Macrophage transcription factor TonEBP promotes systemic lupus erythematosus and kidney injury via damage-induced signaling pathways

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Systemic lupus erythematosus (SLE) is an autoimmune disorder characterized by autoreactive B cells and dysregulation of many other types of immune cells including myeloid cells. Lupus nephritis (LN) is a common target organ manifestations of SLE. Tonicity-responsive enhancer-binding protein (TonEBP, also known as nuclear factor of activated T-cells 5 (NFAT5)), was initially identified as a central regulator of cellular responses to hypertonic stress and is a pleiotropic stress protein involved in a variety of immunometabolic diseases. To explore the role of TonEBP, we examined kidney biopsy samples from patients with LN. Kidney TonEBP expression was found to be elevated in these patients compared to control patients – in both kidney cells and infiltrating immune cells. Kidney TonEBP mRNA was elevated in LN and correlated with mRNAs encoding inflammatory cytokines and the degree of proteinuria. In a pristane-induced SLE model in mice, myeloid TonEBP deficiency blocked the development of SLE and LN. In macrophages, engagement of various toll-like receptors (TLRs) that respond to damage-associated molecular patterns induced TonEBP expression via stimulation of its promoter. Intracellular signaling downstream of the TLRs was dependent on TonEBP. Therefore, TonEBP can act as a transcriptional cofactor for NF-κB, and activated mTOR-IRF3/7 via protein-protein interactions. Additionally, TonEBP-deficient macrophages displayed elevated efferocytosis and animals with myeloid deficiency of TonEBP showed reduced Th1 and Th17 differentiation, consistent with macrophages defective in TLR signaling. Thus, our data show that myeloid TonEBP may be an attractive therapeutic target for SLE and LN.

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KEYWORDS: glomerulonephritis; inflammation; lupus; macrophages; systemic lupus erythematosus

Lay Summary

Systemic lupus erythematosus (SLE) is a complex and clinically heterogeneous autoimmune disease caused by dysregulated innate and adaptive immune systems. Here, we show that tonicity-responsive enhancer-binding protein (TonEBP) is an autoimmune stress protein that mediates damage-associated molecular pattern–mediated signaling in macrophages, leading to skewing of T-cell populations in SLE and lupus nephritis (LN). LN is a common and severe manifestation of SLE associated with both acute kidney injury and chronic kidney disease. In patients with LN, TonEBP expression was elevated in both renal cells and infiltrating immune cells, including macrophages. Elevated renal TonEBP was associated with renal inflammation and local injury, suggesting that it may be an attractive therapeutic target.

Systemic lupus erythematosus (SLE) is a highly complex and heterogeneous chronic autoimmune disease. Lupus nephritis (LN), characterized by severe inflammation in the kidney with deposition of immune complexes, is a major risk factor for morbidity and mortality in SLE.[1] The central

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Table 1 | Comparison of patients' characteristics between the LN group and the control group

	Control (N = 12)	Lupus nephritis (N = 12)	*P* value
Age, yr	39.4 ± 17.7	28.8 ± 10.0	0.071
Female, %	58.3	91.7	0.155
Creatinine, mg/dl	0.8 ± 0.4	0.8 ± 0.3	0.982
eGFR, ml/min per /1.73 m^2	104.0 ± 34.1	101.2 ± 36.3	0.846
Urine protein/creatinine, g/g	0.7 ± 0.6	6.1 ± 3.9	<0.001
Hematuria, %[a]	100	100	>0.999
Pyuria, %[b]	16.7	83.3	0.003
Complement component 3, mg/dl	110.5 ± 14.7	52.6 ± 25.8	<0.001
Complement component 4, mg/dl	22.6 ± 3.8	9.7 ± 7.3	<0.001
CRP, mg/dl	0.5 ± 1.1	0.5 ± 1.1	0.651

CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; HPF, high-power field; LN, lupus nephritis; WBC, white blood cell.
[a]Defined as red blood cell count ≥ 5/HPF.
[b]Defined as WBC ≥ 5/HPF.
Continuous values were expressed as mean ± SD.

function of lymphocytes in SLE is well described: hyperactivated autoantibody-producing B cells and an imbalance of T-cell subsets that contribute to loss of immune tolerance.[2–4] Myeloid cells also contribute to the pathogenesis of SLE. Macrophages are defective in eliminating dying cells via phagocytosis (termed efferocytosis) and drive the proinflammatory milieu in SLE.[5–7] Also, as antigen-presenting cells, they process and present autoantigens to T cells in conjunction with major histocompatibility complex (MHC), or costimulatory molecules on the cell surface,[8–10] leading to T-cell differentiation.

Toll-like receptors (TLRs), widely expressed by various immune cell types, recognize pathogen- and damage-associated molecular patterns and initiate inflammatory responses. Studies in mouse models show that TLR4, TLR7/8, and TLR9 contribute to the pathogenesis of SLE.[11–13] Genetic variations in these genes (*TLR4*, *TLR7*, *TLR8*, and *TLR9*) display a clear linkage to SLE in patients,[14–16] and a gain-of-function genetic variant of *TLR7* causes human SLE.[17] Because most of our understanding of TLR functions in SLE is mainly based on B lymphocytes and dendritic cells, it is important to explore the function of TLRs in macrophages.

Tonicity-responsive enhancer-binding protein (TonEBP), also known as nuclear factor of activated T cells 5, was initially identified as a central regulator of cellular responses to hypertonic stress.[18–20] Recent studies in humans and mice show that upregulated expression of TonEBP mediates the pathogenesis of inflammatory and autoimmune diseases by contributing to the development and activation of immune cells in osmostress-dependent and osmostress-independent contexts.[21] In macrophages, TonEBP is induced by inflammatory signals, such as lipopolysaccharide (LPS) or hyperglycemia,[22,23] driving differentiation into the M1 phenotype via stimulation of nuclear factor (NF)–κB. As a transcriptional suppressor for the *IL-10* and *HO-1* genes,[24,25] TonEBP inhibits the M2 phenotype of macrophages. Given the diverse functions of TonEBP in macrophages, we asked whether TonEBP is involved in the pathogenesis of SLE. We found that patients with LN exhibited higher renal TonEBP expression in infiltrating immune cells, especially macrophages and $CD4^+$ cells, as well as higher renal expression of proinflammatory cytokines, than controls. A mouse model of SLE revealed that TonEBP in macrophages contributes to SLE/LN by suppressing efferocytosis and promoting antigen presentation, thereby affecting $CD4^+$ T-cell differentiation. Macrophage TonEBP is induced by damage-associated molecular patterns and, in turn, mediates activation of NF-κB and mammalian target of rapamycin–interferon regulatory transcription factor 3/7 (mTOR-IRF3/7), leading to expression of inflammatory cytokines and mediators. These data reveal that TonEBP

Table 2 | Clinical features of lupus nephritis patients used in this study

	Classification	Activity index	Chronicity index	Previous immunosuppressant	Anti-ds DNA	C3 (mg/dl)	C4 (mg/dl)	CRP (mg/dl)	Serum creatinine (mg/dl)	Urine protein/ creatinine	SLEDAI score
Patient 1[a]	IV and V	10	4	No	70	34	5	0.08	1.61	6.77	27
Patient 2[a]	IV	5	0	No	59.2	37	1	0.01	0.89	2.56	26
Patient 3[a]	IV	7	1	No	44.5	33	5	0.04	0.75	3.92	25
Patient 4[b]	V	2	1	No	29.5	52	9	0.58	0.31	10.68	15
Patient 5[b]	III and V	2	0	No	14.8	77	18	0.01	0.84	14.53	21
Patient 6[b]	III and V	1	0	No	63.1	107	24	0.23	0.70	4.27	16
Patient 7[c]	III and V	2	0	No	320	74	10	0.08	0.82	4.01	25
Patient 8[c]	IV and V	6	3	No	3.5	22	2	0.05	1.19	9.19	17
Patient 9[d]	III	2	1	No	200	27	3	1.80	0.66	5.72	27
Patient 10[d]	III	1	0	No	42.0	35	2	0.03	0.48	3.07	12
Patient 11[d]	IV	3	0	No	60.1	39	8	3.48	0.67	5.96	20
Patient 12[d]	IV	1.5	0	No	31.5	68	15	0.02	0.90	2.41	8

Anti-dsDNA, anti–double stranded DNA antibodies; C3, complement component 3; C4, complement component 4; CRP, C-reactive protein; ELISA, enzyme-linked immunosorbent assay; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index.
[a]Used both in immunohistochemistry and mRNA expression studies.
[b]Used only in mRNA expression studies.
[c]Used only in immunohistochemistry.
[d]Used only in multiplex ELISA test.

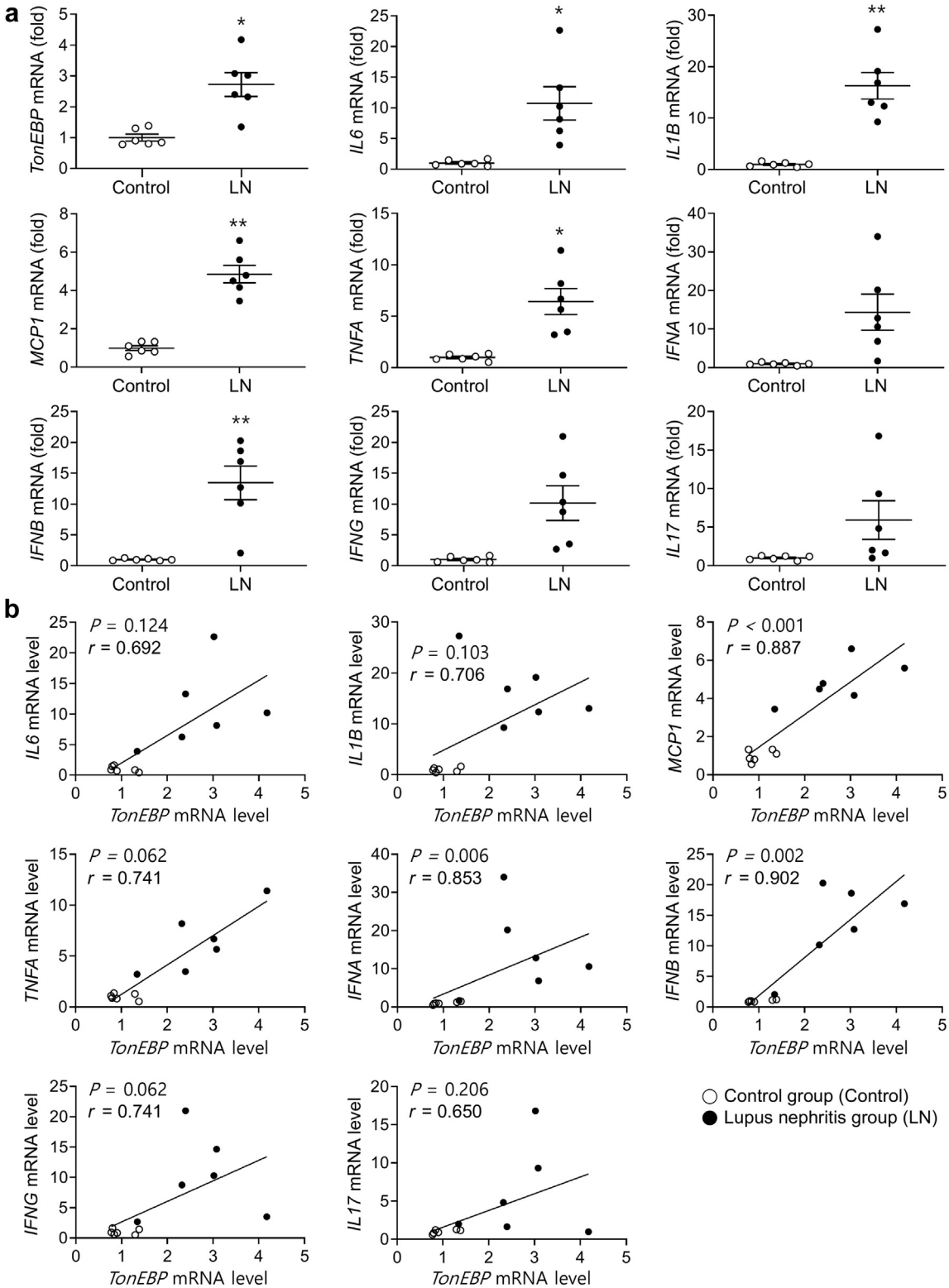

Figure 1 | Renal expression of mRNA encoding TonEBP and proinflammatory cytokines in patients with lupus nephritis (LN). (**a**) Levels of mRNA encoding TonEBP and proinflammatory cytokines were measured by quantitative real-time polymerase chain reaction of frozen kidney biopsy samples from patients with LN and controls (Table 1). mRNA level of each gene was normalized to that of glyceraldehyde-3-phosphate dehydrogenase. Mean ± SEM. *$P < 0.05$, **$P < 0.01$ compared with the controls. (**b**) Correlation between expression of each cytokine mRNA and that of TonEBP.

