Intranuclear delivery of the transcription modulation domain of Tbet-improved lupus nephritis in (NZB/NZW) F1 lupus-prone mice

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Excessive expression of Tbet and IFNγ is evidence of systemic lupus erythematosus (SLE) in lupus patients. In this study, the nucleus-transducible form of Transcription Modulation Domain (TMD) of Tbet (ntTbet-TMD), which is a fusion protein between Protein Transduction Domain Hph-1 (Hph-1-PTD) and the TMD of Tbet comprising DNA binding domain and isotype-specific domain, was generated to inhibit Tbet-mediated transcription in the interactomic manner. ntTbet-TMD was effectively delivered into the nucleus of the cells and specifically inhibited Tbet-mediated transcription without influencing the differentiation of other T cell subsets and signaling events for T cell activation. The severity of nephritis was significantly reduced by ntTbet-TMD as effectively as methylprednisolone in lupus-prone mice. The number of Th1, Th2 or Th17 cells and the secretion of their cytokines substantially decreased in the spleen and kidney of lupus-prone mice by ntTbet-TMD treatment. In contrast to methylprednisolone, the marked increase of Treg cells and the secretion of their immunosuppressive cytokine were detected in the spleen of (NZB/NZW) F1 mice treated with ntTbet-TMD. Thus, ntTbet-TMD can improve nephritis in lupus-prone mice by modulating the overall proinflammatory microenvironment and rebalancing T cell subsets, leading to new immune therapeutics for Th1-mediated autoimmune diseases.

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Lupus nephritis (LN) is one of the major manifestations of systemic lupus erythematosus (SLE), which is characterized by proteinuria, heterogeneous histologic alteration, and immune complex deposition in the kidneys.[1,2] SLE has been known to be initiated by dendritic cells and autoreactive B-cell dysregulation, but recent studies suggest that it is mainly affected by several T-cell subsets,[3] among which autoreactive T helper 1 (Th1) cells in particular have been considered to play a major role in the pathogenesis of LN by creating a proinflammatory microenvironment. However, the direct pathogenic role of Th1 cells in LN remains uncertain.[4]

Tbet (encoded by *Tbx21*) is the main transcription factor for Th1 cells, which has a role in controlling the infection of intracellular pathogens. Tbet directly binds to the *Ifng* promoter for the expression of interferon-γ (IFN-γ), which is important for the differentiation of Th1-cell lineage.[5] Although Th1 cells have a pivotal role in clearing intracellular pathogens, the excessive Th1-mediated immune responses can be the reason for their pathogenesis or exacerbating factors for many chronic inflammatory autoimmune diseases[6] such as multiple sclerosis,[7] type 1 diabetes,[8] and Crohn's disease.[9] Other than Th1 cells, Tbet can be expressed in Th17 cells and B cells, which also participates in the pathogenesis of autoimmune diseases. IFN-γ–producing Th17 cells have been identified as a new subpopulation of pathogenic Th17 cells in several autoimmune models, and Tbet expression in B cells mediates IgG2a production, which may, in turn, activate the development of SLE as well as LN.[10,11]

Recently, various monoclonal antibodies blocking the functions of inflammatory cytokines or their receptors are being considered as the main therapeutic modality in many autoimmune diseases. However, many studies have demonstrated that many autoimmune patients showed therapeutic unresponsiveness to these monoclonal antibodies, and their therapeutic efficacy was gradually reduced due to the production of antibodies to these biological agents or functional redundancy of the inflammatory cytokines during disease progression.[12,13] Therefore, the therapeutic efficacy of the current biological agents for LN is not satisfactory to replace cyclophosphamide or mycophenolate mofetil.[14,15] Therefore, novel therapeutic approaches to modulate the functions of the transcription factor responsible for the initiation and maintenance of Tbet-mediated inflammatory microenvironment are critically needed.

In this study, a novel therapeutic strategy was developed to inhibit the function of endogenous Tbet by delivering the transcription modulation domain (TMD) of Tbet into the nucleus of cells *in vitro* and *in vivo* via protein transduction domain Hph-1 (Hph-1-PTD), thereby preventing Tbet-mediated transcription in an interactomically inhibitory manner without using virus-mediated or genetic methods. The functional specificity of nucleus transducible (nt) Tbet-TMD *in vitro* and *in viv*o was investigated, and its *in vivo* therapeutic efficacy in nephritis in lupus-prone (NZB/NZW) F1 mice was analyzed.

RESULTS

Generation of ntTbet-TMD and its intranuclear delivery kinetics

To modulate the functions of Tbet without genetic alteration and the use of virus-mediated methods, ntTbet-TMD was generated in which Hph-1-PTD enables Tbet-TMD to be delivered into the nucleus of the cells *in vitro* and *in vivo* with high efficacy. Tbet-TMD contains the isotype-specific domain and T-box domain, therefore playing a critical role in isotype-specific DNA binding to the Th1-related gene promoter (Figure 1a).[5,16–18] ntTbet-TMD lacking DNA-binding activity (ntTbet-TMD[R164A]) and Tbet-TMD without Hph-1-PTD were generated as the negative controls (Figure 1a). ntTbet-TMD was expressed in the *Escherichia coli* expression system and purified under native condition, and its identity was confirmed by Western blot using anti-FLAG monoclonal antibody and sodium dodecylsulfate-polyacrylamide gel electrophoresis (Figure 1b).

When primary T cells were treated with different concentrations of ntTbet-TMD or for different periods of time, only 0.2 μM of ntTbet-TMD incubated <1 hour was required for the intranuclear delivery (the upper and middle panels of Figure 1c), whereas Tbet-TMD without Hph-1-PTD failed to penetrate the cells at all. Most of the transduced ntTbet-TMD were detected in the nucleus and maintained stability inside the cells for >24 hours (the lower panels of Figure 1c and d). Treatment of the primary T cells with as much as 4 μM of ntTbet-TMD did not show any cytotoxicity (Figure 1e). Therefore, ntTbet-TMD is effectively delivered into the nucleus of the primary T cells in a dose- and time-dependent manner and retains its stability without any cellular cytotoxicity.

Specific inhibition of Th1-cell differentiation by ntTbet-TMD without influencing the differentiation of other T-cell subsets and T-cell activation

To investigate the functional specificity of ntTbet-TMD to Tbet-mediated transcription, HEK 293 cells were cotransfected with wild-type Tbet- or EOMES-expressing vector and *Ifng* promoter-luciferase vector, and then incubated with 4 μM or 1 μM of ntTbet-TMD. Tbet and EOMES are members of Tbox family, which bind to the different regions of *Ifng* promoter.[19] Luciferase activity induced by wild-type Tbet was substantially reduced by ntTbet-TMD in a dose-dependent manner and ntTbet-TMD(R164A) did not affect luciferase activity (Figure 2a). Interestingly, ntTbet-TMD did not influence luciferase expression induced by EOMES, demonstrating that ntTbet-TMD inhibits the transcription of Tbet-inducible genes in an isotype-specific manner.

To examine whether ntTbet-TMD can specifically block the differentiation of naïve T cells into Th1 cells, the differentiation of naïve T cells into Th1, Th2, Th17, or induced regulatory T cell (iTreg) cells was induced by each T-cell subset–polarizing condition in the presence of ntTbet-TMD. The level of IFN-γ secretion by Th1 cells was significantly reduced by ntTbet-TMD and not by ntTbet-TMD(R164A) (Figure 2b). Surprisingly, ntTbet-TMD did not influence the differentiation of naïve T cells into Th2, Th17, or iTreg nor the level of T-cell activation that was evaluated by an analysis of the inducible surface expression of CD69 and IL-2 secretion (Figure 2c–e). Taken together, ntTbet-TMD inhibits Tbet-mediated transcription in a competitively interactomic manner with a high level of isotype specificity and blocks Th1-cell differentiation in a T-cell subset–specific manner without affecting T-cell activation.

