weeks of age with progression of glomerular injury, and the severity was correlated with Th1 phenotypes.

Although the pathogenic importance of Th1/Th2 polarization in this disease remains unclear,[12–16] IgA production is critically driven by IL-5, a Th2 cytokine.[21] There is also evidence that Th2 cytokines regulate IgA glycosylation, partly via inactivation of β 1,3 galactosyltransferase.[10] Although the molecular structure of murine IgA differs from that of human IgA1, severity of murine IgAN is associated with abnormalities in IgA glycosylation in mice that have a predominant Th2 response (BALB) compared with control (C3HeB) mice.[22] By using transgenic mice of GATA-3 known as a key transcriptional factor of Th2, our recent study demonstrated that Th2-predominant mucosal immune responses are critical for glomerular IgA deposition (Yamanaka *et al.* reported at the annual meeting of the American Society of Nephrology, Philadelphia, PA, USA, 2005). These findings indicate that Th2 predominance may influence mucosal induction of IgA producing cells and thus subsequent mesangial IgA deposition in this disease.[23,24] This study showed that the quiescent mice are Th2 prone, while the onset mice have strong Th1 polarity, suggesting that both Th1 and Th2 polarities may contribute to different processes of this disease, presumably induction and progression steps, respectively.

A previous BMT study using HIGA and B6 mice indicated that BMC plays an important role in the pathogenesis of murine IgAN.[6] In this study, we also applied BMT in the same strain of mice; the IgAN-onset and the quiescent ddY mice. BMC from the quiescent mice abated not only glomerular IgA deposition but also expansion of mesangial matrix and proliferation of mesangial cells, indicating that this improvement may be partly dependent on disappearance of continuous delivery of nephritogenic IgA. Moreover, it is possible that bone marrow-derived renal resident cells may contribute to resolution of the matrix expansion after this BMT, as reported previously.[25] As shown in Figure 4b and d, BMT from the onset mice accelerated the glomerular injury and albuminuria in the recipient-onset mice. We may need to consider the roles of regulatory T cells (Treg) to assess this