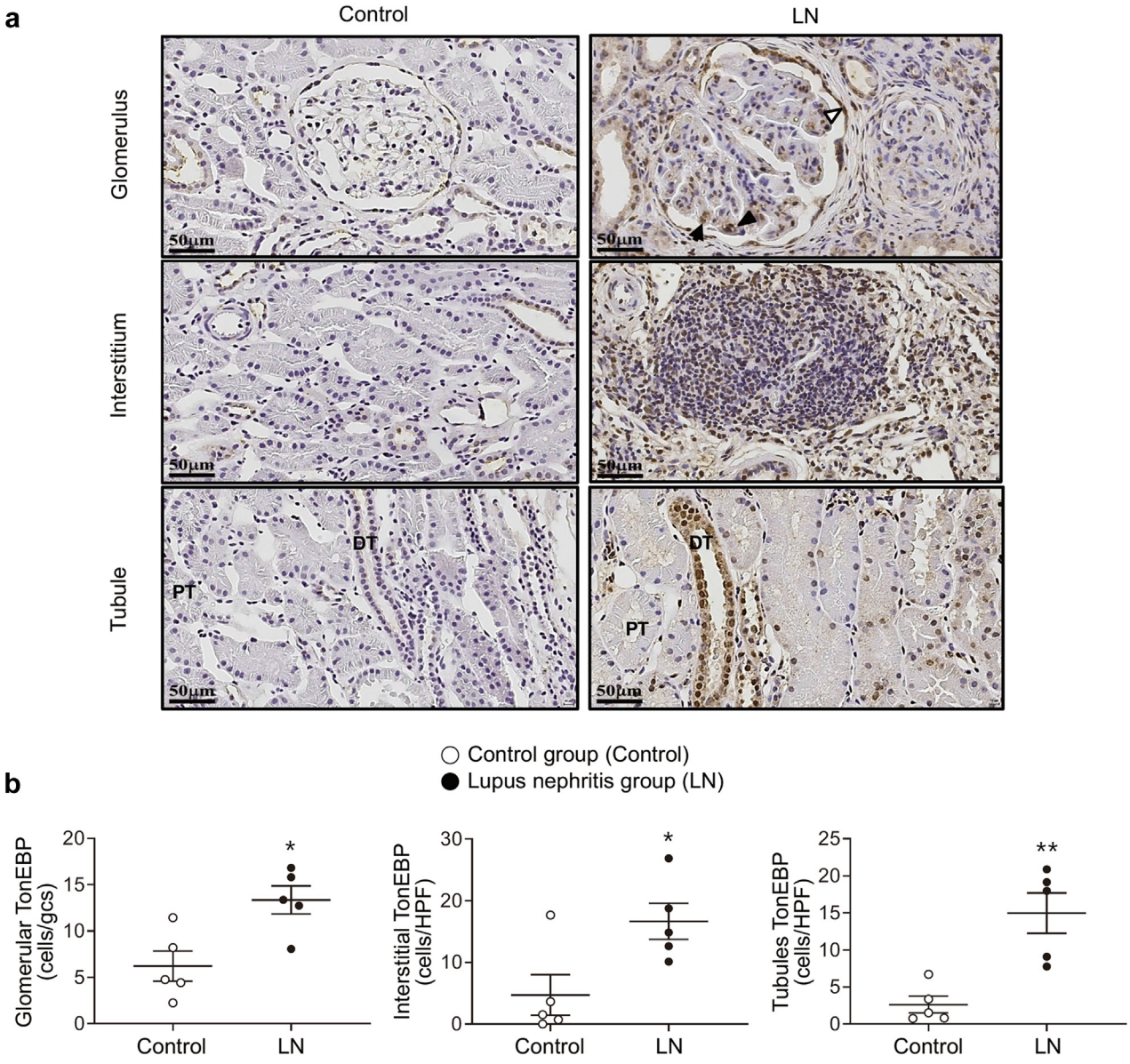

Figure 2| Renal distribution of TonEBP in patients with lupus nephritis (LN). Renal biopsy sections from patients with LN and controls were stained with an anti-TonEBP antibody. (**a**) Representative images of glomerular, interstitial, and tubular regions are shown (bars = 50 μm). Original magnification ×400. (**b**) The number of cells expressing TonEBP per glomerular cross section (gcs) or high-power field (HPF) is shown as mean ± SEM. *$P < 0.05$, **$P < 0.01$ compared with the controls. Arrow indicates a podocyte, arrowhead indicates an endothelial cell, and open arrowhead indicates a parietal epithelial cell. DT, distal tubule; PT, proximal tubule. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

may be an important regulator for the pathogenesis of SLE and LN.

METHODS

Study subjects and clinical data

Among patients with LN who underwent kidney biopsy between 2010 and 2020 at Seoul National University Hospital, patients with type III, IV, or V lupus nephritis without a history of immunosuppressive medication before kidney biopsy were identified. A control group comprised patients who had biopsy-proven thin basement membrane disease. Renal biopsy specimens from patients with LN and controls were analyzed for tissue staining, enzyme-linked immunosorbent assay, and mRNA expression. All patients agreed to donate their kidney biopsy sample to the Korea Biobank with written consent at the time of biopsy. The biospecimens used for this study were provided by the Biobank of Seoul National University Hospital, a member of Korea Biobank Network. The medical records of patients with LN were retrospectively reviewed for demographic and laboratory data, including blood urea nitrogen, serum creatinine, urinalysis, urine protein/creatinine ratio, complement component 3 and 4, and C-reactive protein. Lupus classification, activity index, and chronicity index were determined according to the revised International Society of Nephrology/Renal Pathology

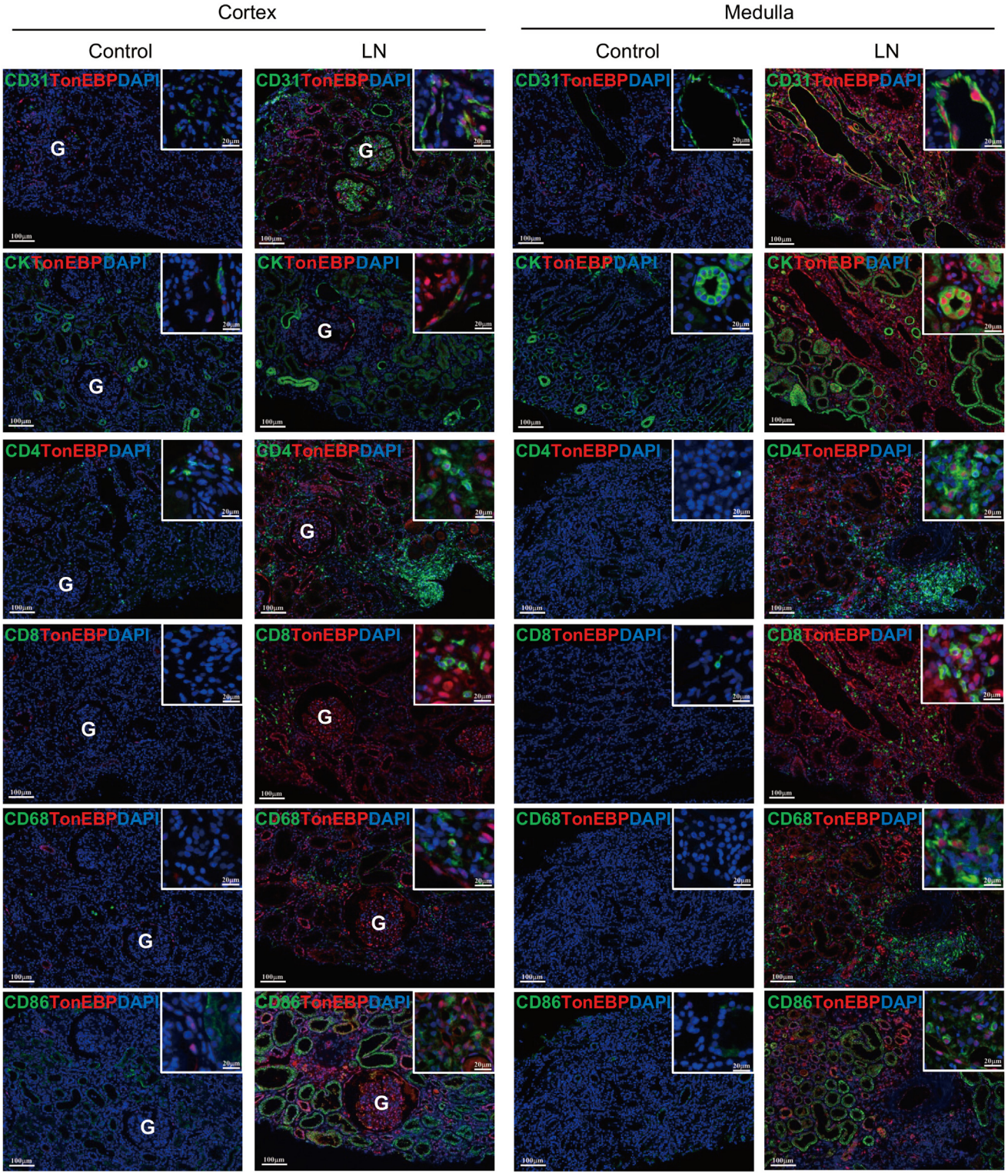

Figure 3| TonEBP expression in endothelial cells, epithelial cells, and infiltrating immune cells in renal biopsy tissues subjected to multiplex immune fluorescence staining. Biopsy sections were double stained for TonEBP (red) and cell surface markers (green): CD31, cytokeratin (CK), CD4, CD8, CD68, or CD86. Nuclei were visualized with 4′,6-diamidino-2-phenylindole (DAPI; blue). Representative pseudocolor merged images of the renal cortex and medulla are shown for both controls and the patients with lupus nephritis (LN). Merged image demonstrates colocalization of TonEBP and DAPI in the nucleus (purple) and cell markers on the cell surface and cytoplasm (green). Original magnification ×200 (bars = 100 μm) and ×400 (boxed areas; bars = 20 μm). G, glomerulus. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