In vivo therapeutic efficacy of ntTbet-TMD for nephritis in lupus-prone mice

The therapeutic potential of ntTbet-TMD for nephritis *in vivo* was evaluated by comparing it with that of methylprednisolone in lupus-prone mice. At 23 weeks of age, all the mice exhibited proteinuria higher than grade 2. Two untreated mice died of lupus exacerbation at 28 weeks of age, whereas all other mice treated with Tbet-TMD, ntTbet-TMD(R164A), ntTbet-TMD, or methylprednisolone survived (Figure 3b). At 30 weeks of age, treatment with ntTbet-TMD significantly reduced proteinuria in a dose-dependent manner, and the therapeutic efficacy of 100 μg per mouse of ntTbet-TMD treatment was comparable to that of methylprednisolone, but Tbet-TMD– or ntTbet-TMD(R164A)–treated mice did not alleviate the severity of lupus symptoms (Figure 3c). Also, the level of serum creatinine in 100 μg of ntTbet-TMD– or methylprednisolone-treated mice considerably decreased compared with that in untreated, Tbet-TMD–treated, or ntTbet-TMD(R164A)–treated mice (Figure 3c). These results

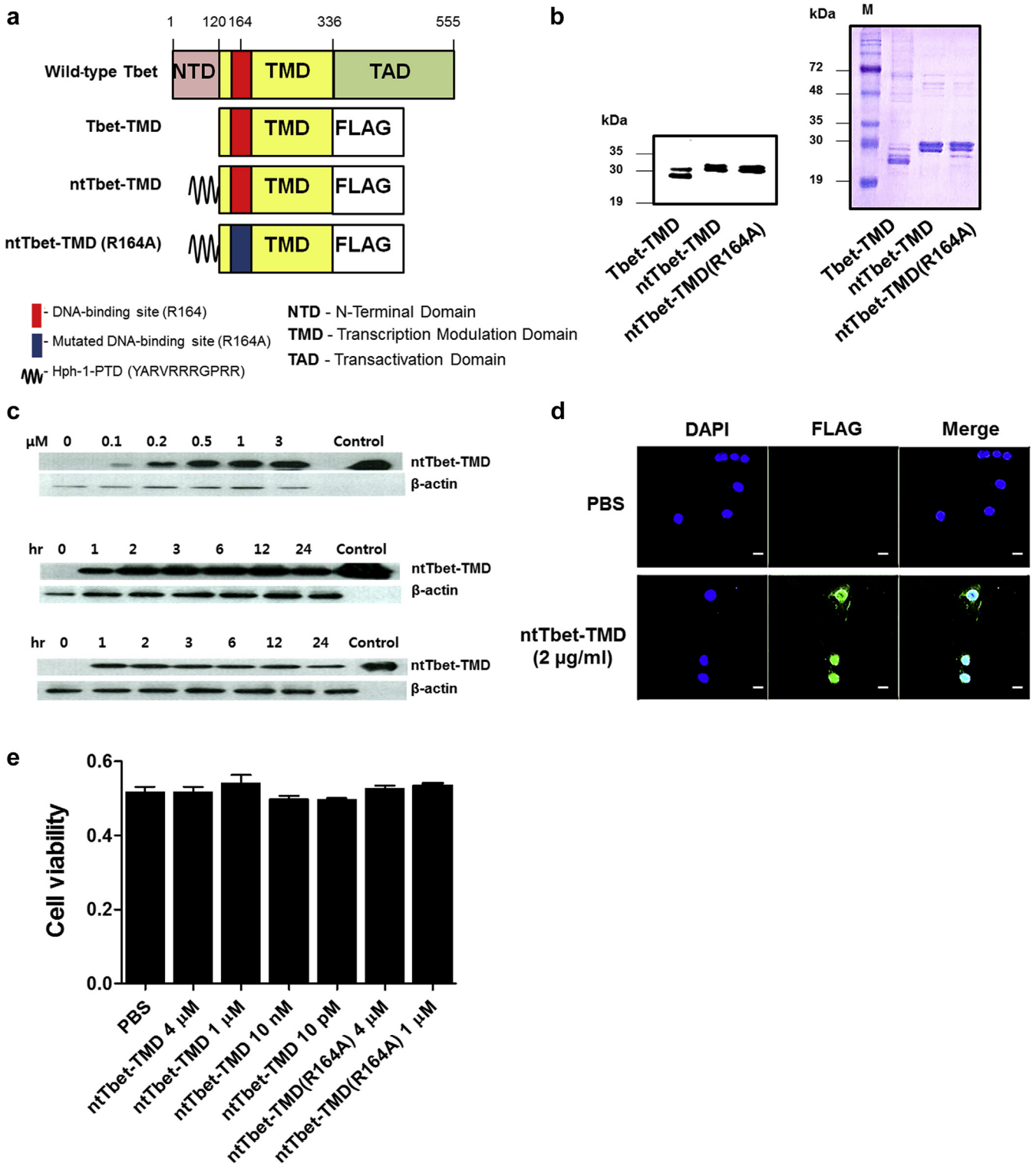

Figure 1 | Generation of nucleus-transducible (nt)Tbet-transcription modulation domain (TMD) and characterization of its intranuclear delivery kinetics. (**a**) DNA construct of 3 types of Tbet-transcription modulation domain (Tbet-TMD); Tbet-TMD without protein transduction domain Hph-1 (Hph-1-PTD), the nucleus-transducible (nt) form of Tbet-TMD (ntTbet-TMD), and a mutant form of ntTbet-TMD (ntTbet-TMD [R164A]). (**b**) ntTbet-TMD was purified, and its identity was confirmed by Western blot and sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE). (**c**) Dose- (upper panel) or time- (middle panel) dependent intracellular transduction kinetics of ntTbet-TMD were examined with mouse primary T cells by Western blot. Protein stability was confirmed in primary T cells (lower panel). β-Actin was detected as the control. (**d**) Intranuclear localization of ntTbet-TMD was analyzed by immunofluorescence staining with anti-FLAG antibody or 4′,6-diamidino-2-phenylindole (DAPI) after 1 hour of protein transduction. The cells were visualized by confocal microscopy. Bar = 10 μm. (**e**) Cellular cytotoxicity of ntTbet-TMD with different concentrations was examined by cell counting kit-8 analysis. PBS, phosphate-buffered saline. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

