ORIGINAL RESEARCH

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Transglutaminase is a Critical Link Between Inflammation and Hypertension

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***Background*—**The pathogenesis of essential hypertension is multifactorial with different underlying mechanisms contributing to disease. We have recently shown that TNF superfamily member 14 LIGHT (an acronym for homologous to lymphotoxins, exhibits inducible expression, and competes with herpes simplex virus glycoprotein D for herpes virus entry mediator, a receptor expressed by T lymphocytes, also known as TNFSF14) induces hypertension when injected into mice. Research reported here was undertaken to examine the role of transglutaminase (TGase) in LIGHT-induced hypertension.

***Methods and Results*—**Initial experiments showed that plasma and kidney TGase activity was induced by LIGHT infusion (13.91 ± 2.92 versus 6.75 ± 1.92 mU/mL and 19.86 ± 3.55 versus 12.00 ± 0.97 mU/10 μg) and was accompanied with hypertension (169 ± 7.16 versus 117.17 ± 11.57 mm Hg at day 14) and renal impairment (proteinuria, 61.33 ± 23.21 versus 20.38 ± 9.01 μg/mg; osmolality, 879.57 ± 93.02 versus 1407.2 ± 308.04 mmol/kg). The increase in renal TGase activity corresponded to an increase in RNA for the tissue TGase isoform, termed TG2. Pharmacologically, we showed that LIGHT-induced hypertension and renal impairment did not occur in the presence of cystamine, a well-known competitive inhibitor of TGase activity. Genetically, we showed that LIGHT-mediated induction of TGase, along with hypertension and renal impairment, was dependent on interleukin-6 and endothelial hypoxia inducible factor-1α. We also demonstrated that interleukin-6, endothelial hypoxia inducible factor-1α, and TGase are required for LIGHT-induced production of angiotensin receptor agonistic autoantibodies.

***Conclusions*—**Thus, LIGHT-induced hypertension, renal impairment, and production of angiotensin receptor agonistic autoantibodies require TGase, most likely the TG2 isoform. Our findings establish TGase as a critical link between inflammation, hypertension, and autoimmunity. **(*J Am Heart Assoc*. 2016;5:e003730 doi: 10.1161/JAHA.116.003730)**

Key Words: angiotensin receptor • AT_1-AA • hypertension • inflammation • transglutaminase

The pathogenesis of essential hypertension is multifactorial, with different underlying mechanisms contributing to the disease. In recent years, there has been an increased effort to determine the role of the immune system in the development of hypertension.[1–5] Research concerning the immunological basis of hypertension has revealed that both the innate and adaptive arms of the immune system contribute to the pathophysiology of hypertension, features explored by research presented here. The inflammatory response that accompanies activation of the innate immune system has received considerable attention and has revealed a prominent role for proinflammatory cytokines in the development of hypertension.[6–8] Human hypertension is associated with highly elevated levels of key proinflammatory markers, including C-reactive protein, tumor necrosis factor (TNF), interleukin (IL)-1β, IL-6, and IL-17, among others.[6–8] The molecular mechanisms linking inflammation to hypertension are poorly understood.

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We have examined the role of inflammatory cytokines in an experimental model of hypertension in mice.[9] Specifically we have recently shown that TNF superfamily member 14, LIGHT (an acronym for homologous to lymphotoxins, exhibits inducible expression, and competes with herpes simplex virus glycoprotein D for herpes virus entry mediator [HVEM], a receptor expressed by T lymphocytes, also known as TNFSF14) is elevated in women with preeclampsia (a serious hypertensive condition of pregnancy) and induces hypertension when injected into pregnant or nonpregnant mice.[9] LIGHT is produced by cells of the innate and adaptive immune system, including granulocytes, monocytes, macrophages, dendritic cells, and T cells.[10,11] It is initially present on the cell membrane as a homotrimer but can be released from the cell membrane via proteolytic cleavage and exist in a soluble form.[12] Whether membrane bound or free, LIGHT can bind to two receptors, HVEM, which is present on T cells, and lymphotoxin β receptor, present on nonlymphoid hematopoietic cells including natural killer cells.[13] LIGHT-mediated activation of HVEM receptors on T cells results in a costimulatory response leading to T-cell activation, cell proliferation, and cytokine production.[11] LIGHT-deficient mice have reduced T-cell immunity associated with impaired allograph rejection.[14] Thus, considerable evidence supports a role for LIGHT in adaptive T-cell immunity and cytokine production. The molecular mechanisms responsible for LIGHT-induced hypertension are not understood, a knowledge gap addressed by experiments reported here.