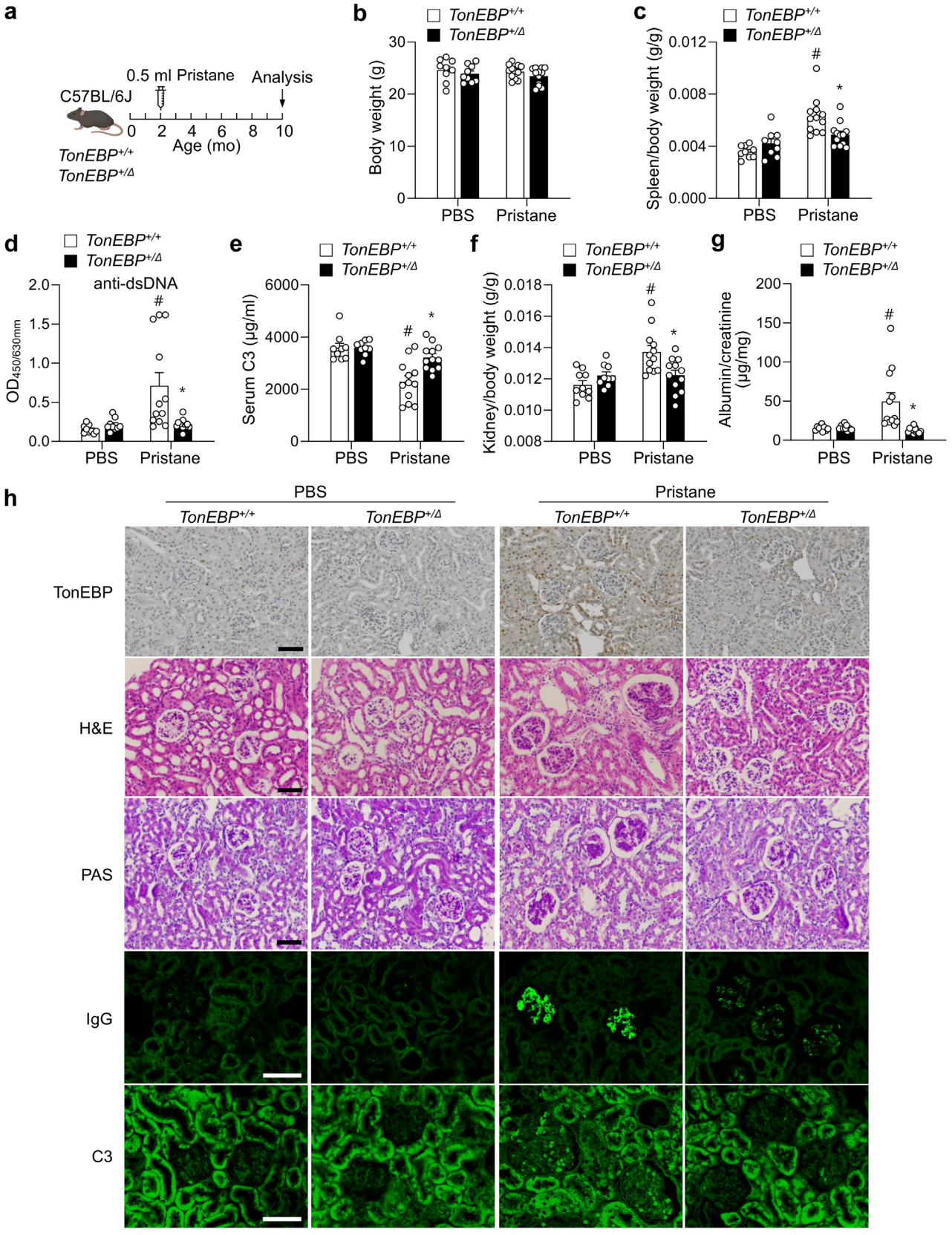

Figure 4 | TonEBP haplodeficiency prevents development of systemic lupus erythematosus (SLE) and renal injury. (**a**) Experimental scheme for pristane-induced SLE. The 2-month-old female *TonEBP*$^{+/\Delta}$ mice and their *TonEBP*$^{+/+}$ littermates received a single i.p. injection of pristane (n = 12) or phosphate-buffered saline (PBS; n = 9). Animals were analyzed 8 months later. (**b**) Body weight. (**c**) Spleen/body weight ratio. (**d**) Serum autoantibodies against double-stranded DNA (dsDNA) in serum samples were quantified by enzyme-linked (continued)

Society classification.[26] The Systemic Lupus Erythematosus Disease Activity Index score, an index of lupus disease activity, was calculated on the basis of medical records, as previously described.[27] This study followed the Declaration of Helsinki and was approved by Seoul National University Hospital Institutional Review Board (H-1806-176-956).

Quantitative real-time polymerase chain reaction

TRIzol (Invitrogen) was used to extract total RNA from frozen kidney biopsy specimens that had been stored at −70°C in RNA-later solution (Invitrogen). Next, cDNA was synthesized from 1 μg of RNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems). Real-time polymerase chain reaction (PCR) was performed using a QuantStudioTM3 PCR system with Power SYBR Green PCR master mix (Applied Biosystems). The PCRs were run for 20 cycles. All samples were analyzed in triplicate. mRNA expression of each target gene was normalized to that of glyceraldehyde-3-phosphate dehydrogenase, and the relative mRNA expression levels in the lupus nephritis group compared with those in the control group were calculated using the −ΔΔCt (cycle threshold) method.

Animal model

All experiments involving live animals were performed in accordance with approved guidelines. All experimental protocols were authorized by the Institutional Animal Care and Use Committee of the Ulsan National Institute of Science and Technology. Heterozygous TonEBP mice (TonEBP$^{+/\Delta}$; Nfat5$^{+/tm1Snh}$), which had been back-crossed to the C57BL/6 strain,[18] and their wild-type littermates (TonEBP$^{+/+}$) were used. Mice carrying loxP sites, targeting TonEBP gene (TonEBP$^{fl/fl}$; Nfat5$^{tm1.1Chku/tm1.1Chku}$), were reported previously.[28] Transgenic mice expressing Cre recombinase, specifically in myeloid lineage cell (LysM-cre), were purchased from The Jackson Laboratory. TonEBP$^{fl/fl}$ and LysM-cre mice were crossed to generate mice with myeloid-specific deletion of TonEBP (TonEBP$^{fl/fl}$, LysM-cre). To induce SLE-like disease, 8-week-old female mice received a single i.p. injection of 0.5 ml pristane (2,6,10,14-tetramethylpentradecane; Sigma-Aldrich), whereas control mice received phosphate-buffered saline alone. Mice were analyzed after 4 or 8 months. Blood was obtained by retro-orbital bleeding, and spleens and kidneys were harvested for analysis.

Statistical analysis

All data are expressed as the mean ± SEM. Categorical values were compared using χ^2 test or Fisher exact test, as appropriate. Continuous values were compared by an unpaired *t* test for comparisons between 2 conditions, whereas Bonferroni correction was performed for multiple comparisons. The correlation between TonEBP expression and cytokine expression, proteinuria, serum levels of anti–double-stranded DNA (dsDNA) antibodies, and complements was analyzed using Pearson or Spearman correlation, as appropriate according to variables' distribution. One-way analysis of variance and Tukey *post hoc* test were used for multiple comparisons of data from the pristane-induced murine model and the *in vitro* experiments. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using GraphPad Prism 8.2 software (GraphPad Software Inc).

RESULTS

Clinical characteristics of the patients with LN

First, we asked whether TonEBP plays a role in SLE and LN. In this case, we decided to investigate patients with LN. Twelve patients with type III, IV, or V LN were identified, along with 12 control patients with thin basement membrane disease. Although all patients displayed hematuria, the LN group showed higher levels of proteinuria and lower levels of serum complement than the control group (Table 1). The clinical characteristics of the patients with LN are summarized in Table 2. All had active LN with overt proteinuria, reduced complement levels, elevated anti-dsDNA antibodies, and normal serum creatinine levels, except for one patient who had a value of 1.61 mg/dl. The activity index ranged from 1 to 10, and the SLE disease activity index ranged from 15 to 27.

Expression of proinflammatory cytokines is upregulated and correlates with TonEBP mRNA expression in the kidneys of patients with LN

Because upregulated expression of TonEBP, which drives transcription of proinflammatory cytokines,[22,29] is the key feature of inflamed tissues in rheumatoid arthritis[30,31] and hepatitis,[32] we first analyzed mRNA expression encoding TonEBP and cytokines in frozen kidney biopsy samples. *TonEBP* mRNA expression was higher in the LN group than in the control group (Figure 1a). Expression of *IL6*, *IL1B*, *MCP1*, *TNFA*, and *IFNB* mRNA was also significantly higher in the LN group. In addition, expression of *MCP1*, *IFNA*, and *IFNB* mRNA correlated with that of *TonEBP* mRNA (Figure 1b), which is consistent with the role of TonEBP in transcription of proinflammatory cytokines. The protein levels of these cytokines also tended to be higher in the LN group than in the control group, although the difference was not statistically significant (Supplementary Figure S1). Furthermore, the level of *TonEBP* mRNA correlated significantly with the degree of proteinuria (Supplementary Figure S2A) and showed a trend toward a negative correlation with serum complement component 3 levels (Supplementary Figure S2C).

Renal TonEBP is elevated in patients with LN

Next, we performed immunohistochemistry to examine renal TonEBP. TonEBP was clearly detected in both nuclear and cytoplasmic regions (Figure 2a). TonEBP was localized to a variety of cell types, including podocytes, parietal epithelial

Figure 4 | (continued) immunosorbent assay (ELISA). (**e**) Serum complement component 3 (C3) levels were analyzed by ELISA. (**f**) Kidney/body weight ratio. (**g**) Albumin/creatinine ratio in spot urine samples. (**h**) Representative images of kidney sections: immunohistochemical staining of TonEBP, hematoxylin and eosin (H&E) staining, and periodic acid–Schiff (PAS) staining, and immunofluorescence images of IgG and C3 (bars = 50 μm). Mean + SEM. #$P < 0.05$ versus corresponding PBS, *$P < 0.05$ versus corresponding *TonEBP*$^{+/+}$. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