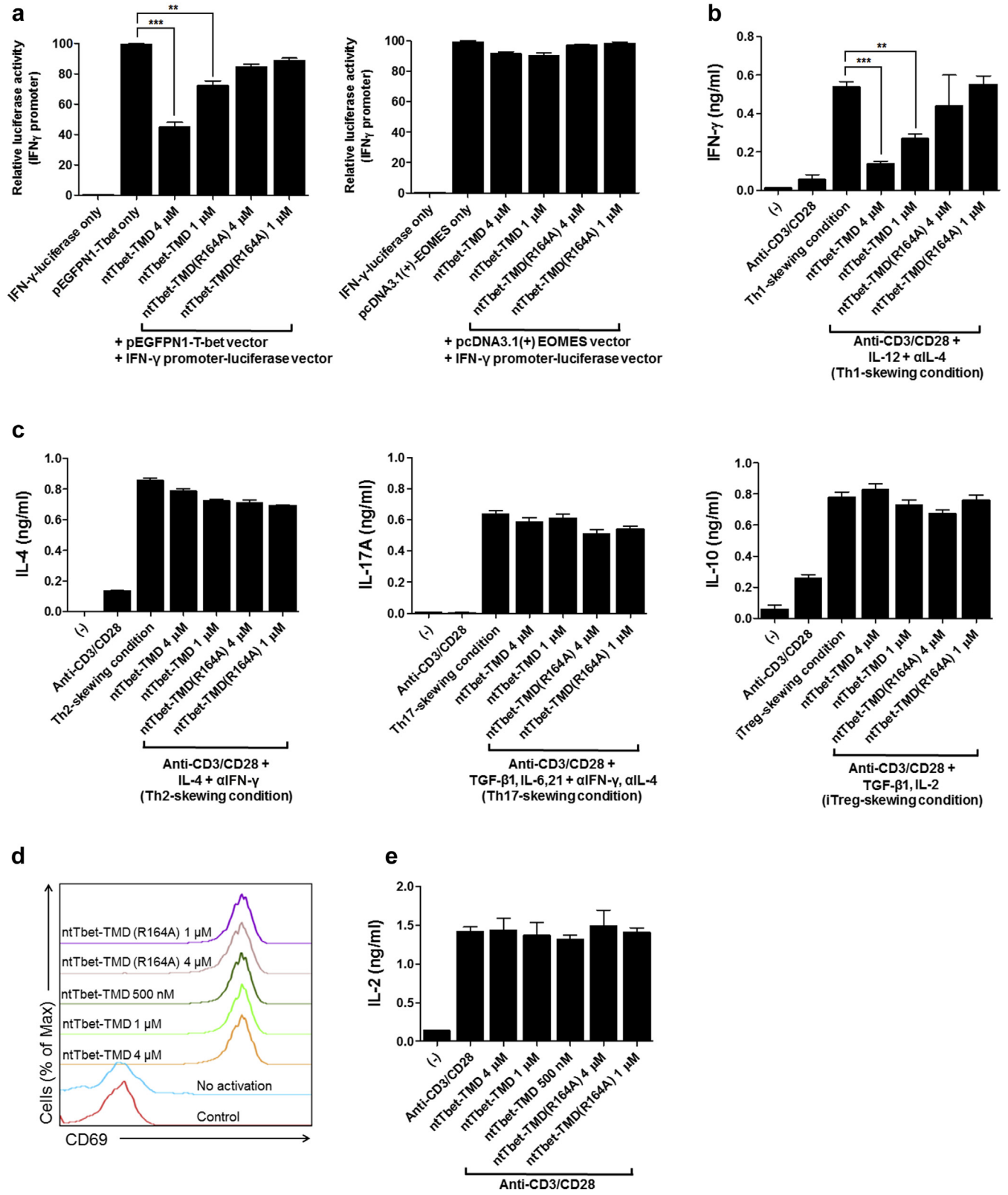

Figure 2 | Functional specificity of nucleus-transducible (nt)Tbet-transcription modulation domain (TMD) *in vitro*. (**a**) HEK 293 cells were transiently cotransfected with pEGFP-N1-Tbet or pcDNA3.1(+)-EOMES and the *Ifng* promoter–luciferase vector followed by the treatment with various forms of ntTbet-TMD with different concentrations for 24 hours. Luciferase activity was measured by a luminometer, and relative measurement was calculated by *Renilla* activity. (**b**) Differentiation of mouse $CD4^+CD62L^+$ naïve T cells into T helper 1 (Th1) cells was induced by Th1-polarizing condition for 72 hours in the presence of 4 or 1 μM or 500 nM of ntTbet-TMD or 4 μM of ntTbet-TMD(R164A). The level of interferon-γ (IFN-γ) in the culture media was analyzed by enzyme-linked immunosorbent assay (ELISA). (**c**) Differentiation of mouse $CD4^+CD62L^+$ naïve T cells into Th2, Th17, or Treg cells was induced by each subset-polarizing condition in the presence of (continued)

showed that ntTbet-TMD clearly improved nephritis in lupus-prone mice with a therapeutic efficacy similar to that of methylprednisolone.

Reduction of histologic and immunologic abnormality related to nephritis by ntTbet-TMD in lupus-prone mice

Next, the therapeutic effect of ntTbet-TMD on the histologic and immunologic abnormality of lupus-prone mice was examined. Kidney tissue of untreated mice, Tbet-TMD–treated mice, and a mutant form (R164A) of ntTbet-TMD–treated mice showed severe glomerular expansion and cell proliferation, inflammatory cell infiltration, and focal crescent formation, whereas the mice treated with ntTbet-TMD or methylprednisolone exhibited a substantial improvement of glomerular, tubular, and vascular damage (Figure 4a). Heavy accumulation of IgG (green) and C3 (red) as immune-complex formation (merged yellow) was observed in the mesangium and in capillary loops of kidney tissue of untreated, Tbet-TMD–treated, and ntTbet-TMD(R164A)–treated mice, which was significantly reduced by treatment with ntTbet-TMD or methylprednisolone (Figure 4b). The level of improvement of histologic and immunologic damage by 100 μg of ntTbet-TMD was comparable to that of methylprednisolone. These results indicated that the functional modulation of Tbet in T cells by ntTbet-TMD can attenuate nephritis in lupus-prone mice by reducing glomerular immune-complex deposition.

Functional rebalance of splenic T-cell subsets and reduction of pathogenic molecules in lupus-prone mice treated with ntTbet-TMD

To investigate whether ntTbet-TMD treatment can influence the immunologic status of T-cell subsets in lupus-prone mice, the number of each T-cell subset in the spleen of the treated animals was examined. The total number of $CD4^+$ T cells was downregulated in ntTbet-TMD– and methylprednisolone-treated mice (Figure 5a). ntTbet-TMD treatment also significantly reduced $CD4^+IFN\text{-}\gamma^+$ T cells and induced an increase in $CD4^+Foxp3^+$ T cells in a dose-dependent manner and a substantial decrease in the number of $CD4^+IL\text{-}17A^+$ T cells and $CD4^+IL\text{-}4^+$ T cells. However, methylprednisolone reduced the level of all T-cell subsets (Figure 5b and Supplementary Figure S1A–D). Consistent with the alteration in the number of inflammatory T-cell subsets, ntTbet-TMD or methylprednisolone treatment inhibited spontaneous progression of splenomegaly and reduced spleen weight (Figure 5c). Consistent with the results shown in Figure 5a–c, 100 μg of ntTbet-TMD treatment considerably lowered the levels of IFN-γ, IL-17A, and IL-6 in the serum; the reduction of these inflammatory cytokines in the serum by ntTbet-TMD was dose dependent. Interestingly, the level of immunosuppressive cytokine IL-10 in the serum was markedly upregulated by ntTbet-TMD treatment, which was not observed with methylprednisolone treatment (Figure 5d).

To examine whether alteration of the ratio of T-cell subsets *in vivo* can modulate the population of pathogenic B cells or the production of pathogenic molecules for lupus onset and maintenance, the number of $CD19^+$ B cells in the spleen or the levels of anti–double-stranded DNA autoantibody and inflammatory cytokines in the serum were examined. Autoantibody-producing plasmablasts or plasma cells share the characteristic of expressing CD19 on the cell surface.[20] To confirm whether ntTbet-TMD could influence the functions of pathogenic B cells, which are the inducer of LN, the level of $CD19^+$ cells in the spleen was examined by flow cytometry. The reduced percentage of splenic $CD19^+$ B cells was observed in ntTbet-TMD- or methylprednisolone-treated mice (Figure 6a). Furthermore, methylprednisolone or ntTbet-TMD treatment dose-dependently reduced the level of anti–double-stranded DNA autoantibody in serum compared with no treatment, Tbet-TMD, and ntTbet-TMD(R164A), and an especially significant decrease in serum anti–double-stranded DNA autoantibody with IgG2a and IgG3 subclass was observed with treatment with 100 μg of ntTbet-TMD, which are well-known pathogenic molecules for LN (Figure 6b–f).[21,22] These results imply that functional modulation of Tbet in the T-cell subset by ntTbet-TMD can suppress the generation of pathogenic B cells and autoantibody with lupus-pathogenic IgG subclasses and the secretion of inflammatory cytokines in the lupus-prone mice while inducing more immunosuppressive cytokine IL-10. Therefore, ntTbet-TMD may alleviate nephritis in lupus-prone mice by converting the pathogenic status of inflammatory T-cell subsets to a nonpathogenic status or increasing $CD4^+$ Treg cells via functional modulation of Tbet.