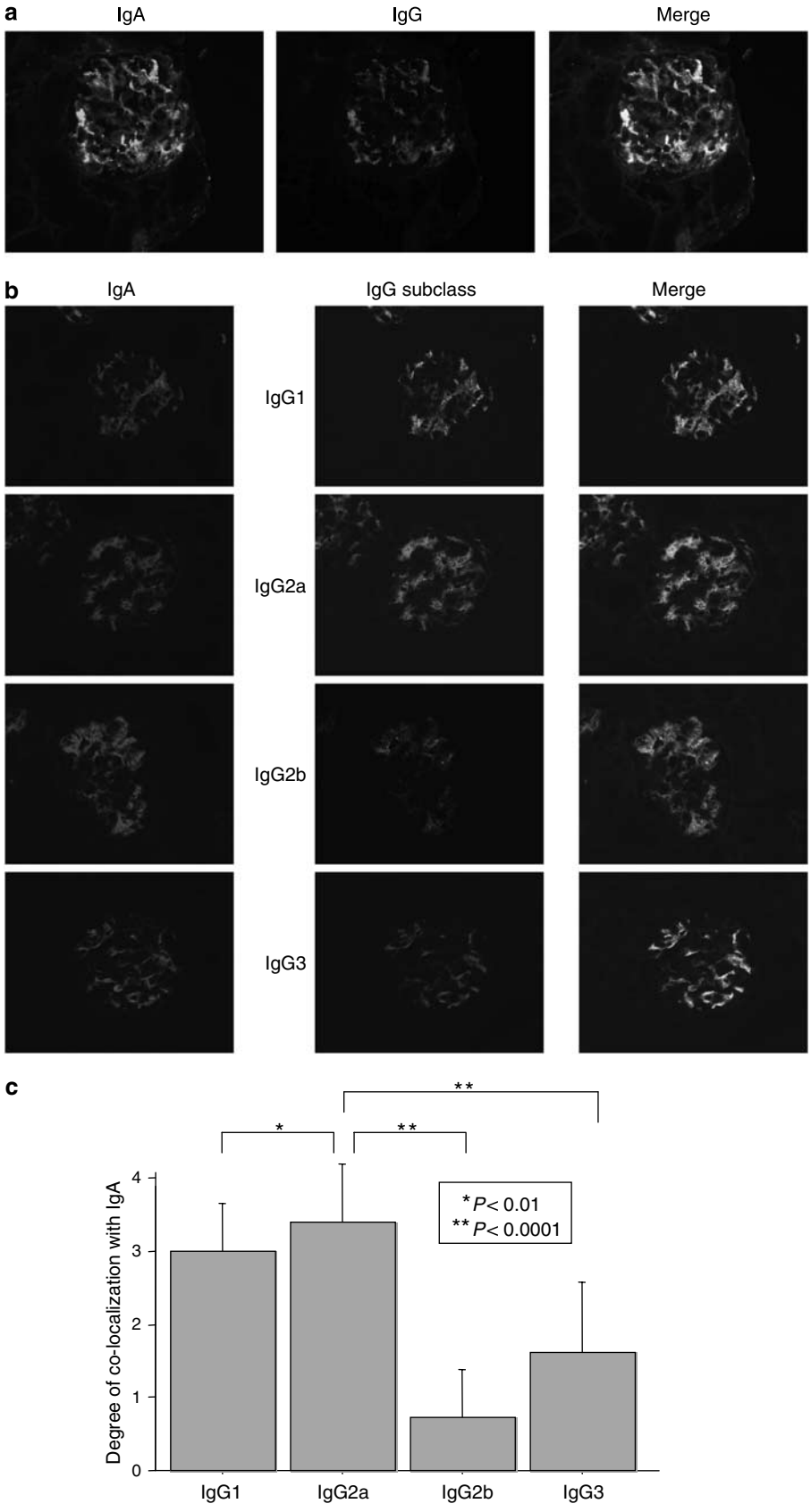

Figure 2 | Immunostaining of IgA, IgG, and each IgG subclass in the IgAN-onset ddY mouse. (**a**) Glomerular IgA deposition was colocalized with IgG. (**b**) Especially, IgG2a was well colocalized with IgA. Each original magnification is $\times 400$. (**c**) Glomerular IgA–IgG colocalization was semi-quantitated. Glomerular IgA was well colocalized with IgG2a, followed by IgG1, IgG3, and IgG2b. Data are mean $\pm$ s.d.