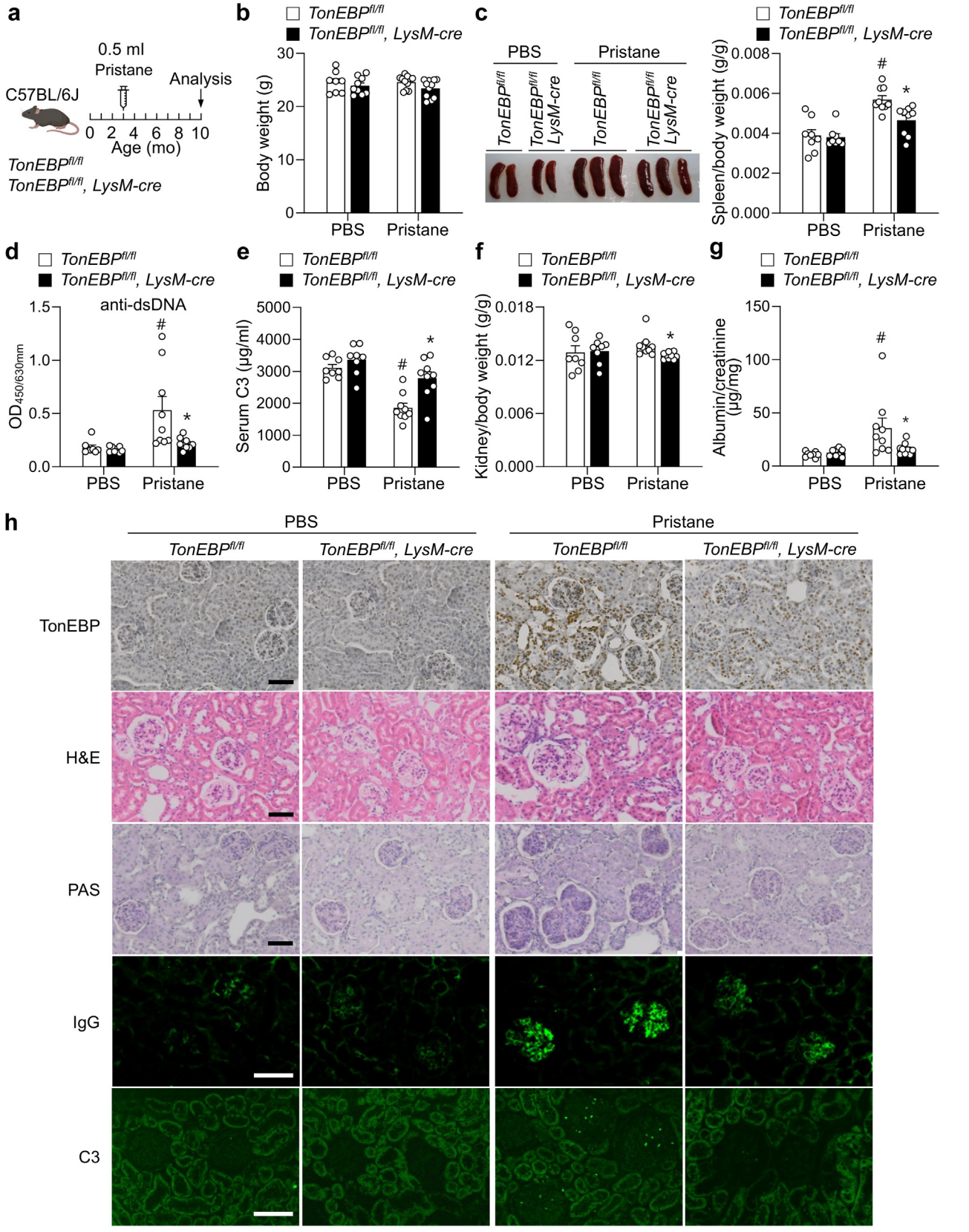

Figure 5 | Myeloid TonEBP deficiency reduces the severity of systemic lupus erythematosus and renal injury. (**a**) The experimental scheme is as shown in Figure 4a; *TonEBP*$^{fl/fl}$, *LysM-cre* mice and their *TonEBP*$^{fl/fl}$ littermates received a single i.p. injection of pristane (n = 9) or phosphate-buffered saline (PBS; n = 8). (**b**) Body weight. (**c**) Representative images of spleens and spleen/body weight ratio. (**d**) Serum autoantibodies against double-stranded DNA (dsDNA). (**e**) Serum complement component 3 (C3) levels. (**f**) Kidney/body weight (continued)

cells, endothelial cells, and neutrophils, in the glomeruli of patients with LN. In the LN group, renal interstitial areas were heavily infiltrated with immune cells. Among renal tubules, TonEBP was prominently expressed in distal tubules with positive uromodulin staining and collecting duct with negative uromodulin staining, whereas the TonEBP expression was weak in proximal tubules (Figure 2a and Supplementary Figure S3). The number of TonEBP-positive cells was significantly higher in all areas of the kidney (glomerular, interstitial, and tubular regions) in the LN group than in the control group (Figure 2b). Thus, renal protein expression of TonEBP is clearly elevated in LN, in line with its mRNA levels.

Next, we used multiplex immune fluorescence staining to localize TonEBP expression to various renal and immune cells. The number of TonEBP-positive cells was clearly elevated in the LN group (Figure 3 and Supplementary Figure S4A). The increase was observed in both epithelial (cytokeratin-positive) and endothelial (CD31-positive) cells. The same was seen with the immune cells (Figure 3 and Supplementary Figure S4B and C): $CD4^+$ T cells, $CD8^+$ T cells, $CD68^+$ macrophages, and $TonEBP^+CD86^+$ M1 macrophages. However, we could not adequately assess renal $TonEBP^+CD20^+$ B cells, $TonEBP^+CD11c^+$ dendritic cells, and $TonEBP^+CD206^+$ M2 macrophages, because the numbers of these cells were too low. In sum, TonEBP expression is elevated in renal cells and infiltrating immune cells, including $CD4^+$ T cells and macrophages, in LN.

TonEBP haplodeficiency prevents pristane-induced lupus

Next, we asked what triggers renal expression of TonEBP. To answer this question, we used a mouse model of SLE induced by a single injection of pristane (Figure 4a).[33,34] We then compared TonEBP haplodeficient ($TonEBP^{+/\Delta}$) mice with their wild-type ($TonEBP^{+/+}$) littermates; this is because haplodeficient animals are resistant to experimentally induced rheumatoid arthritis[30] and diabetic nephropathy.[23] Wild-type mice displayed a clear SLE/LN phenotype, which includes splenomegaly, elevated levels of circulating anti-dsDNA autoantibodies, decreased serum complement component 3 levels, and renal hypertrophy and glomerular injury in association with glomerular deposition of IgG and complement component 3 (Figure 4b–h). In addition, renal expression of TonEBP was markedly elevated (Figure 4h and Supplementary Figure S5A–C), similar to patients with LN. More important, this phenotype was absent from TonEBP haplodeficient animals, indicating that TonEBP may be involved in the development of SLE and associated renal injury.

There was little immune complex deposition in the glomeruli of TonEBP halplodeficient animals, as well as the absence of anti-dsDNA antibodies, suggesting that systemic events important for the development of SLE are blocked. To examine the systemic events, we analyzed alveolar hemorrhage and immune cell profiles in the peritoneal cavity and lung at 2 weeks after pristane treatment (Supplemental Figure S6). There was clear alveolar hemorrhage, which was not affected by TonEBP haplodeficiency. By contrast, influx of $Ly6C^{hi}$ inflammatory monocytes/macrophages into the peritoneal cavity was less pronounced. Likewise, influx of $CD11b^+$ cells, $CD11b^+Ly6G^+$ neutrophils, and $CD11b^+F4/80^+$ macrophages into lung was lower. Thus, we speculated that TonEBP in myeloid cells is associated with the pathogenesis of SLE and LN.

Myeloid TonEBP is required for development of pristane-induced lupus

Myeloid TonEBP is required for dendritic cell maturation[35] and macrophage activation[31] in rheumatoid arthritis. Given that we observed elevated TonEBP in macrophages in patients with LN (Figure 3 and Supplementary Figure S4C) and its role in systemic immune responses with reduced in myeloid cell populations in pristane-induced TonEBP haplodeficient mice (Supplementary Figure S6), we explored the role of myeloid TonEBP using mice with myeloid-specific deletion of TonEBP ($TonEBP^{fl/fl}$, *LysM-cre*) and their $TonEBP^{fl/fl}$ littermates (Figure 5a). We found that mice with myeloid-specific deletion of TonEBP did not develop SLE and LN (Figure 5b–h), similar to mice with TonEBP haplodeficiency. Increased renal TonEBP expression was also blunted in mice with myeloid TonEBP deficiency (Figure 5h and Supplementary Figure S5D–F).

Myeloid TonEBP is required for expansion and activation of myeloid cells in pristane-induced lupus

Next, we asked whether TonEBP deficiency in myeloid cells affects immune cell populations in the spleen. For this, animals were analyzed early (i.e., at 4 months after pristane administration rather than 8 months; Supplementary Figure S7A vs. Figure 5a). At this point, no kidney hypertrophy was observed (Supplementary Figure S7C) but splenomegaly with an increase in the total number of splenocytes was observed in a TonEBP-dependent manner (Supplementary Figure S7D and E). Although the number of macrophages ($CD11b^+F4/80^+$ cells) or neutrophils ($CD11b^+Ly\text{-}6G^+$ cells) in $TonEBP^{fl/fl}$ animals increased markedly, no increase was observed in $TonEBP^{fl/fl}$, *LysM-cre* littermates (Figure 6a and b).

Surface expression of MHCII and costimulatory molecules (CD80 and CD86) is important for antigen presentation and T-cell differentiation induced by antigen-presenting cells. Expression of CD86 and MHCII on the surface of $CD11b^+$ splenic cell population was elevated in the $TonEBP^{fl/fl}$

Figure 5 | (continued) ratio. (**g**) Albumin/creatinine ratio in spot urine samples. (**h**) Representative images of kidney sections: immunohistochemical staining of TonEBP, hematoxylin and eosin (H&E) staining, and periodic acid–Schiff (PAS) staining, and immunofluorescence images of IgG and C3 (bars = 50 μm). Mean + SEM. #$P < 0.05$ versus corresponding PBS, *$P < 0.05$ versus corresponding $TonEBP^{fl/fl}$. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