Reduced infiltration of proinflammatory T cells in the kidney by treatment with ntTbet-TMD

Furthermore, the intrarenal composition of inflammatory T cells in (NZB/NZW) F1 mice was measured to confirm the inhibitory effects of ntTbet-TMD on the infiltration of inflammatory T cells into the target organ. Infiltration of $CD4^+$ T cells into kidney was significantly increased in untreated, Tbet-TMD-treated, or Tbet-TMD(R164A)-treated mice, whereas the number of intrarenal $CD4^+$ T cells was very small in 25 μg or 100 μg of ntTbet-TMD or methylprednisolone-treated mice (Figure 7a). Consistent with the change in splenic inflammatory T cells, the number of $CD4^+IFN\text{-}\gamma^+$ T cells, $CD4^+IL\text{-}4^+$ T cells, or $CD4^+IL\text{-}17A^+$ T cells was markedly decreased in ntTbet-TMD–treated mice as well as in methylprednisolone-treated mice (Figure 7b and Supplementary Figure S2A and S2B). In

Figure 2 | (continued) 4 μM or 1 μM of ntTbet-TMD or ntTbet-TMD(R164A) for 72 hours. The level of interleukin (IL)-4, IL-17A, or IL-10 was evaluated by ELISA. (**d**,**e**) Primary T cells were activated by anti-CD3/CD28 monoclonal antibody in the presence of ntTbet-TMD or ntTbet-TMD(R164A) with the designated concentrations. The induced level of CD69 expression or the secretion of IL-2 was analyzed by staining with anti-CD69-fluorescein isothiocyanate antibody or ELISA, respectively. Error bars represent SEM (**$P < 0.01$, ***$P < 0.001$). TGFβ1, transforming growth factor-β1.

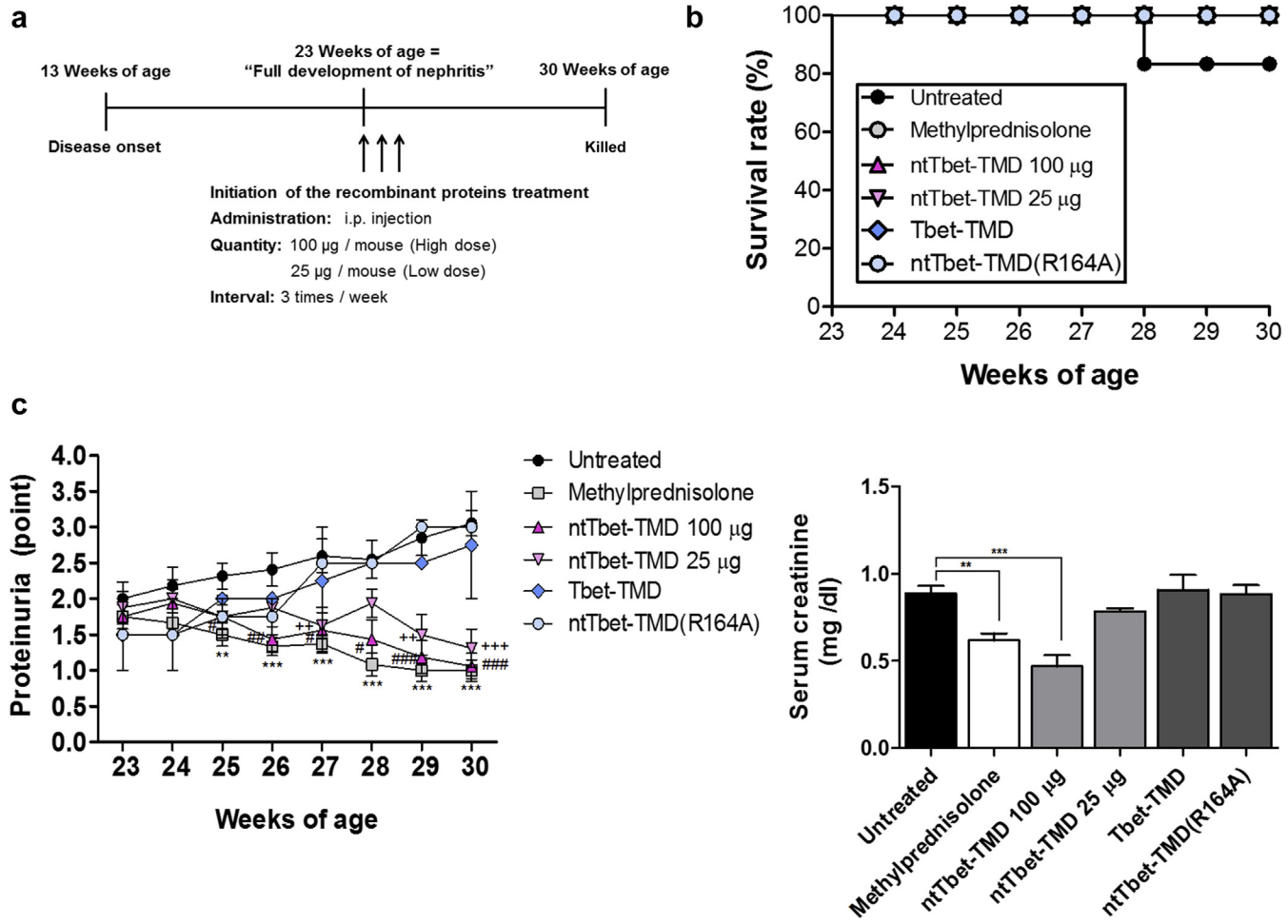

Figure 3 | Nucleus-transducible (nt)Tbet-transcription modulation domain (TMD) alleviated lupus nephritis in lupus-prone mice. (**a**) Treatment scheme of (NZB/NZW) F1 mice with phosphate-buffered saline (untreated), a high or low dose (100 or 25 μg/mouse) of ntTbet-TMD, methylprednisolone (140 μg/mouse), Tbet-TMD (100 μg/mouse), or ntTbet-TMD(R164A) (100 μg/mouse) is represented. (**b**) Survival rate of the lupus-prone mice treated with ntTbet-TMD or methylprednisolone. (**c**) Proteinuria level in the treated mice was measured twice weekly by urine dipstick. The level was determined based on a scale of 0 (none or trace) to 4+ (>1000 mg/dl). Creatinine concentration was measured by a kinetic colorimetric method using a creatinine assay kit. The results are the mean ± SEM. #$P < 0.05$; **, ##, ++$P < 0.01$; ***, ###, +++$P < 0.001$ were significant. *Untreated and methylprednisolone group. #Untreated and ntTbet-TMD 100 μg. +Untreated and 25 μg ntTbet-TMD.

contrast to the significant increase in splenic Treg cells, the number of intrarenal Treg cells was relatively low in lupus-prone mice treated with ntTbet-TMD. In comparison, the number of Treg cells in the kidneys was increased in all 3 groups (untreated, Tbet-TMD-treated, or ntTbet-TMD [R164A]-treated) in contrast to that in the spleen (Figure 7b and Supplementary Figure S2C). Thus, ntTbet-TMD can control the balance between each subset of inflammatory T cells in the spleen and kidneys to maintain overall immune homeostasis in a different manner.