Transglutaminases (TGases) are a widely distributed family of enzymes that modify proteins through the hydrolytic deamination or transamidation of the carboxamide moiety of glutamine residues on proteins.[15] Several lines of evidence link TGases with inflammation and hypertension.[16–21] Initial evidence that TGase contributes to hypertension came from studies showing that cystamine, a transglutaminase inhibitor, reduced blood pressure in the spontaneously hypertensive rat.[22] More recently, we showed that circulating TGase activity is significantly elevated in women with preeclampsia and in an experimental model of preeclampsia in mice.[23] A pathogenic role for TGase in preeclampsia is suggested by our experiments showing that the TGase inhibitor cystamine or small interfering RNA (siRNA) to tissue TGase (TG2, a member of the TGase family of enzymes) mRNA significantly attenuated the hypertension and proteinuria in this mouse model. Data linking TGase to inflammation are based on reports showing that *Tgm2* gene expression (encodes TG2) is induced by transforming growth factor (TGF)-β,[24] TNF-α,[19] and IL-6,[20] proinflammatory cytokines that are highly elevated in hypertensive disease,[6–8] including preeclampsia.[25–27] Cytokine-induced *Tgm2* gene transcription is mediated by nuclear factor-κB[28,29] and hypoxia inducible factor (HIF)-1α.[30] Experiments reported here test the hypothesis that TGase is a critical link between inflammation and hypertension.

Other factors potentially linking inflammation with hypertension are agonistic autoantibodies to the AT_1 angiotensin receptor (AT_1R) that are associated with hypertensive conditions in humans.[31–33] These autoantibodies, termed AT_1-AAs, were initially identified in preeclampsia where they are present in the maternal circulation of a large majority of affected women.[34,35] These autoantibodies cause hypertension and proteinuria when introduced into pregnant or nonpregnant mice[36] and presumably contribute to these features in the women with preeclampsia from whom they were obtained. In addition to preeclampsia, these pathogenic autoantibodies are also associated with hypertensive conditions outside of pregnancy including malignant hypertension,[37,38] refractory hypertension,[39–41] and primary aldosteronism.[42–45] An interesting feature of these autoantibodies is that they uniformly recognize the same epitope (AFHYESQ) located on the second extracellular loop of AT_1Rs. Evidence linking AT_1-AAs with inflammation is provided by experiments showing that proinflammatory cytokines (TNF, IL-6, IL-17, and LIGHT) induce their production in pregnant animals.[46–49] Proinflammatory cytokines also contribute hypertension in nonpregnant animals,[9,50] but the possibility of pathogenic autoantibody production in these cases has not been examined. We show here that LIGHT-induced hypertension in nonpregnant mice is accompanied with the production of AT_1-AAs and that the production of these pathogenic autoantibodies required IL-6 and endothelial HIF-1α–dependent induction of TGase. Overall, our results show that TGase is a critical factor in cytokine-induced hypertension and the production of AT_1-AAs, pathogenic autoantibodies associated with hypertension.