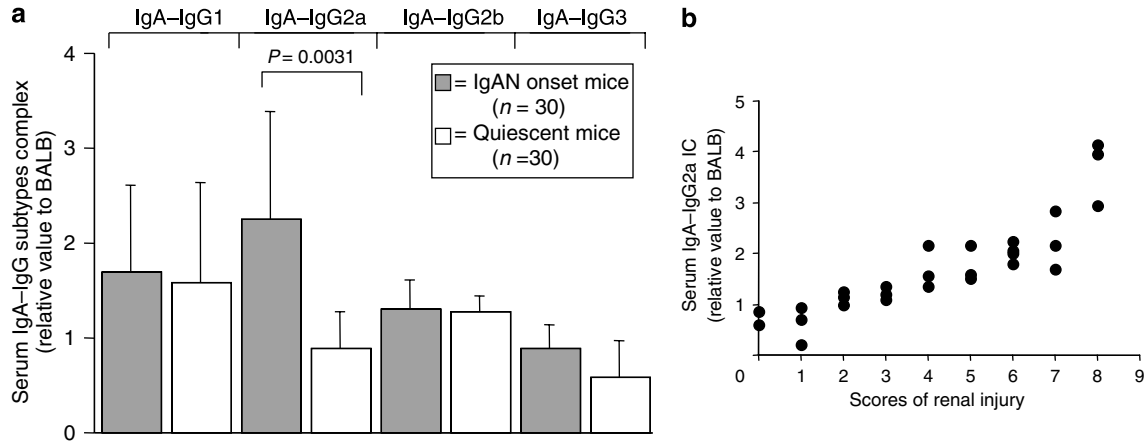

Figure 3 | Serum levels of IgA/IgG IC. (**a**) Serum levels of each IgA–IgG IC were expressed by relative value to those of BALB. Serum levels of IgA–IgG2a complex in the IgAN-onset ddY mice ($n = 30$) were significantly higher than those in the quiescent mice ($n = 30$) ($P = 0.0031$). Data are mean ± s.d. (**b**) Serum levels of IgA–IgG2a complex correlated with the severity of glomerular injury ($P < 0.0001$).

result. Treg play a suppressive role in many autoimmune diseases and can potentially affect various steps in the progression of disease.[26–28] Importantly, the balance between Treg and Th1 effector T cells is important in determining the outcome of disease development.[29,30] Indeed, some IgAN-onset mice showed rapid progression like the recipient-onset mice transplanted from the onset mice (data are not shown). Since Treg accumulated in secondary lymph nodes[31] might be depleted by total body irradiation in recipient-onset mice, Th1 effector function might be enhanced. In results, dramatically increased Th1 cytokine might accelerate progression of renal injuries. It is our urgent task to investigate how Treg regulate the murine IgAN for this hypothesis.

Although the reconstituted IgAN by BMT was accompanied with Th1 polarization, it is still unclear whether Th1 polarization is causal or resultant of renal injuries. Dendritic cells are unique bone marrow-derived antigen-presenting cells that play a key role in the initiation and modulation of immune responses. They also regulate $CD4^+$ T-cell priming and thus Th1/Th2 polarization.[32,33] Previous studies postulated that microbial antigens are involved in the pathogenesis of IgAN,[34,35] partly via dendritic cells function.[36] However, since all BMT in our study were performed under specific pathogen-free conditions, environmental factors such as additional exogenous microbial antigens could be neglected in the reconstitution after BMT. To examine whether $CD4^+$ T cells regulate the IgA production, $CD4^+$ T cells from the onset mice were adoptively transferred into nude mice. We also injected serum from the onset mice to exclude the possibilities that serum protein such as IgA–IgG2a IC is required for T- or B-cell functions. However, no mice showed any glomerular IgA deposition or renal injuries. Accordingly, bone marrow may include B-cell lineages that independently of $CD4^+$ T cells and continuously provide the nephritogenic IgA. Indeed, abnormal IgA1 in human IgAN is considered to be produced in bone marrow.[37,38] Moreover, our group recently reported that BMT from the onset mice into alymphoplasia mutant mice (aly/aly) lack all systemic lymph nodes, Payer's patch, and serum IgA-reconstituted glomerular IgA deposition, but not disease progression and Th1 polarization (Aizawa M *et al*. reported at the annual meeting of American Society of Nephrology, San Diego, CA, USA, 2006). These uncoupling data suggest that Th1 polarization may require secondary lymphoid tissues after the IgA deposition. Interestingly, serum levels of IgA–IgG2a IC correlated with the severity of renal injury in this disease. In rodents, it is known that deposition of antigen-specific IgG2a and glomerular IgG2a is linked to Th1-dominant immune regulation.[39,40] Although the underlying mechanisms for the formation of IgA–IgG2a IC and following glomerular deposition are still unclear, there is ample evidence for the presence of abnormal IgA in complexes with IgG even in IgAN patients.[41–45] In addition, it is noteworthy that human IgAN was originally described by Berger and Hinglais[1] as 'nephropathy with mesangial IgA–IgG co-deposits'. These reports suggest that IgA–IgG IC formation may be partly due to antigen–antibody interaction with altered IgA1 as the antigen.[45,46] Importantly, Tomana *et al*.[45] reported that the antibody subtype to the altered IgA1 in human IgAN was predominantly IgG2.