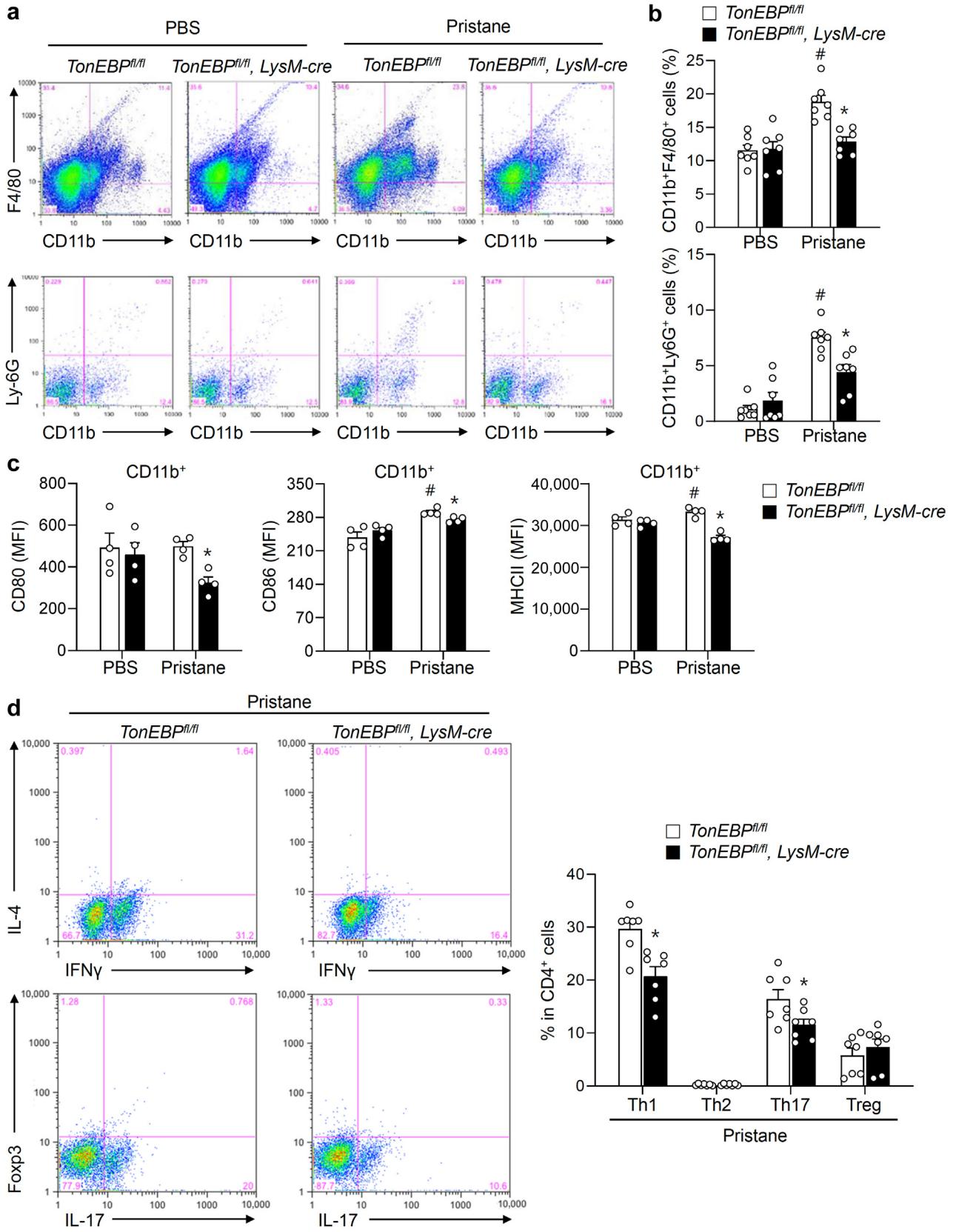

Figure 6| Myeloid TonEBP deficiency blocks expansion of macrophages and neutrophils, and differentiation of T-helper cell 1 (Th1) and T-helper cell 17 (Th17) cells. Cells in the spleens were analyzed 4 months after pristane injection, as described in Supplementary Figure S7A. (**a,b**) Representative flow cytometry plots and calculated percentage of $CD11b^+F4/80^+$ macrophages and $CD11b^+Ly6G^+$ neutrophils are shown (n = 7). (**c**) Surface expression of CD80, CD86, or major histocompatibility complex (MHC) II in $CD11b^+$ cell (continued)

littermates in response to pristane treatment, which was lower in cells from the *TonEBP*$^{fl/fl}$, *LysM-cre* animals (Figure 6c). Similar TonEBP-dependent expression of CD80 or MHCII was observed in CD11c^{+} dendritic cells (Supplementary Figure S7F and G). At 8 months after pristane injection, the number of infiltrating F4/80^{+} macrophages observed in kidney sections from mice with myeloid-specific TonEBP deletion was lower than that in *TonEBP*$^{fl/fl}$ littermates (Supplementary Figure S8), which is consistent with a lack of inflammation.

Myeloid TonEBP is required for Th1 and Th17 cell responses in pristane-induced lupus

Antigen presentation to T cells by antigen-presenting cells activates and triggers differentiation of naïve T cells, a key event in the pathogenesis of SLE. Because myeloid TonEBP is required for expansion of antigen-presenting cells and expression of molecules associated with antigen presentation, we asked whether myeloid TonEBP contributes to T-cell differentiation. First, we analyzed lymphoid cell subsets from whole splenocytes at 4 months after pristane treatment. Although the percentage of CD19^{+}B220^{+} B cells was reduced by TonEBP deficiency, those of CD4^{+} and CD8^{+} T-cell populations were not affected (Supplementary Figure S9). Further analyses of CD4^{+} T cells revealed a reduced population of T-helper cell 1 (Th1; interferon [IFN]-γ^{+}) and T-helper cell 17 (Th17) cells (interleukin [IL]-17^{+}) in myeloid-specific TonEBP-deficient mice, with no changes in the T-helper cell 2 (IL-4^{+}) and regulatory T-cell (forkhead box P3 [Foxp3^{+}]) populations (Figure 6d). Splenic and renal gene expression profile was consistent with defects in Th1/Th17 differentiation (Supplementary Figure S10A and B; i.e., induction of Th1/Th17 cytokines [IL-6, IL-17A, IL-17F, and IFN-γ] as well as type I IFNs [IFN-α and IFN-β] was blocked). Interestingly, splenic and renal expression of M2 phenotype genes (Arginase-1 and IL-10) tended to increase in myeloid-specific TonEBP-deficient mice (Supplementary Figure S10A and B). Thus, myeloid TonEBP deficiency is associated with suppression of T-cell differentiation to the Th1 or Th17 phenotypes, as well as suppression of inflammatory responses. This is consistent with the resistance of these animals to pristane-induced SLE.

TonEBP mediates TLR-mediated activation of macrophage *via* NF-κB and mTOR-IRF3/7 phosphorylation

To better understand the role of TonEBP in myeloid cells, we decided to investigate macrophages. We used peritoneal macrophages (PMs) isolated from female *TonEBP*$^{fl/fl}$, *LysM-cre* mice and their *TonEBP*$^{fl/fl}$ littermates. PMs were treated with LPS (a TLR4 ligand), R848 (a TLR7/8 ligand), or CpG-B (a TLR9 ligand) because TLR4,[11,14] TLR7/8,[12,17,36,37] and TLR9[13,37] form a part of the receptor systems for damage-associated molecular patterns and play critical roles in the pathogenesis of SLE. We found that these ligands induced expression of proinflammatory cytokines associated with Th1/Th17 differentiation and type I IFNs in a TonEBP-dependent manner (Figure 7a), as observed in spleens of the mice treated with pristane. The same pattern of TonEBP-dependent induction of CD80, CD86, and MHCII genes (Figure 7b) and proteins (Figure 8) was also observed. These changes were associated with elevated expression of TonEBP (Figure 7a and Supplementary Figure S11A).

In addition, we sought to uncover the molecular mechanisms underlying the actions of TonEBP. First, we asked whether induction of TonEBP is associated with activation of the TonEBP promoter. To address this, we constructed a pGL3 luciferase reporter vector containing an ≈5-kb human TonEBP promoter sequence. LPS, R848, and CpG-B stimulated expression of TonEBP promoter-driven luciferase >5-fold in RAW264.7 cells (Figure 9a). These data demonstrate that engagement of TLRs drives TonEBP expression via the promoter.

Next, we examined activation of NF-κB and phosphorylation of IRF3 and IRF7 because these molecules are critical transcription factors involved in the pathogenesis of SLE.[38–43] Our previous studies show that TonEBP stimulates the transcriptional activity of NF-κB in response to LPS by acting as a cofactor for NF-κB: TonEBP brings in the histone acetyltransferase p300 to the p65 subunit of NF-κB *via* protein-protein interactions.[22,29] Here, we asked whether TonEBP stimulates NF-κB in response to other TLR ligands. In RAW264.7 cells, R848 and CpG-B induced *Il12b*, *Il1b*, and *Tnfa* mRNA expression, as well as *TonEBP* mRNA, in association with activation of NF-κB–like LPS (Supplementary Figure S11A and B). In addition, siRNA-mediated knockdown of TonEBP blocked induction of mRNA expression encoding cytokines and activation of NF-κB. By contrast, nuclear translocation and DNA binding of p65 were not affected by TonEBP knockdown (Supplementary Figure S11C and D), which is consistent with the role of TonEBP as a transcriptional cofactor. We also examined mouse embryonic fibroblast cells from *TonEBP*$^{\Delta/\Delta}$ mice in which the gene product of the *TonEBP*$^{\Delta}$ allele is incapable of stimulating NF-κB because it does not interact with p65.[22] In these cells, activation of NF-κB in response to TLR ligands was blocked (Figure 9b), and the TonEBP-p65 interaction was absent (Figure 9c). Taken together, the data suggest that TonEBP stimulates NF-κB in response to TLR ligands *via* protein-protein interactions with p65.

We also examined IRF3 and IRF7 in RAW264.7 cells stimulated by TLR ligands. mRNA expression of *Ifnb1*, a type

Figure 6 | (continued) populations was analyzed and expressed as mean fluorescence intensity (MFI; n = 4). (**d**) Each panel depicts a representative flow cytometry plot (left). The percentage of interferon (IFN)-γ^{+} Th1 cells, interleukin (IL)-4^{+} T-helper cell 2 (Th2) cells, IL-17^{+} Th17 cells, and forkhead box P3 (Foxp3^{+}) regulatory T cells (Tregs) within the CD4^{+} cell population (right) was calculated (n = 7). Mean + SEM. #$P < 0.05$ versus corresponding phosphate-buffered saline (PBS), *$P < 0.05$ versus corresponding *TonEBP*$^{fl/fl}$.

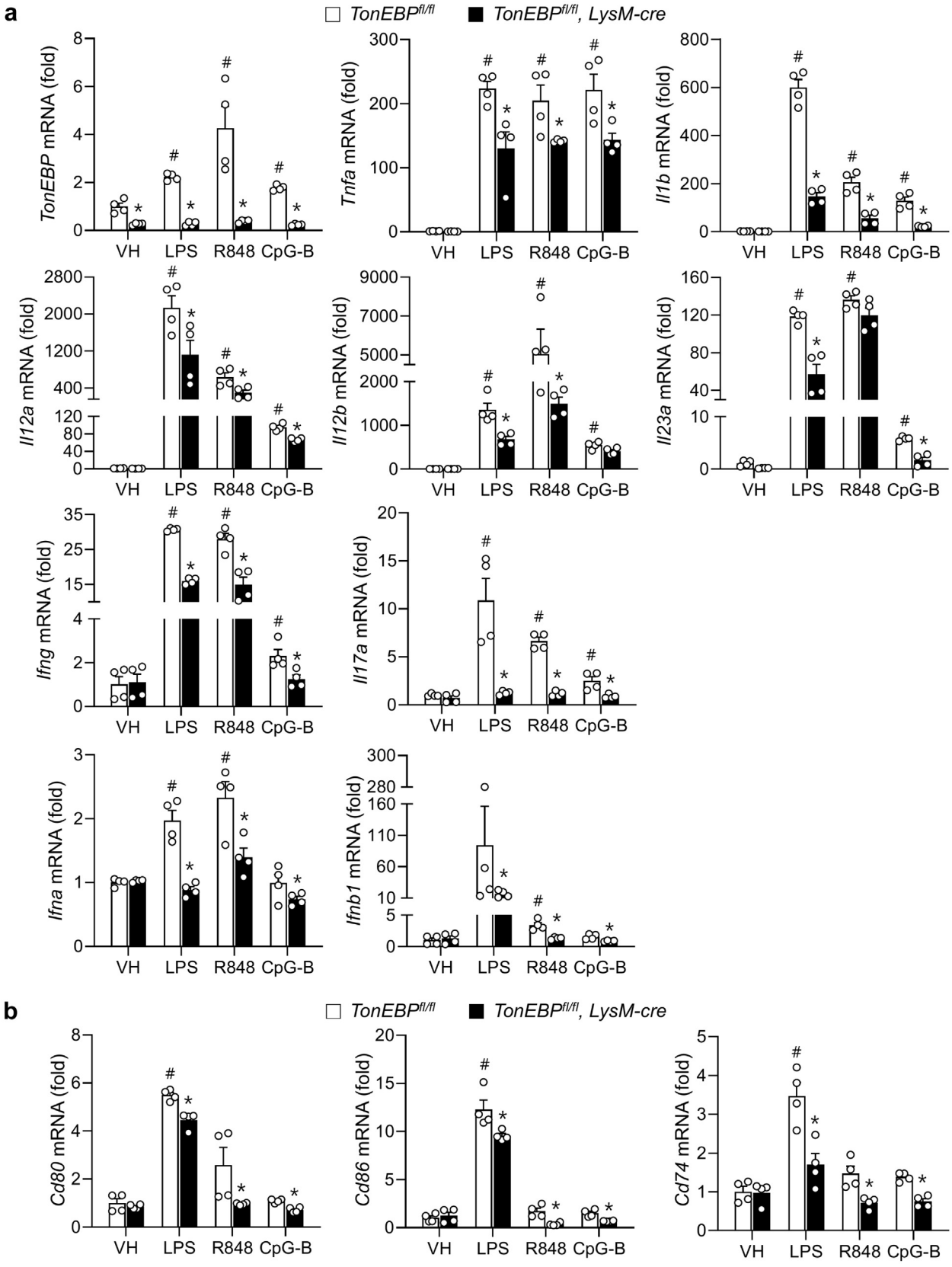

Figure 7 | TonEBP regulates genes associated with toll-like receptor–mediated immune responses and antigen presentation in macrophages. Peritoneal macrophages isolated from 8-week-old female *TonEBP*$^{fl/fl}$, *LysM-cre* mice and their *TonEBP*$^{fl/fl}$ littermates were treated with lipopolysaccharide (LPS; 100 ng/ml), R848 (10 μg/ml), CpG class B oligodeoxynucleotide (CpG-B; 1 μg/ml), or vehicle (VH) for 6 hours (n = 4). (**a**) Quantitative real-time polymerase chain reaction (RT-PCR) was performed to measure mRNA expression of *TonEBP* and (continued)