DISCUSSION

LN can be categorized into 6 classes from minimal mesangial LN (class I) to sclerosing LN (class VI). Among these classes, proliferative LN (classes III and IV) shows the most severe clinical features and disease course, and without proper treatment, end-stage renal disease may develop in a considerable number of patients.[23] Treatment with glucocorticoid plus either cyclophosphamide or mycophenolate mofetil is the currently recommended standard induction therapeutic regimen for class III and IV LN.[24] Also, a low dose of glucocorticoid with or without immunosuppressive agents such as azathioprine, mycophenolate mofetil, and tacrolimus can be administered for a relatively long period as maintenance therapeutic regimens for relapse prevention.[24] However, these traditional medications may induce serious and unwanted complications including infection, pancytopenia, bladder cancer, and infertility. In addition, despite improved outcomes of these therapeutic regimens, end-stage renal disease still can occur in 30% of patients during the course of the disease.[25] Furthermore, several biological agents such as rituximab and belimumab have been introduced and used in clinical practice, but their efficacy was not satisfactory in LN refractory to traditional medications.[26,27] Therefore, new

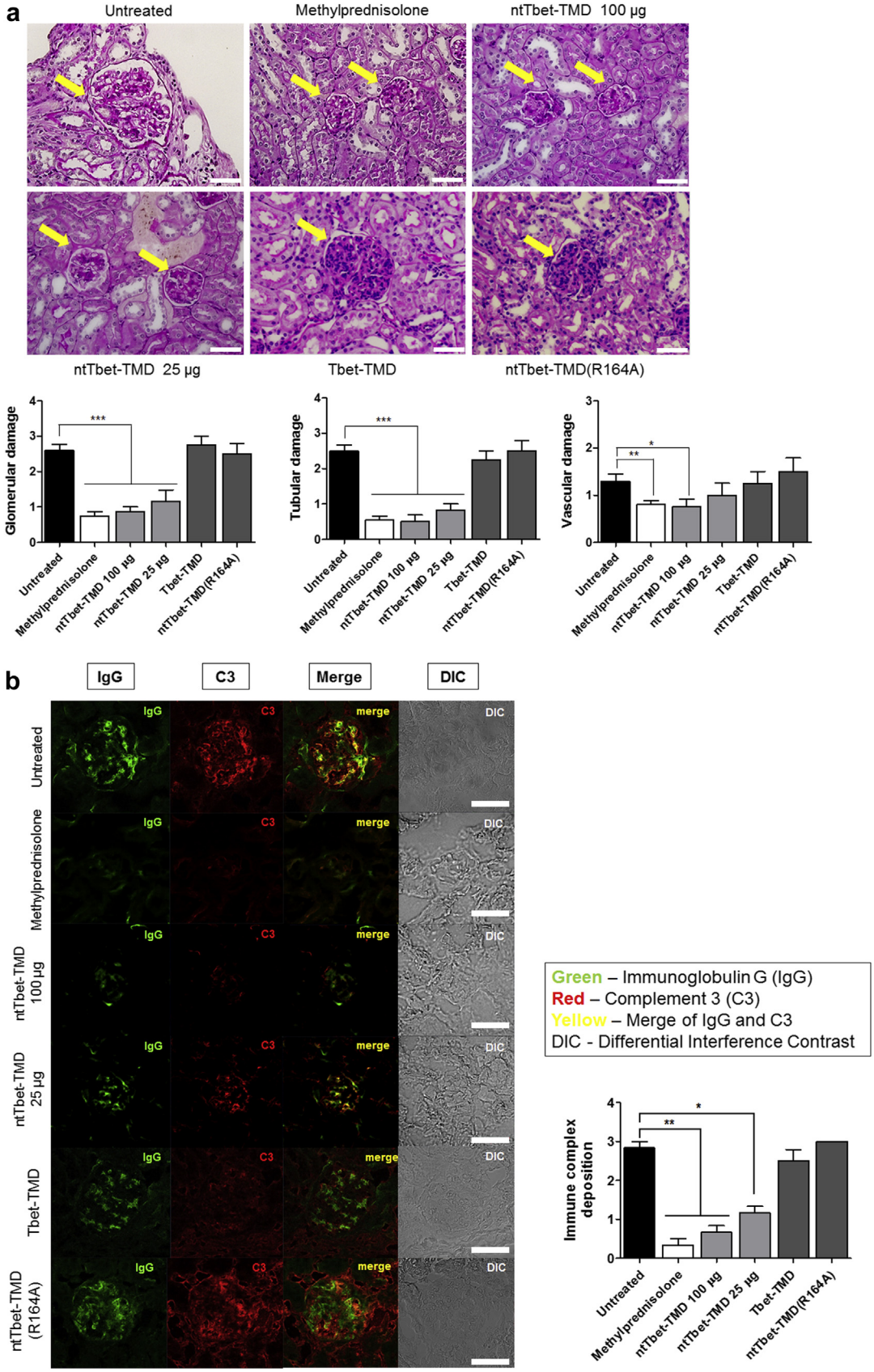

Figure 4 | Changes of histologic and immunologic abnormality associated with lupus nephritis by nucleus-transducible (nt)Tbet-transcription modulation domain (TMD) treatment. (**a**) Kidney tissue was collected from the treated mice. Immunohistochemical staining of the kidney tissue was conducted to assess the renal histologic abnormalities. Yellow arrows indicate vascular, glomerular, and (continued)

therapeutic modalities to overcome these limitations are still necessary.

Recently, the altered regulation of the immune network, unbalanced ratio of various T-cell subsets, and autoantibody production of B cells regulated by T cells with immune-complex deposition have been considered as major elements in the pathogenesis of LN.[25] It is quite evident that the early onset of LN was accelerated in lupus-prone (NZB/NZW) F1 mice receiving IFN-γ,[28] and delayed in (NZB/NZW) F1 mice treated with anti–IFN-γ antibody.[29] Also, a greatly increased level of Th1-related genes was detected in patients with LN[30] and correlated well with the severity of LN.[31] Although the exact action mechanisms of Th1 cells expressing Tbet in the development or aggravation of LN remain uncertain, it could be speculated that Th1 cells and IFN-γ might be mainly involved in the pathogenesis of LN.

In this study, a novel therapeutic approach was attempted for the treatment of LN, in which the functions of Tbet were inhibited by intranuclear delivery of TMD of Tbet using Hph-1-PTD. Tbet-TMD contains the isotype-specific domain and DNA binding domain so that, on delivery into the nucleus, it interferes with the interaction of the endogenous Tbet with the cognate DNA sequences in the promoter and other transcription factors for the formation of Tbet-specific transcriptome in an isotype-specific manner. As shown in Figure 1, ntTbet-TMD was efficiently delivered into the nucleus of the primary T cells in a dose- and time-dependent fashion, and its stability in the nucleus was maintained for >24 hours. In particular, differentiation of naïve T cells into Th1 cells was selectively prevented by ntTbet-TMD without influencing differentiation into other T-cell subsets (Th2, Th17, or Treg cells). T-cell receptor–mediated signaling events such as IL-2 secretion and CD69 induction as well as cell viability were not affected by ntTbet-TMD treatment (Figure 2).

In the experiments using (NZB/NZW) F1 lupus-prone mice, we found that ntTbet-TMD clearly alleviated nephritis, improved the histologic damage in the kidneys, and reduced the glomerular immune-complex deposition. The pathogenic roles of B cells were critical in the initiation and promotion of LN. Pathogenic $CD19^+$ B cells including memory B cells and plasma cells were reduced, and the concentration of autoantibodies significantly decreased with ntTbet-TMD treatment, demonstrating that ntTbet-TMD can directly influence the reduction of pathogenic antibodies by modulating the functions of autoreactive B cells. ntTbet-TMD can specifically downregulate Tbet-mediated gene expression in Th1 cells, leading to the inhibition of IFN-γ secretion by binding to its promoter without influencing the differentiation of naïve T cells into Th2, Th17, and iTreg cells *in vitro*. On the other hand, ntTbet-TMD showed the therapeutic potential for LN by reducing the population and functions of Th1, Th2, and Th17 cells comparable to methylprednisolone treatment in (NZB/NZW) F1 mice. Although it has been reported that different T-cell subsets are present in various stages of lupus progression,[32] the appearance order of T-cell subsets and their functional importance during lupus progression have not been investigated thoroughly. From our results, it can be hypothesized that at the early stage of LN Tbet-driven immune microenvironment mainly contributed by Th1 cells triggers the differentiation or proliferation of different inflammatory T-cell subsets including Th17 and especially T follicular helper cells (Tfh cells). In this inflammatory microenvironment, the number of Treg cells decreases due to the inhibition of Treg cell differentiation or proliferation. When the inflammatory condition is lifted by ntTbet-TMD treatment, the overall homeostasis of the T-cell subset ratio and their functions are restored. Alternatively, ntTbet-TMD may alleviate nephritis in lupus-prone mice by converting the pathogenic status of Th1 cells to a nonpathogenic status, followed by subsequent functional conversion of other inflammatory T-cell subsets.