This study demonstrated that progression of murine IgAN is linked to systemic Th1 immune response accompanied by elevation of serum IgA–IgG2a IC levels. This polarization might be indirectly regulated by bone marrow-derived nephritogenic B cells followed by priming of $CD4^+$ T cells. To design new therapeutic strategies for IgAN, additional studies using this model are required to identify the responsible cell types and define the exact underlying mechanisms.

MATERIALS AND METHODS

Mice

Female ddY mice (SLC Japan, Shizuoka, Japan) were maintained at the animal facility of Juntendo University and provided with regular

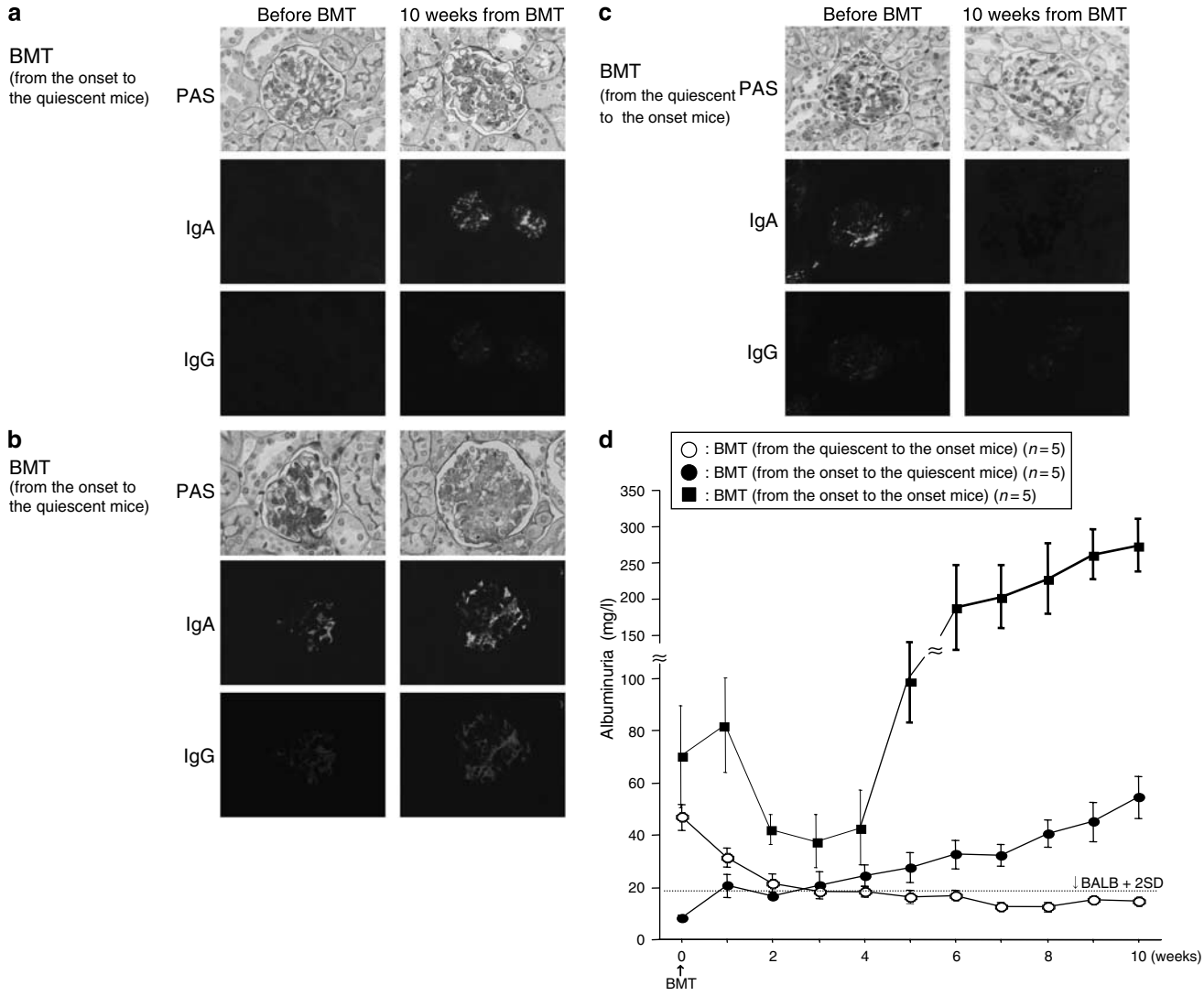

Figure 4 | BMC are responsible for the induction of IgAN. (**a**–**c**) Renal pathology. (**a**) BMT from the onset mice resulted in mesangioproliferative glomerular injury with mesangial deposition of IgA and IgG in the quiescent recipients. (**b**) BMT from the onset mice accelerated the progression of glomerular injury with IgA and IgG deposition in the onset mice. (**c**) In contrast, BMT from quiescent mice resulted in reduction of not only glomerular injury but also mesangial deposition of IgA and IgG in the recipient-onset mice. Each original magnification is × 400. (**d**) Urinary