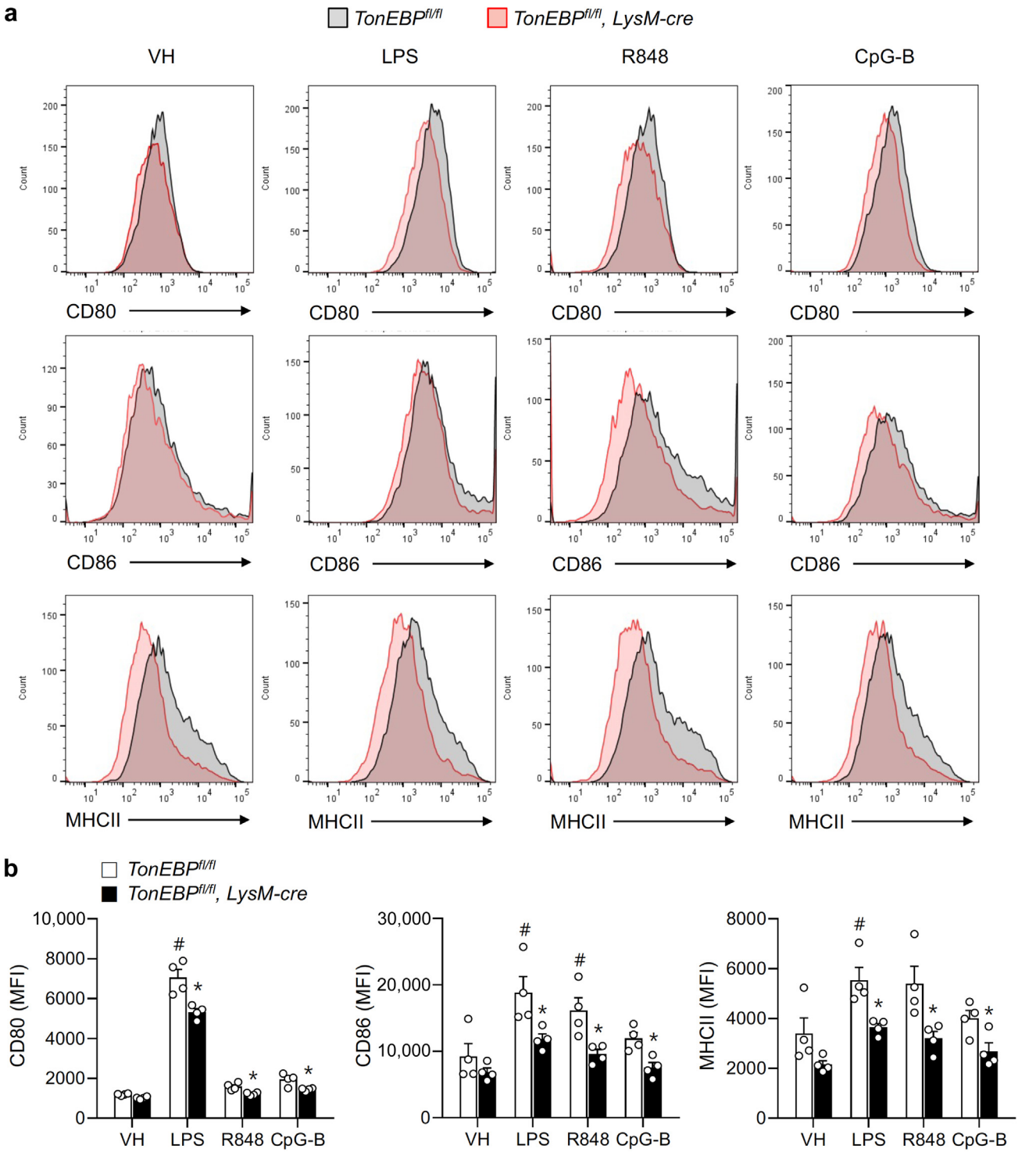

Figure 8 | TonEBP promotes expression of molecules involved in toll-like receptor–mediated activation and antigen presentation by macrophages. Peritoneal macrophages obtained from *TonEBP*$^{fl/fl}$ or *TonEBP*$^{fl/fl}$, *LysM-cre* mice were treated with lipopolysaccharide (LPS; 100 ng/ml), R848 (10 μg/ml), CpG class B oligodeoxynucleotide (CpG-B; 1 μg/ml), or vehicle (VH) for 24 hours. (**a**) Surface expression of CD80, CD86, or major histocompatibility complex (MHC) II was analyzed by flow cytometry, and representative histograms are shown. (**b**) Mean fluorescence intensity (MFI) of cell surface expression of CD80, CD86, and MHCII was calculated (n = 4). Mean + SEM. #$P < 0.05$ versus corresponding VH, *$P < 0.05$ versus corresponding *TonEBP*$^{fl/fl}$.

Figure 7 | (continued) cytokines associated with T-helper cell 1/T-helper cell 17 differentiation (*Tnfa*, *Il1b*, *Il12a*, *Il12b*, *Il23a*, *Ifng*, and *Il17a*) and type 1 interferon (*Ifna* and *Ifnb1*). (**b**) mRNA expression of costimulatory molecules (*Cd80* and *Cd86*) and major histocompatibility complex II–related gene (*Cd74*) was measured by quantitative RT-PCR. Mean + SEM. #$P < 0.05$ versus corresponding VH, *$P < 0.05$ versus corresponding *TonEBP*$^{fl/fl}$.

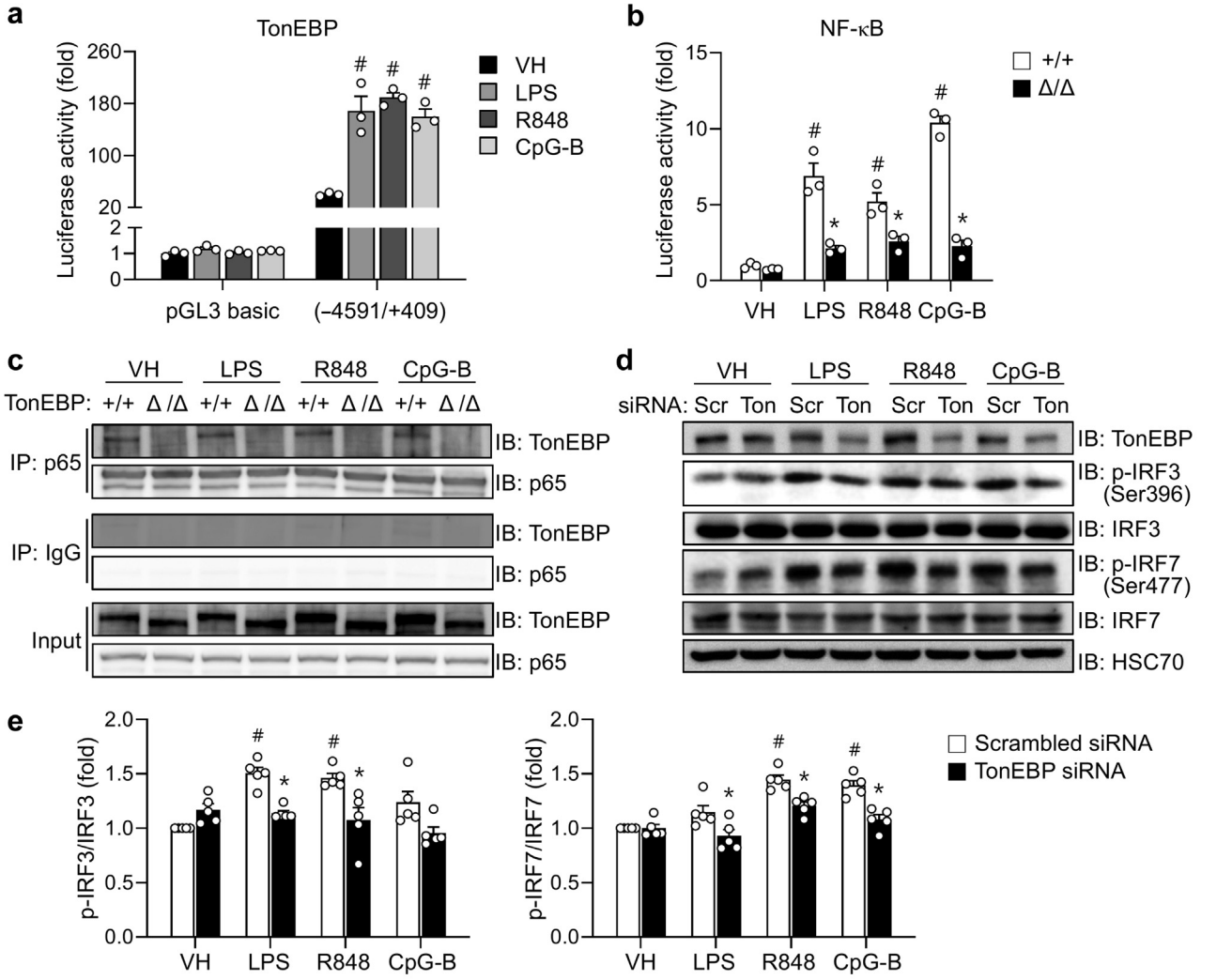

Figure 9 | TonEBP mediates nuclear factor (NF)–κB transcriptional activity and interferon regulatory transcription factor (IRF3/7) phosphorylation in response to toll-like receptor ligands. (**a**) RAW264.7 cells were transfected with a plasmid construct containing a DNA fragment covering –4591 to 409 bp of the human TonEBP promoter in pGL3 basic (–4591/+409) or pGL3 basic for 24 hours. Luciferase activity was measured 8 hours after treatment with lipopolysaccharide (LPS; 100 ng/ml), R848 (10 μg/ml), CpG class B oligodeoxynucleotide (CpG-B; 1 μg/ml), or vehicle (VH). (**b**) Mouse embryonic fibroblast (MEF) cells from TonEBP$^{+/+}$ (+/+) and TonEBP$^{\Delta/\Delta}$ (Δ/Δ) mice were transfected with an NF-κB reporter construct, followed by treatment with LPS (100 ng/ml), R848 (10 μg/ml), CpG-B (1 μg/ml), or VH for 8 hours. Luciferase activity was measured in a luminometer (n = 3). (**c**) MEF cells were treated with LPS, R848, CpG-B, or VH for 1 hour. Proteins were immunoprecipitated (IP) with anti-p65 IgG or normal IgG. Precipitates and cell lysates were immunoblotted (IB) to detect p65 and TonEBP. (**d**) RAW264.7 cells were transfected with scrambled (Scr) or TonEBP (Ton) siRNA and then stimulated with LPS, R848, CpG-B, or VH for 30 minutes. Cells were immunoblotted to detect TonEBP, phosphorylated IRF3 (p-IRF3), IRF3, phosphorylated IRF7 (p-IRF7), IRF7, or heat shock cognate protein 70 (HSC70). (**e**) Quantification of p-IRF3 or p-IRF7 levels normalized to total IRF3 or IRF7 levels. Mean + SEM. #$P < 0.05$ versus corresponding VH, *$P < 0.05$ versus corresponding +/+.