Furthermore, we assume that this functional discrepancy of ntTbet-TMD between *in vitro* and *in vivo* conditions may be caused by cross-regulation between Tbet and other transcription factors including GATA3 for Th2 cells or RORγt for Th17 cells. In lupus-prone mice, ntTbet-TMD can competitively inhibit the binding of endogenous Tbet to the promoter of Tbet-inducible genes including IFN-γ. Unbound Tbet (free Tbet) is likely to exist in high levels in naïve T cells in lupus-prone mice. Unlike the *in vitro* condition, the cells that are blocked to differentiate into Th1 cells by ntTbet-TMD are in a proinflammatory microenvironment that can push these cells to differentiate into other proinflammatory T-cell subsets *in vivo*. For naïve T cells in the differentiation microenvironment into Th2 cells, free Tbet may bind to GATA3 or RUNX3 and limit the binding of GATA3 to the promoter of Th2-related genes.[33] In the case of Th17 cells, free Tbet may bind to RUNX1, which is a cooperative transcription factor for RORγt, and limit its functions, leading to the suppression of the expression of RORγt-inducible genes.[34] Thus, ntTbet-TMD could reduce the population of Th1, Th2, and Th17 cells by functional cross-regulation of Tbet on transcription factors specific to Th2 or Th17 cells in lupus-prone mice. Alternatively, the inflammatory proteins encoded by Tbet-inducible genes may be the early initiator of the subsequent differentiation of naïve T cells into inflammatory T-cell subsets such as Th2 and Th17 cells. Also, a previous study

Figure 4 | (continued) tubular changes in the kidney tissue. Glomerular, tubular, and vascular damage was quantified by a 4-point scale independently by 2 pathologists. Bar = 50 μm. (**b**) Immune-complex deposition was observed by laser scanning confocal microscopy. Immunofluorescence staining of IgG (green) or C3 (red) was observed in the kidney tissue and scored for fluorescence intensity based on a scale ranging from 0 (no staining) to 3 (strong staining intensity) by 2 independent pathologists for each group. Bar = 50 μm. The results are mean ± SEM (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

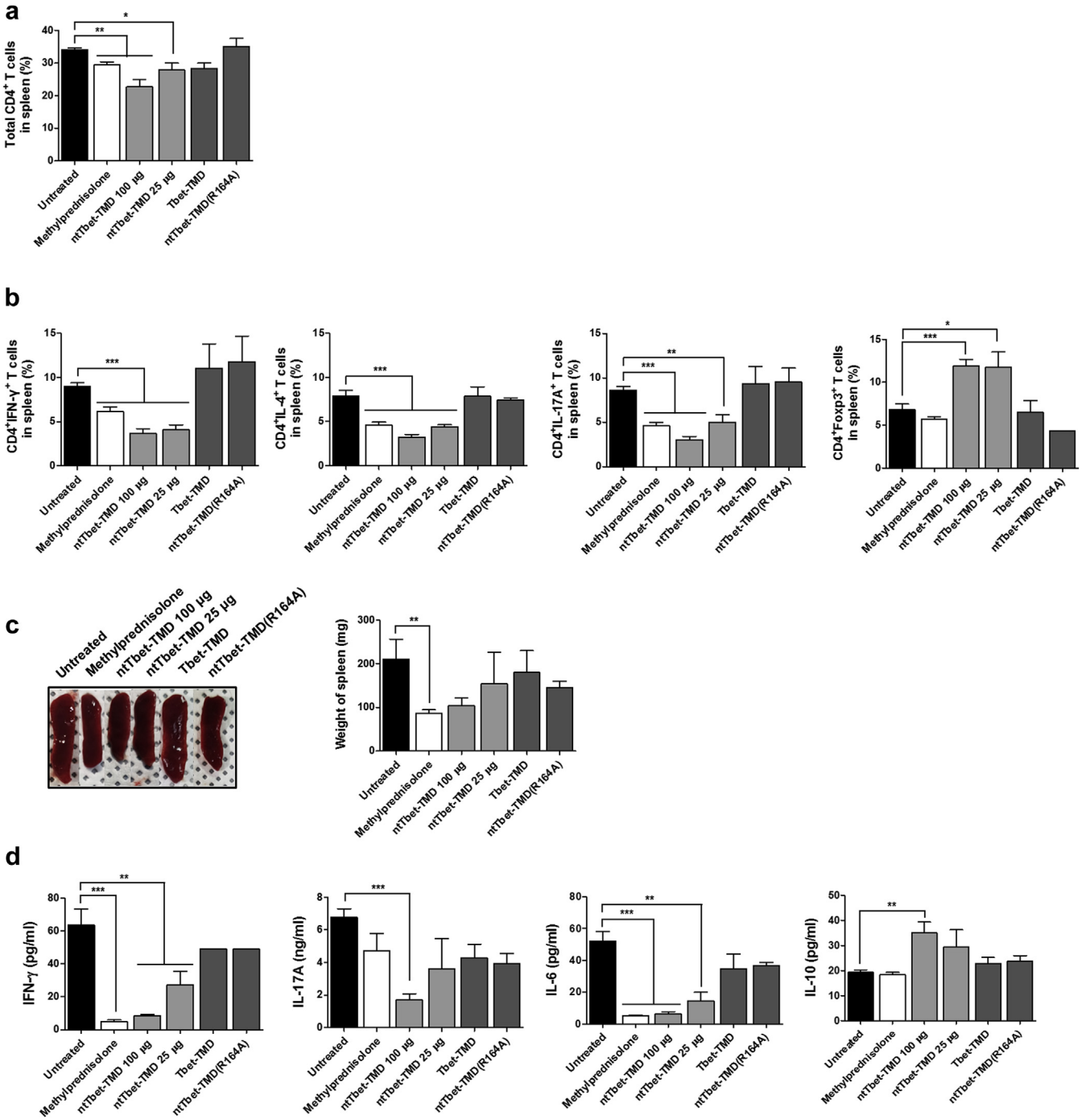

Figure 5 | Functional rebalance of T-cell subsets in the spleen of lupus-prone mice treated with nucleus-transducible (nt)Tbet-transcription modulation domain (TMD). (**a**,**b**) Mouse splenocytes were prepared from the spleen of the treated mice at the end of the experiment, and activated with anti-CD3/CD28 for 72 hours. Total number of splenic $CD4^+$ T cells was analyzed by the anti-CD4 antibody. The number of $CD4^+$ T cells secreting interferon (IFN)-γ, interleukin (IL)-4 or IL-17A, or expressing Foxp3 was detected by intracellular staining. Quantitative data were analyzed by Flowjo V10 program. (**c**) The spleens were harvested from lupus-prone mice treated with ntTbet-TMD, methylprednisolone, Tbet-TMD or ntTbet-TMD(R164A), and the size and weight of the spleens were compared. (**d**) The level of inflammatory cytokines such as IFN-γ, IL-17A, IL-6, and IL-10 in the serum was measured by sandwich enzyme-linked immunosorbent assay. The values are the mean ± SEM (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

reported that the reduced expression of IFN-γ could increase the $CD4^+Foxp3^+$ T cells in lupus mice. Therefore, in the microenvironment with fewer Tbet-inducible proteins such as IFN-γ, the naïve T cells cannot differentiate into Th2 or Th17 cells properly, and their differentiation into Treg cells is enhanced.