albumin levels. BMT from the onset mice caused progression of albuminuria in the recipient-quiescent mice. BMT from the onset mice caused marked enhancement of albuminuria in the recipient-onset mice. Conversely, levels of albuminuria were decreased in recipient-onset mice after BMT from the quiescent mice. Data are mean ± s.d.

Table 1 | Features of the IgAN onset and the quiescent mice and summary of results from BMT studies using those mice

Bone marrow transplantation		Kidney			Serum			Systemic
Donor	Recipient	Glomerular injury IgA deposition	Mesangial co-deposition	IgA–IgG	IgA–IgG2a IC level	IgA level	IgG level	Th1 polarity
IgAN onset	Quiescent	+	+	+	↑	→	→	↑
Quiescent	IgAN onset	↓	↓	↓	↓	→	→	–
IgAN onset	IgAN onset	++	++	++	↑↑	→	→	↑↑
IgAN onset mice	–	+	+	+	↑	→	→	↑
Quiescent mice	–	–	–	–	→	→	→	–

BMT, bone marrow transplantation; IgAN, IgA nephropathy.

chow (MF; Oriental Yeast, Tokyo, Japan) in a specific pathogen-free room. The original ddY mice were maintained as outbreed animal and thus genetically heterogeneous. However, we evaluated their renal histology by serial renal biopsies and found that they were in three different groups dependent on the renal injuries.[17] The ddY mice at 20 weeks of age were divided into two groups, the