Materials and Methods

Animals

Wild-type 8- to 10-week-old C57BL6 mice were purchased from Harlan Laboratories. IL-6–deficient mice (IL-6$^{-/-}$) congenic with the C57BL6 background were generated and genotyped as described.[51] We generated mice with specific endothelial HIF-1α deficiency by mating floxed HIF-1α mice (*HIF-1*$\alpha^{f/f}$) with the *VE-cadherin-Cre*$^+$ mice containing a transgene expressing Cre recombinase only in the endothelial cells. *Hif-1*$\alpha^{f/f}$ mice and *Hif-1*$\alpha^{f/f}$*/VE-cadherin cre*$^+$ mice, also congenic with C57BL6 mice, were originally from Dr Holger Eltzchig's laboratory at the University of Colorado at Denver and have been described in a previous publication.[52] Six to 8 mice for each group were infused with LIGHT via minipump. Mice were anesthetized with isoflurane (2%), and osmotic minipumps were implanted subcutaneously in the neck.

Recombinant mouse LIGHT (R&D Systems) was delivered at a rate of 4 ng/d into mice for 14 days. Cystamine-treated mice were provided drinking water containing 0.9 g/L cystamine dihydrochloride throughout the 14 days. Control mice were infused with saline. We collected urine on days 3, 5 and 14 and measured blood pressure at 0, 3, 5, 7, 10, and 14 days. After treatment for 14 days, mice were killed. All protocols involving animal research were reviewed and approved by the Institutional Animal Welfare Committee of the University of Texas Health Science Center at Houston. All animal procedures were in accordance with institutional guidelines.

Plasma TGase Activity

TGase activity in human and mouse samples was determined using in vitro TGase assay kits (Covalab; Sigma-Aldrich) following the manufacturer's instructions. The increased TGase activity we see in LIGHT-infused animals is unlikely to be factor XIIIa transglutaminase because the use of EDTA as an anticoagulant in plasma collection results in the cleavage and inactivation of factor XIIIa by thrombin.[53]

Real-Time Polymerase Chain Reaction of Kidney RNA

RNA was extracted using Trizol reagent (Invitrogen). Transcript levels were quantified using real-time quantitative reverse transcription–polymerase chain reaction. Syber green was used for analysis of *Tgm2* and *Gapdh* mRNAs by using the following primers: mouse: *Tgm2*, forward: 5′-ggtggcaggccattgacccc-3′, reverse: 5′-gccccacgaccaaggaacgg-3′, *Gapdh,* forward: 5′-tgacctcaactacatggtctaca-3′, reverse: 5′-cttcccattctcggccttg-3′. Polymerase chain reaction was performed using an ABI Prism 7700 sequence Detector (Applied Biosystems). Each cDNA sample was run in triplicate. For data analysis, the $\Delta\Delta$Ct method was used. For each gene, the fold change was calculated as a difference in kidney gene expression.

IL-6 ELISA of Mice

We detected IL-6 levels in mice blood using a BD OptEIA™ Mouse IL-6 ELISA Kit (BD Biosciences) following the instructions provided by the vendor.

Blood Pressure Measurement and Quantification of Urinary Protein

The systolic blood pressure was noninvasively measured by determining the tail blood volume with a volume pressure recording sensor and an occlusion tail-cuff (CODA System; Kent Scientific). We routinely adapt the mice to blood pressure measurements by training them 3 or 4 times in the device before initiating the actual experiments and have successfully used this approach many times in recent years.[9,36,54–56] Blood pressure was measured at the same time daily ($\pm$1 hour) while the mice were kept warm using a warming pad. In previous experiments, we have validated blood pressure measurements through invasive monitoring of intracaroitid arterial pressure in mice on the last day of tail-cuff measurement.[9] Further, published results show that this method is in good agreement with radiotelemetry measurements of blood pressure.[57]

Twenty-four-hour urine specimens were collected for analysis through the use of metabolic cages (Nalgene). Mice were trained in metabolic cages for 2 days before urine collection. All of the mice were killed on GD18.5 before delivery, when their serum and organs were collected. We quantified urinary albumin using ELISA (Exocell) and measured urinary creatinine with the use of a picric acid colorimetric assay (Exocell). We used the ratio of urinary albumin to urinary creatinine as an index of urinary protein. Urine osmolality was measured by using a vapor pressure osmometer (Wescor).