From an analysis of the level of Foxp3 protein, a previous report suggested that the lack of IFN-γ receptor signaling in

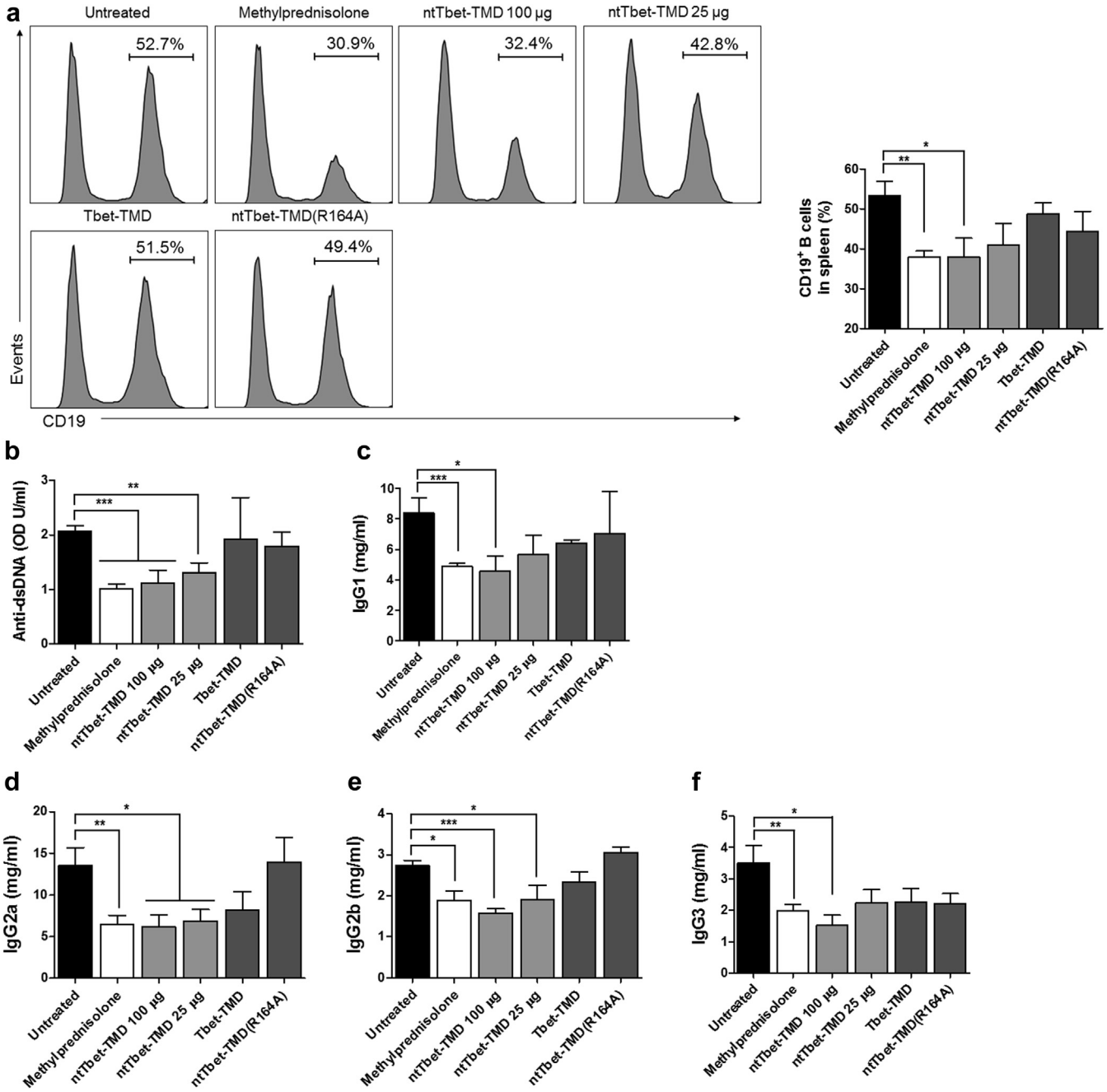

Figure 6 | Reduction of pathogenic CD19$^+$ B cells and the level of anti–double-stranded DNA (anti-dsDNA) autoantibody with different IgG subclasses in the serum of nucleus-transducible (nt)Tbet-transcription modulation domain (TMD)–treated lupus-prone mice. (**a**) Mouse splenocytes were isolated, and CD19$^+$ B cells in the total splenocytes were detected by flow cytometry using an anti-mouse CD19 antibody. Representative images were presented, and the quantified percentage of CD19$^+$ cells was measured by Flowjo V10. (**b–f**) The serum was collected from the lupus-prone mice treated with phosphate-buffered saline, Tbet-TMD, ntTbet-TMD(R164A), ntTbet-TMD, or methylprednisolone at the time the mice were killed. (**b**) The serum concentration of total anti–double-stranded DNA autoantibody was measured by sandwich enzyme-linked immunosorbent assay. (**c–f**) Anti-dsDNA autoantibody with different IgG subclasses was analyzed by a multiplex assay for mouse IgG isotypes. The results are the mean ± SEM (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

the lupus-prone environment could mediate the increase of the Treg cell population in Roquin$^{san/san}$*Ifngr*$^{-/-}$ mice.[35] Possibly, ntTbet-TMD inhibited IFN-γ secretion and therefore would induce the downregulation of IFN-γ receptor signaling in (NZB/NZW) F1 mice. Therefore, it can be suggested that the number of Treg cells and IL-10 secretion in the spleen of lupus-prone mice could be increased by the reduced IFN-γ–related signaling by ntTbet-TMD treatment.

There is much evidence that intrarenal T cells can mediate the initiation and progress of nephritis in an direct and indirect manner.[28] Therefore, the identification of kidney-infiltrating T cells is necessary to understand the degree of