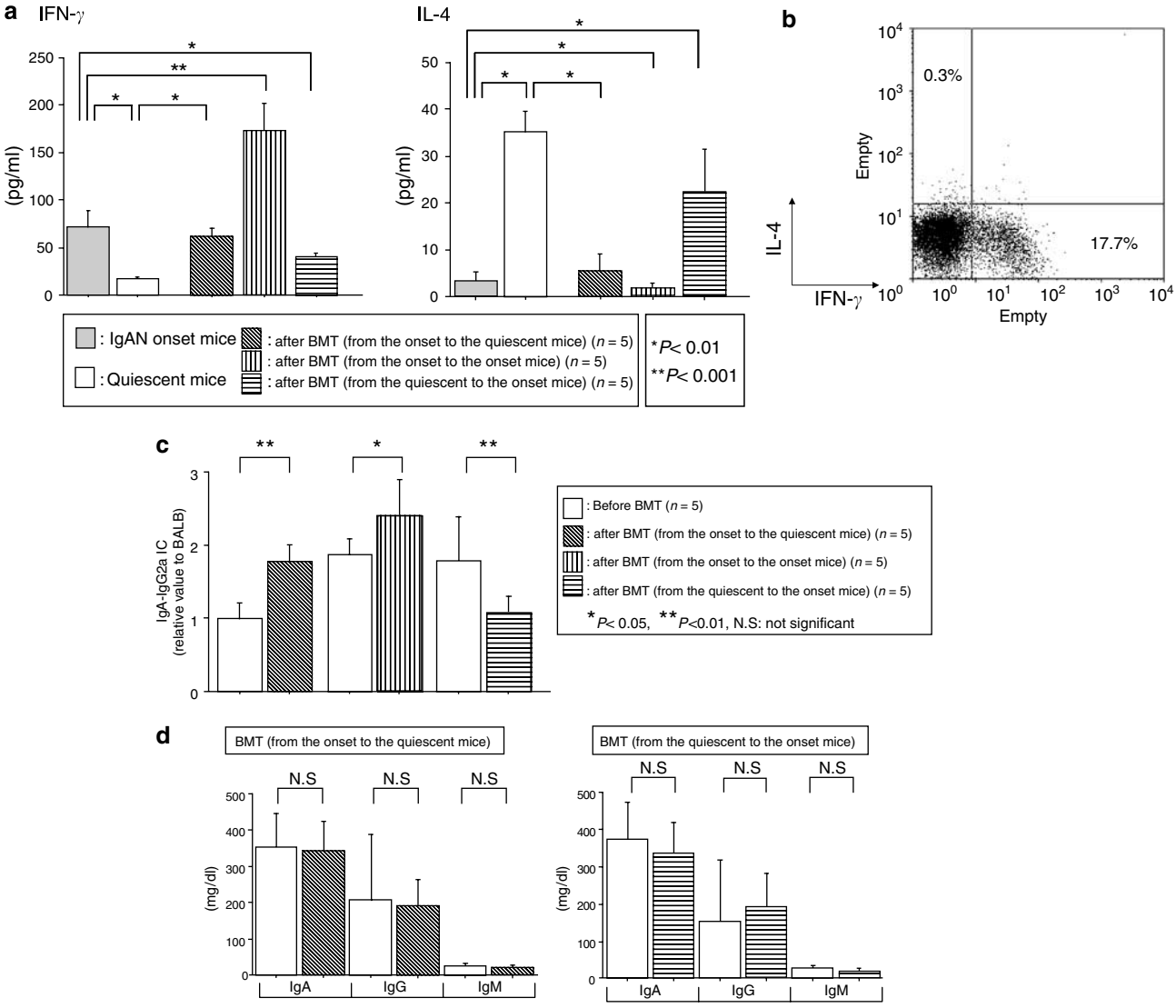

Figure 5 | BMT reconstituted IgAN with Th1 phenotypes. (**a**) Levels of IFN-γ increased and IL-4 levels decreased in recipient-quiescent mice after BMT from the onset mice ($P<0.01$). After BMT from the onset mice, IFN-γ levels further increased in recipient-onset mice ($P<0.001$). Conversely, the splenic productions of IFN-γ decreased and that of IL-4 increased in recipient-onset mice after BMT from the quiescent mice ($P<0.01$). (**b**) FACS analysis for the intracellular cytokine production revealed strong IFN-γ expression in $CD4^+$ T cells in the transplanted control mice from the onset mice. (**c**) Serum levels of IgA–IgG2a IC were expressed relative to those of BALB. BMT from the onset mice to both the quiescent** and the onset mice* resulted in increase in serum IgA–IgG2a complex (*$P<0.05$ and **$P<0.01$, respectively), while those levels were decreased after BMT from the quiescent to the onset mice. (**d**) Serum levels of IgA, IgG, and IgM did not change after each BMT. Data are mean $\pm$ s.d.