I IFN regulated by IRF3 and IRF7, was stimulated by TLR ligands in a TonEBP-dependent manner (Supplementary Figure S11A). Phosphorylation of IRF3 was stimulated by LPS and R848, whereas phosphorylation of IRF7 was stimulated by R848 and CpG-B in a TonEBP-dependent manner (Figure 9d and e).

To understand the molecular pathways underlying TonEBP-mediated regulation of IRF3/7, we decided to explore mTOR, which belongs to the TonEBP interactome,[44] because it is a key regulator of the IRF family.[45,46] First, we confirmed the TonEBP-mTOR interaction in mutual immunoprecipitation experiments (Supplementary Figure S12A). Interestingly, the TLR ligands stimulated phosphorylation of mTOR and its downstream target S6K in a TonEBP-dependent manner (Supplementary Figure S12B and C). To confirm the role of TonEBP, we expressed various forms of TonEBP in cells, in which TonEBP had been knocked down. Full-length TonEBP restored S6K phosphorylation in response to the TLR ligands, but a TonEBP molecule lacking the Rel-homology domain did not (Supplementary Figure S12D and E). These data demonstrate that TonEBP mediates activation of mTOR/S6K in response to TLR engagement in a manner dependent on its Rel-homology domain.

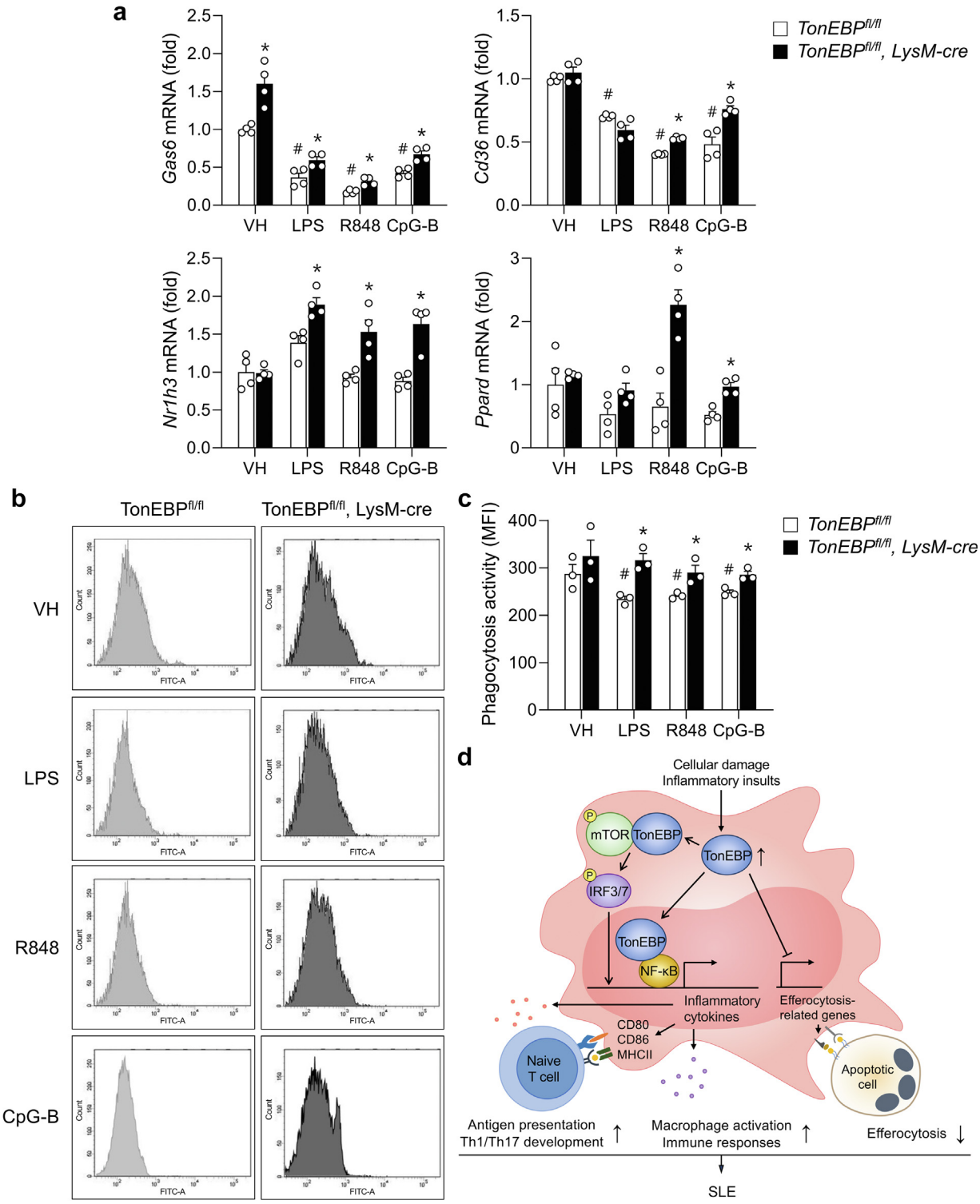

Figure 10 | Myeloid TonEBP deficiency blocks toll-like receptor–mediated deterioration of apoptotic cell clearance. Peritoneal macrophages (PMs) were isolated from 8-week-old female *TonEBP*$^{fl/fl}$ and *TonEBP*$^{fl/fl}$, *LysM-cre* mice. (**a**) mRNA expression of efferocytosis-related genes was measured by quantitative real-time polymerase chain reaction after stimulation with lipopolysaccharide (LPS; 100 ng/ml), R848 (10 μg/ml), CpG class B oligodeoxynucleotide (CpG-B; 1 μg/ml), or vehicle (VH) for 6 hours (n = 4). (**b,c**) PMs were treated with LPS, R848, CpG-B, or VH for 24 hours, and then cocultured with pHrodo-labeled apoptotic thymocytes at a ratio of 1:4 for 30 minutes at 37 °C. Phagocytosis was assessed by flow cytometry. Representative histogams are shown (**b**), and the mean fluorescence intensity (MFI) of fluorescein isothiocyanate within the $CD11b^{+}F4/80^{+}$ cell populations (**c**) was quantified (n = 3). (**d**) Proposed model for the role of macrophage TonEBP in systemic lupus erythematosus (SLE). Mean + SEM. #$P < 0.05$ versus corresponding VH, *$P < 0.05$ versus corresponding *TonEBP*$^{fl/fl}$. IRF, interferon regulatory transcription factor; MHC, major histocompatibility complex; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor-κB; Th1, T-helper cell 1; Th17, T-helper cell 17.

TonEBP blocks efferocytosis by macrophages

Given the importance of apoptotic cell clearance in the pathogenesis of SLE,[5–7] we examined the role of TonEBP in efferocytosis by PMs. First, we assessed expression of bridging molecules (*Gas6* and *CD36*) and transcription factors that regulate engulfment receptors (*Nr1h3* and *Ppard*) responsible for efferocytosis.[47,48] TonEBP-deficient PMs displayed higher expression of genes associated with these transcription factors and bridging molecules in response to TLR ligands (Figure 10a). Next, we measured phagocytosis of apoptotic cells by PMs and found that TonEBP-deficient PMs exhibited much higher phagocytic activity (Figure 10b and c). These data demonstrate that TonEBP in macrophages blocks engulfment of apoptotic cells in association with lower expression of genes related to phagocytic activity.

In sum, the data obtained from mice demonstrate that TonEBP in macrophages contributes to the pathogenesis of SLE/LN by stimulating autoimmune responses, antigen presentation, and Th1/Th17 cell development *via* NF-κB activation and IRF3/7 phosphorylation and suppressing efferocytosis (Figure 10d).

DISCUSSION

The data presented here suggest that TonEBP may be an important mediator in SLE. Activation of TLRs (TLR4, TLR7/8, and TLR9) by damage-associated molecular patterns is an early event in the pathogenesis of SLE in both mice[11–13] and humans.[14–17] We found that the TonEBP promoter in macrophages is stimulated by each of these activated TLRs, leading to upregulated expression of TonEBP. This upregulation is required for the pathogenesis of SLE. Upregulation of TonEBP is also important for macrophages[31] and dendritic cells[35] in rheumatoid arthritis. Thus, myeloid TonEBP appears to be a general stress protein responsive to autoimmune signals and stresses.

In rheumatoid arthritis, TonEBP drives the monocyte chemoattractant protein-1 (MCP-1) production, leading to sustained activation of macrophages and unrelenting inflammation,[31] and the maturation of dendritic cells for Th1/Th17 differentiation.[35] This study demonstrates that macrophage TonEBP promotes SLE *via* 3 separate pathways. First, TonEBP suppresses efferocytosis by reducing the expression of molecules involved in efferocytosis, thereby contributing to accumulation of damaged cells and autoimmune reactions. Two key transcriptional pathways, NF-κB and IRF3/7, are stimulated by TonEBP, leading to Th1/Th17 differentiation and macrophage activation. TonEBP interacts directly with the p65 subunit of NF-κB and stimulates its transcriptional activity. Likewise, TonEBP directly interacts with mTOR, leading to phosphorylation of IRF3/7.

Elevated TonEBP expression is associated with several inflammatory diseases, including rheumatoid arthritis,[30,31] diabetic nephropathy,[23] hepatitis,[32] and brain inflammation.[29] TonEBP leads to induction of proinflammatory genes via TonEBP-mediated stimulation of their promoters by NF-κB.[22] Here, we found that this pathway is active in LN as TonEBP expression was elevated in virtually all cell types in the kidney of patients and mice. Renal TonEBP expression correlates with the degree of proteinuria, indicating that TonEBP contributes to renal injury most likely *via* inflammation. These data suggest that TonEBP may be involved in the pathogenesis of SLE, as well as renal damage, *via* local inflammation.

The prevention of SLE In the TonEBP haplodeficient animals was remarkable. Even more remarkable is the finding that these animals are also resistant to experimentally induced rheumatoid arthritis,[30] diabetic nephropathy,[23] and hepatitis.[32] It is possible that a 50% reduction of TonEBP expression in myeloid cells is sufficient for this phenotype. Alternatively, TonEBP haplodeficiency in multiple cell types/tissues might be needed. Immunofluorescence data from patients with LN and murine model and the absence of anti-dsDNA autoantibodies by TonEBP haplodeficiency suggest a role of TonEBP in T and B cells. Even so, it appears that partial inhibition of TonEBP is sufficient to block inflammation. TonEBP may be a good target for treating LN because drugs rarely inhibit the activity of a biologic mediator completely.