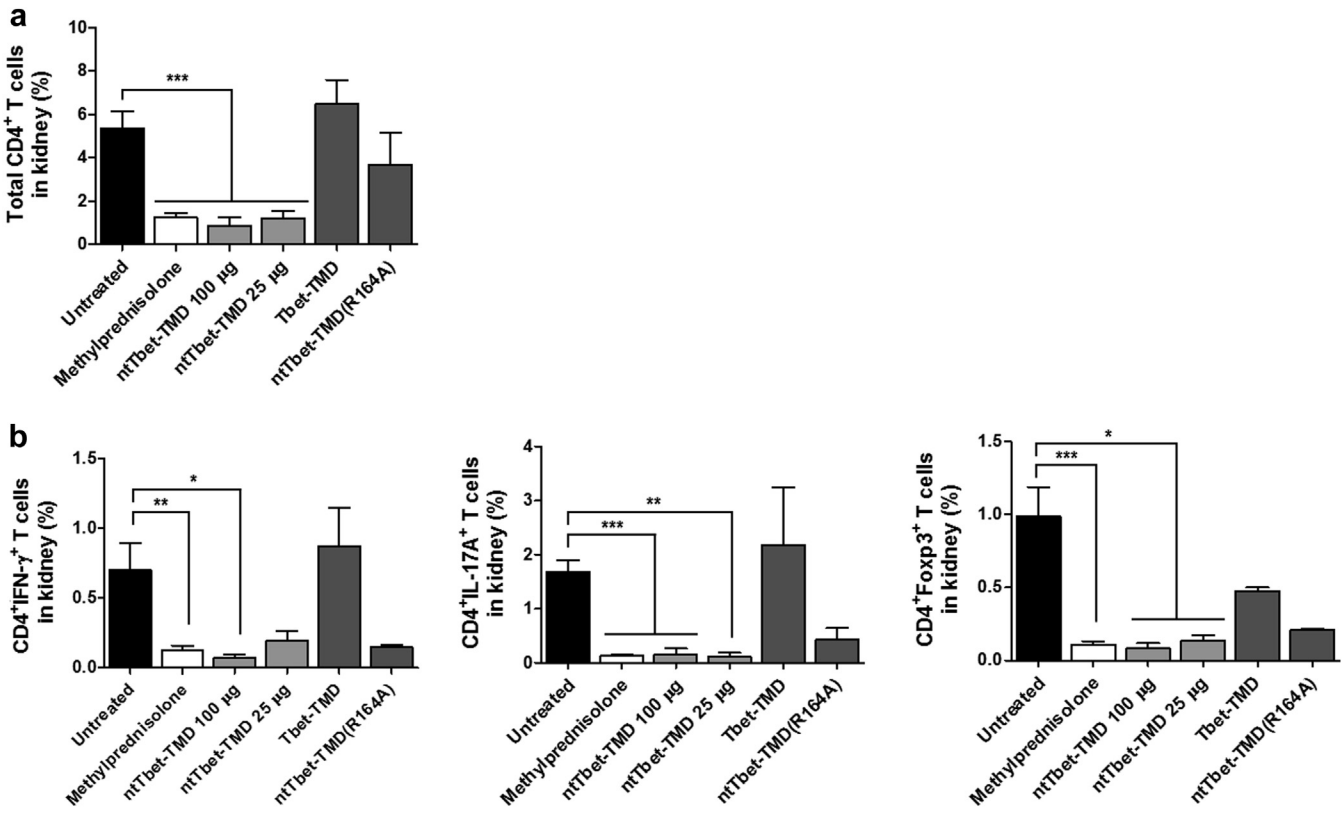

Figure 7 | Decrease in the number of kidney-infiltrating T cells by nucleus-transducible (nt)Tbet-transcription modulation domain (TMD) treatment. (**a**) Mouse lymphocytes in the kidney were isolated by kidney-tissue digestion and activated with an anti-CD3/CD28 antibody for 72 hours. After 72 hours of incubation, the cells were re-stimulated and stained with anti-CD4 antibody for the surface staining. (**b**) For the intracellular staining, re-stimulated cells were fixed/permeabilized and stained with anti–interferon (IFN)-γ, anti–interleukin (IL)-17A, or anti-Foxp3 antibody by flow cytometry. Quantitative data were analyzed using the Flowjo V10 program. The results are the mean ± SEM (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

inflammation in (NZB/NZW) F1 mice. ntTbet-TMD reduced the number of total $CD4^+$ T cells and proinflammatory T cells in the kidney consistent with that in the spleen. However, the number of Treg cells in the kidney was relatively increased in all 3 groups (untreated, Tbet-TMD–treated, and ntTbet-TMD[R164A]–treated) contrary to that in spleen, whereas the number of Treg cells in the kidneys of the ntTbet-TMD–treated group was relatively low. This might be accounted for by the fact that in a secondary lymphoid organ such as the spleen, there are many naïve T cells or T cells undergoing subset differentiation that can be functionally converted into nonpathogenic T cells or suppressive T cells by ntTbet-TMD. However, the functional conversion of these T cells in a local organ such as the kidney may be relatively low. We assume that Treg cells infiltrate the kidney where the severe inflammatory responses occur to counterbalance the inflammatory immune responses in untreated, Tbet-TMD–treated, or ntTbet-TMD(R164A)–treated mice. Alternatively, Treg cells in the kidney may be the nonfunctional Treg cells that remained. This phenomenon has been also observed in the joints of the animal model of rheumatoid arthritis. On the other hand, migration of Treg cells into the kidney where immune homeostasis is already maintained in ntTbet-TMD–treated mice is not required.

In conclusion, this is the first report showing that the inflammatory microenvironment induced by Tbet is critical for the lupus-associated pathogenesis in the early stage of the disease. Importantly, in contrast to methylprednisolone and immunosuppressive agents, ntTbet-TMD can be a novel and highly specific immunotherapy for the treatment of LN without affecting the differentiation of other T-cell subsets and T-cell activation events. Furthermore, ntTbet-TMD can allow us to investigate the role of Th1 cells in many Th1-associated autoimmune diseases and the novel functions of Tbet in new T-cell subsets coexpressing Tbet and other transcription factors together. This fundamental technology to deliver the wild-type or dominant negative form of the transcription factor into the nucleus of the cells *in vitro* and *in vivo* can be a core technology in developing novel therapeutics for various autoimmune diseases in which specific transcription factors play a key role in the pathogenesis.

METHODS

Purification and *in vitro* characterization of ntTbet-TMD protein

Expression and purification of ntTbet-TMD are described in detail in the Supplementary Material. Briefly, ntTbet-TMD protein was expressed in the *E coli* strain BL-21 (DE3) star pLysS (Novagen) and

purified by affinity and ion-exchange chromatography under native conditions. Purified ntTbet-TMD protein was characterized for using therapeutic protein drug with immunoblot, immunocytochemistry, cytotoxicity assay, luciferase reporter assay, *in vitro* $CD4^+$ T-cell differentiation, and enzyme-linked immunosorbent assay. The details of these methods can be found in the Supplementary Methods.

Treatment protocol and measurement of proteinuria

All lupus-prone mice were treated in accordance with the Guidelines and Regulations for the Use and Care of Laboratory Animals of Yonsei University, Seoul, Republic of Korea. Twenty-one-week-old female (NZB/NZW) F1 mice were purchased and housed individually in a specific pathogen-free barrier facility under standard sterile conditions. Ten mice were assigned to the untreated group, 2 mice to the Tbet-TMD 100-μg– or ntTbet-TMD(R164A) 100-μg–treated group, 8 mice each to the 25-μg and 100-μg ntTbet-TMD–treated groups, and 10 mice to 7-mg/kg methylprednisolone–treated group. Treatment with 2 doses of ntTbet-TMD or methylprednisolone began at 23 weeks of age, with the mice being injected i.p. 3 times per week until 30 weeks of age (Figure 3a).[36] Phosphate-buffered saline was injected i.p. in the untreated mice according to the same schedule. Twice per week during the experimental period, proteinuria in spot urine collected from each mouse was measured using albumin reagent strips (Yongdong Pharmaceutical Co., Yongin-si, Gyeonggi-do, Republic of Korea). Proteinuria was expressed semiquantitatively as follows: 0, none or trace; 1+, ≤100 mg/dl; 2+, ≤300 mg/dl; 3+, ≤1000 mg/dl; and 4+, >1000 mg/dl. In addition, details of the method for histologic assessment, immunofluorescence staining, flow cytometric analysis of splenic and kidney T cells, and the analysis of serum concentrations of anti–double-stranded, IgG subclasses, or cytokines are provided in the Supplementary Methods.

Statistical analysis

Data are presented as a mean ± SEM. Statistical analysis was examined using an unpaired Student *t* test. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ were considered statistically significant.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Figure S1. Functionally balanced population of T-cell subsets by nucleus-transducible (nt) Tbet–transcription modulation domain (TMD) in the spleen. (**A–D**) Mouse splenocytes were isolated from the spleens of the treated-mice and activated with plate-bound anti-CD3/CD28 for 72 hours. The number of $CD4^+$ T cells secreting interferon (IFN)-γ, interleukin (IL)-17A, or IL-4 or expressing Foxp3 was detected by staining with anti-CD4 by surface staining and IFN-γ, IL-17A, or IL-4 or expressing Foxp3 by intracellular staining. Analysis of each population was performed with the Flowjo V10 program. Representative images are presented.

Figure S2. Reduced number of T cells by nucleus transducible (nt) Tbet–transcription modulation domain (TMD) treatment in the kidney. (**A–C**) Kidney-infiltrating lymphocytes were separated by digestion of kidney tissues from all of the treated mice and activated with anti-CD3 and 28 antibodies. Activated lymphocytes were re-stimulated with cell restimulation cocktail and stained with anti-CD4 antibody for the surface staining. Sequentially, the fixed and permeabilized cells were stained with anti–interferon-γ (IFN-γ), anti–interleukin (IL)-17A or anti-Foxp3 antibody for the intracellular staining. The stained cells were detected with flow cytometry and analyzed by Flowjo V10 program. Representative images were presented.

Supplementary Materials and Methods.

Supplementary material is linked to the online version of the paper at www.kidney-international.org.

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