IgAN-onset and the quiescent groups by semi-quantitative analysis of biopsy specimens, as described previously by our group.[17] Same-age female BALB/c (BALB) and C57Bl/6 (B6) mice were used for control. Female BALB/cAJcl-*nu*/*nu* (nude) mice were used for adoptive transfer from the onset ddY mice. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Juntendo University School of Medicine.

Histological examination of renal tissues

For light microscopy, the renal specimens were fixed in 10% neutral phosphate-buffered formalin, embedded in paraffin, and sliced at 2 μm thickness. The slices were stained with hematoxylin and eosin, periodic acid-Schiff, and Azan. Snap frozen 4-μm thick renal sections were used for immunofluorescence with fluorescein isothiocyanate-conjugated goat anti-mouse IgA (BD Biosciences, San Diego, CA, USA; Pharmingen, San Diego, CA, USA) and rhodamine-conjugated goat anti-mouse IgG antisera (Cappel Research Reagents, Costa Mesa, CA, USA). For analysis of IgG subclasses, we used fluorescein isothiocyanate-conjugated rat anti-mouse IgG1, IgG2a, IgG2b, IgG3 (BD Biosciences, Pharmingen), and rhodamine-conjugated goat anti-mouse IgA antisera (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Semi-quantitative analysis of biopsy specimens

Renal injuries in the biopsy specimens were scored in a triple-blind manner by two nephrologists and one pathologist. Biopsy specimens

that contained more than 10 glomeruli were used for histopathological analysis. All biopsy specimens were quantitatively analyzed to determine the percentages of glomeruli with (a) segmental and global sclerosis and/or (b) mesangial cell proliferation and/or (c) increase in mesangial matrix. Each specimen was scored semi-quantitatively for the percentages of the aforementioned lesioned glomeruli (score 0 = 0%, score 1 = 1–24%, score 2 = 25–49%, and score 3 $\geqslant$50% of all glomeruli).[17,47,48] Total scores in each specimen maximally became nine using our scoring system. We defined renal injury as renal lesion whose histology score is more than the mean + 2 s.d. of the score in BALB mice. Since the average score in BALB mice was 1.424 ± 0.969, cases whose histology scores are more than four were regarded as renal injury in this study. For evaluation of degree of IgA–IgG colocalization, 10 glomeruli on 10 biopsy specimens from the onset mice were semi-quantitatively analyzed (score 0 = 0%, score 1 = 1–24%, score 2 = 25–49%, score 3 = 50–75%, and score 4 $\geqslant$75% of all glomeruli).

Measurement of serum IgA–IgG IC and urinary albumin excretion

Blood samples were obtained from the orbital venous plexus using capillary tubes. Serum IgA–IgG IC concentrations were measured by sandwich enzyme-linked immunosorbent assay. Enzyme-linked immunosorbent assay plate was coated overnight with 5 μg/ml purified rat anti-mouse IgG1, IgG2a, IgG2b, and IgG3 antisera (BD Biosciences, Pharmingen) for overnight at 4°C. Coated plates were blocked with 2% bovine serum albumin in phosphate-buffered saline, and duplicated serum samples were incubated overnight at 4°C. After incubation with horseradish peroxidase-conjugated goat anti-mouse IgA (ZYMED Laboratories, San Francisco, CA, USA), for 3 h at room temperature, wells were developed with tetramethylbenzidine (TMB) substrate reagent set (BD Biosciences, San Jose, CA, USA), and the absorbance was measured at 450 nm. Data were expressed relatively to the value of BALB. Urinary albumin was measured using an enzyme-linked immunosorbent assay kit (Albuwell, Exocell, Philadelphia, PA, USA). Serum and urine samples from BALB were used as control.