Because of pristane-induced alveolar hemorrhage in the C57BL/6J background used in this study, further studies should examine other strains that do not display alveolar hemorrhage, such as BALB/c mice. Nevertheless, the present study shows that targeting TonEBP might be an attractive strategy for treatment or prevention of not only SLE but also LN. Given the multipronged pathways of TonEBP, there could be multiple strategies: inhibition of the TonEBP promoter or interference with TonEBP–NF-κB and TonEBP-mTOR interactions. A combination of drugs targeting different pathways might greatly enhance overall efficacy.

In conclusion, this study uncovers that myeloid TonEBP mediates multiple cellular pathways involved in the pathogenesis of SLE and LN. Because TonEBP is also involved in rheumatoid arthritis and other autoimmune diseases,[21] it may be a potential therapeutic target as a general autoimmune mediator.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Supplementary Methods.

Supplementary Figure S1. Renal expression levels of TonEBP mRNA and proinflammatory cytokines in patients with lupus nephritis (LN). Protein levels of proinflammatory cytokines in

kidneys were measured by multiplex enzyme-linked immunosorbent assay (ELISA) from frozen kidney biopsy samples of patients with LN and control patients. mRNA levels of TonEBP were measured by quantitative real time-polymerase chain reaction (qRT-PCR). Mean ± SEM. *$P < 0.05$, **$P < 0.01$ compared with control. IL, interleukin; IFN, interferon; MCP1, monocyte chemotactic protein-1; TNF, tumor necrosis factor.

Supplementary Figure S2. Correlation of renal mRNA expression levels of TonEBP and clinical parameters of lupus nephritis: proteinuria (**A**), and serum levels of anti–double-stranded DNA (dsDNA; **B**), complement component 3 (C3; **C**), and complement component 4 (C4; **D**). mRNA expression levels of TonEBP were normalized to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). n = 2 to ≈6 per group.

Supplementary Figure S3. Serial sections of a biopsy from a patient with lupus nephritis (LN) were stained for uromodulin (**A**) or TonEBP (**B**) using immunohistochemical staining. Bars = 100 μm. Original magnification ×400. Red arrow indicates renal tubules with TonEBP expression as well as uromodulin expression, and black arrow indicates renal tubules with TonEBP expression in the absence of uromodulin expression. DT, distal tubule; PT, proximal tubule.

Supplementary Figure S4. TonEBP expression in various cell types analyzed from the images of Figure 3. Counts of positive cells per high-power field (HPF) (×200) normalized to 4′,6-diamidino-2-phenylindole (DAPI) signal were shown for both cortical and medullary area. Mean ± SEM. *$P < 0.05$, **$P < 0.01$ compared with control group.

Supplementary Figure S5. TonEBP expression in spleens and kidneys from the pristane-induced mouse model of Figure 4 and 5. (**A**–**C**) Splenic and renal mRNA (**A**) and protein (**B,C**) expression of TonEBP from *TonEBP*$^{+/+}$ and *TonEBP*$^{+/\Delta}$ mice 8 months after pristane (n = 7) or phosphate-buffered saline (PBS) (n = 5) injection. (**D**–**F**) Splenic and renal mRNA (**D**) and protein (**E,F**) expression of TonEBP from *TonEBP*$^{fl/fl}$ and *TonEBP*$^{fl/fl}$, *LysM-cre* mice 4 months after pristane (n = 5) or PBS (n = 4) injection. Mean + SEM. #$P < 0.05$ versus corresponding PBS, *$P < 0.05$ versus corresponding TonEBP$^{+/+}$ or *TonEBP*$^{fl/fl}$.

Supplementary Figure S6. TonEBP haplodeficiency prevents expansion of myeloid cell populations. (**A**) Diffuse alveolar hemorrhage incidence of pristane-treated *TonEBP*$^{+/\Delta}$ mice and their *TonEBP*$^{+/+}$ littermates at day 14. (**B**) Flow cytometry analysis and quantification of the percentages of Ly6G-Ly6C^{hi} inflammatory monocytes/macrophages, Ly6G-Ly6C^{lo} monocytes, and Ly6G$^+$ cells in CD11b$^+$ subsets from peritoneal exudate cells (PECs) at day 14. (**C**) Flow cytometry analysis and quantification of the alveolar cell populations of CD11b$^+$ subsets, CD11b$^+$Ly6G$^+$ neutrophils, CD11b$^+$F4/80$^+$ macrophages, CD4$^+$ T cells, CD8a$^+$ T cells, and B220$^+$ B cells from pristane-treated *TonEBP*$^{+/\Delta}$ mice and their *TonEBP*$^{+/+}$ littermates at day 14. Mean + SEM. #$P < 0.05$ versus corresponding phosphate-buffered saline (PBS), *$P < 0.05$ versus corresponding *TonEBP*$^{+/+}$.

Supplementary Figure S7. Myeloid TonEBP deletion inhibits splenomegaly and maturation of dendritic cells. (**A**) Experimental scheme. (**B**–**D**) Body weight (**B**), kidney/body weight ratio (**C**), and spleen/body weight ratio (**D**) were calculated from *TonEBP*$^{fl/fl}$ and *TonEBP*$^{fl/fl}$, *LysM-cre* mice 4 months after pristane or phosphate-buffered saline (PBS) injection (n = 7). (**E**) Splenocytes isolated from spleens were counted (n = 7). (**F**) Percentage of CD11c$^+$ dendritic cells in splenocytes was quantified by flow cytometry (n = 4). (**G**) Surface expression of CD80, CD86, or major histocompatibility complex (MHC) II in CD11c$^+$ dendritic cells was analyzed by flow cytometry, and mean fluorescence intensity (MFI) was calculated (n = 4). Mean + SEM. #$P < 0.05$ versus corresponding PBS, *$P < 0.05$ versus corresponding *TonEBP*$^{fl/fl}$.

Supplementary Figure S8. TonEBP expression in renal infiltrating macrophages in pristane-induced lupus model. Immunostaining was performed with kidney sections from *TonEBP*$^{fl/fl}$ and *TonEBP*$^{fl/fl}$, *LysM-cre* mice after 8 months of pristane administration. Representative images for F4/80 and TonEBP were shown (bars = 50 μm). Arrowheads denote TonEBP-positive macrophages.

Supplementary Figure S9. Flow cytometric analysis of splenic B cells and T cells in pristane-induced lupus model. Splenocytes obtained from *TonEBP*$^{fl/fl}$ and *TonEBP*$^{fl/fl}$, *LysM-cre* mice were analyzed by flow cytometry 4 months after pristane treatment, as described in Supplementary Figure S7A. (**A**–**C**) Percentages of B220$^+$CD19$^+$ B cells (**A**), CD4$^+$ helper T cells (**B**), and CD8a$^+$ cytotoxic T cells (**C**) within CD3$^+$ T-cell population were quantified (n = 7). Mean + SEM. *$P < 0.05$ versus corresponding *TonEBP*$^{fl/fl}$.

Supplementary Figure S10. Myeloid TonEBP deficiency reduces immune responses in spleens of pristane-induced lupus mice. Spleens and kidneys were isolated from *TonEBP*$^{fl/fl}$, *LysM-cre* mice and their *TonEBP*$^{fl/fl}$ littermates 8 months after phosphate-buffered saline (PBS) (n = 4) or pristane (n = 5) administration. (**A**) Splenic mRNA expression encoding TonEBP, type I interferon (IFN) (IFN-α and IFN-β), T-helper cell 1 (Th1)/T-helper cell 17 (Th17) cytokines (interleukin [IL]-6, IL-17A, IL-17F, and IFN-γ), and M2 phenotype (Arginase-1 and IL-10) was measured by quantitative real time-polymerase chain reaction (RT-PCR). (**B**) Renal mRNA expression encoding TonEBP, type I IFN (IFN-α and IFN-β), Th1/Th17 cytokines (IL-6, IL-17A, IL-17F, and IFN-γ), and M2 phenotype (Arginase-1 and IL-10) was measured by quantitative RT-PCR. Mean + SEM. #$P < 0.05$ versus corresponding PBS, *$P < 0.05$ versus corresponding *TonEBP*$^{fl/fl}$.

Supplementary Figure S11. TonEBP promotes nuclear factor (NF)–κB transcriptional activity without altering nuclear translocation and DNA binding of p65. (**A**) RAW264.7 cells were transfected with scrambled (Scr) or TonEBP (Ton) siRNA. mRNA expression of *TonEBP*, *Il12b*, *Il1b*, *Tnfa*, and *Ifnb1* was analyzed after treatment with lipopolysaccharide (LPS) (100 ng/ml), R848 (10 μg/ml), CpG-B (1 μg/ml), or vehicle (VH) for 6 hours using quantitative real time-polymerase chain reaction (RT-PCR) (n = 4). (**B**) siRNA transfected cells were transfected again with an NF-κB reporter construct. Luciferase activity was measured after 8 hours of treatment with LPS, R848, CpG-B, or VH (n = 3). (**C**) Cells were transfected with siRNA, followed by stimulation with LPS, R848, CpG-B, or VH for 1 hour. Cytoplasmic and nuclear extracts were separated using the nuclear extraction kit and were immunoblotted for TonEBP, p65, or Lamin B. (**D**) Cells were transfected with siRNA, followed by stimulation with LPS, R848, CpG-B, or VH for 1 hour. Nuclear extracts were used to analyze DNA binding of p65 to an NF-κB binding site. Mean + SEM. #$P < 0.05$ versus corresponding VH, *$P < 0.05$ versus corresponding Scr.

Supplementary Figure S12. TonEBP interacts with mTOR and regulates mTOR/S6K signaling pathway in a Rel-homology domain (RHD)–dependent manner. (**A**) HEK293T cells were transfected with a plasmid expressing FLAG-tagged mTOR and Myc-tagged Yc1 for 24 hours. Cell lysates were immunoprecipitated with anti-Myc or anti-FLAG antibodies, followed by immunoblotting. (**B**) Immunoblot analyses of phosphorylated mTOR (p-mTOR), mTOR, phosphorylated S6K (p-S6K), S6K, and TonEBP after 30 minutes of treatment with lipopolysaccharide (LPS) (100 ng/ml), R848 (10 μg/ml), CpG-B (1 μg/ml), or vehicle (VH) were performed. (**C**) Quantification of p-mTOR or p-S6K level normalized to total mTOR or S6K level. Mean + SEM. #$P < 0.05$ versus corresponding VH, *$P < 0.05$ versus corresponding scrambled (Scr). (**D**) HEK293T cells were transfected with TonEBP siRNA followed by transfection with empty vector (pCMV-Tag 2A) or expression vector for flag-tagged Yc1, ΔRHD, or TonEBP. The cells were then treated with LPS, R848, or CpG-B for 30 minutes before immunoblotting. (**E**) Quantification of p-S6K level normalized to total S6K

level. Mean + SEM. #$P < 0.05$ versus corresponding TonEBP siRNA, *$P < 0.05$ versus corresponding TonEBP siRNA + pCMV-Tag 2A, $$P < 0.05$ versus corresponding TonEBP siRNA + FLAG-ΔRHD.

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