Bone marrow transplantation

The bone marrow was harvested from the tibia, femur, and humerus under sterile conditions. Red blood cells were removed by ACK lysing buffer (0.15 mol/l NH_4Cl, 1.0 mmol/l $KHCO_3$, and 0.1 mmol/l Na_2EDTA, pH 7.2) for 5 min at room temperature, and washed in RPMI-1640 media supplemented with 1% fetal calf serum (FCS). Recipient mice were exposed to 10 Gy total body irradiation to deplete own BMC. To prevent the effect of irradiation on kidney, the lower abdomen was covered with a lead protector. After total body irradiation, 1×10^7 donor BMC were injected into the tail vein on day 0. The resulting chimeric mice were housed under specific pathogen-free conditions for 10 weeks to allow bone marrow reconstitution. Environmental factors were not altered before and after each BMT. Urinalysis was performed every week after BMT. After 10 weeks from BMT, renal histology was analyzed and blood samples were collected for measurement of immunoglobulin and IgA–IgG2a IC.

Measurement of cytokines produced by splenic T cells

To assess the production of IFN-γ and IL-4 by spleen cells, spleens were removed from the mice. Spleen cell suspensions were prepared by compression with the handle of a syringe in RPMI-1640 media with 1% FCS followed by passage through a 100 μm nylon mesh. Red blood cells were removed by ACK lysing buffer (0.15 mol/l NH_4Cl, 1.0 mmol/l $KHCO_3$, and 0.1 mmol/l Na_2EDTA, pH 7.2) for 5 min at room temperature, and washed in RPMI-1640 media with 1% FCS. Spleen cells (1×10^6 cells/ml) were cultured in RPMI-1640 media with 10% FCS for 48 h at 37°C. IFN-γ and IL-4 were measured using enzyme-linked immunosorbent assay kit (ENDOGEN, Pierce Biotechnology, Rockford, IL, USA). To further confirm that $CD4^+$ T cells in the diseased mice mainly produce IFN-γ, we analyzed intracellular IFN-γ in $CD4^+$ T cells by FACS. Splenic $CD4^+$ T cells were isolated by positive selection using anti-CD4 (L3T4) MicroBeads (MACS®, Miltenyi Biotec, Bergisch Gladbach, Germany), according to the supplier's instructions. Briefly, spleen cells were resuspended at 1×10^7 cells/90 μl and 10 μl of anti-CD4 MicroBeads was added to cells for 15 min at 4°C. Then, $CD4^+$ T cells were isolated by magnetic separation. $CD4^+$ T cells (1×10^6/ml) were analyzed for IFN-γ production by intracellular staining. For IFN-γ staining, purified $CD4^+$ T cells were cultured in RPMI-1640 media with 10% FCS, stimulated for 2 h with 1 μg/ml of ionomycin, and 10 μg/ml Breferdin-A (Sigma-Aldrich, St Louis, MO, USA). After permiabilizing (BD FACS Permeabilizing solution 2, BD Biosciences), fluorescein isothiocyanate-conjugated rat anti-mouse IFN-γ, rat anti-mouse CD4-PerCP-Cy5.5 (BD Pharmingen™ BD Biosciences, San Jose, CA, USA) were used for staining. Samples were acquired using an FACScan (BDIS™, BD Biosciences, San Jose, CA, USA), and the data were analyzed by WinMDI (Windows Multiple Document Interafce for Flow Cytometry).

Adoptive transfer of CD4T cells

$CD4^+$ T cells (1×10^6) were isolated from the spleen of onset ddY mice at 20 weeks of age using CD4 (L3T4) MicroBeads (MACS®). Serum was also collected at the same time. $CD4^+$ T cells with or without serum and serum alone were injected into nude mice. The serum (100 μl) was injected to each mouse once a week for three times. After 3 weeks, renal specimens were histologically analyzed for the induction of IgAN.

Statistical analysis

Correlations between the different parameters were analyzed by the Student's *t*-test. Analysis of variance was used to determine differences in the characteristics among multiple groups. Data are expressed as mean ± s.d. or median values. $P < 0.05$ were considered significant. All statistical analyses were performed using the Macintosh version of StatView 5.0 software (Abacus Concept Inc., Berkeley, CA, USA).

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