Measurement of AT_1-AAs

ELISA

AT_1-AAs were measured as previously described[49] using a commercially available sandwich ELISA (CellTrend GmbH) that is now marketed by One Lambda. The ELISA from CellTrend was supplied to measure human AT_1-AAs. For measurement of murine AT_1-AAs, the HRP-linked antihuman IgG that was supplied with the kit was replaced with horseradish peroxidase–linked antimouse IgG secondary antibody (Jackson ImmunoResearch Labs). Other steps in this measurement were carried out according to vendor instructions. We have recently described the use of the original assay to detect human AT_1-AAs and its modification to measure mouse AT_1-AAs.[49]

Peptide display

Plasma samples from mice were assayed for binding to the 7–amino acid epitope peptide by using an assay in which the epitope sequence AFHYESQ is displayed in a high-avidity format on the bacterial cell surface.[58] The assay detects only mouse IgG antibodies that bind to the 7–amino acid epitope peptide. This is because after incubation with mouse plasma (1:100 dilution), whatever binds to the 7–amino acid epitope peptide is detected with the use of biotinylated goat antimouse IgG antibody, diluted 1:200 (Vector Labs). The latter is detected with the use of a streptavidin-conjugated *R*-phycoerythrin (bright red-orange fluorescence), diluted 1:333. All of these incubations are carried out with reagents diluted in PBS with 0.1% BSA. AT_1-AA binding activity is

measured as fold fluorescence over background (a negative control consisting of bacteria with an empty scaffold, ie, no displayed peptide). Thus, this assay only detects mouse antibodies that bind to the empty scaffold and to the scaffold with the 7–amino acid epitope peptide displayed. Further, another step of preincubating the plasma with the negative control bacteria is also used to remove anything binding to bacteria with the empty scaffold. Antibody binding to the 7–amino acid epitope peptide is expressed as fold increase over background. This assay was initially described by Elliott et al[58] and subsequently used by Liu et al.[49] This methodology does not require purified antibodies as additional work on bacterial displayed peptide libraries has used diluted plasma with an Ig-specific secondary reagent.[59] Detecting plasma Ig with a secondary reagent is consistently used wth ELISA and protein microarray studies as well.[60]

Statistical Analyses

All data are expressed as mean±SEM. Measurements of blood pressure, urinary protein concentrations, and plasma concentrations of autoantibodies and enzymes typically approximated a normal distribution. Unpaired Student *t* tests were applied in 2-group analysis. Differences between the means of multiple groups were compared by using 1-way ANOVA, followed by Tukey's multiple comparisons test. Blood pressure measurements data are mean values taken at different time points (days 0, 3, 5, 7, 10, and 14). The longitudinal data were analyzed by using a repeated-measures ANOVA followed by Tukey's multiple comparisons test. AT_1-AA epitope binding activity data were analyzed by using 1-way ANOVA followed by Dunnett's multiple comparisons test. A value of $P<0.05$ was considered significant. Statistical programs were run with GraphPad Prism 5 software (GraphPad Software).

Results

LIGHT Infusion Results in an IL-6– and Endothelial HIF-1α–Dependent Increase in Plasma and Renal TGase Activity

We have previously shown that LIGHT induces hypertension in pregnant and nonpregnant animals.[9] To determine if elevated TGase is also associated with LIGHT-induced hypertension, we infused recombinant mouse LIGHT into mice for 5 or 14 days and examined mice for elevated TGase. The results show that plasma TGase activity (Figure 1A) was significantly increased after 14 days of LIGHT infusion. Kidney TGase activity showed a small increase at day 5 and a significantly greater increase

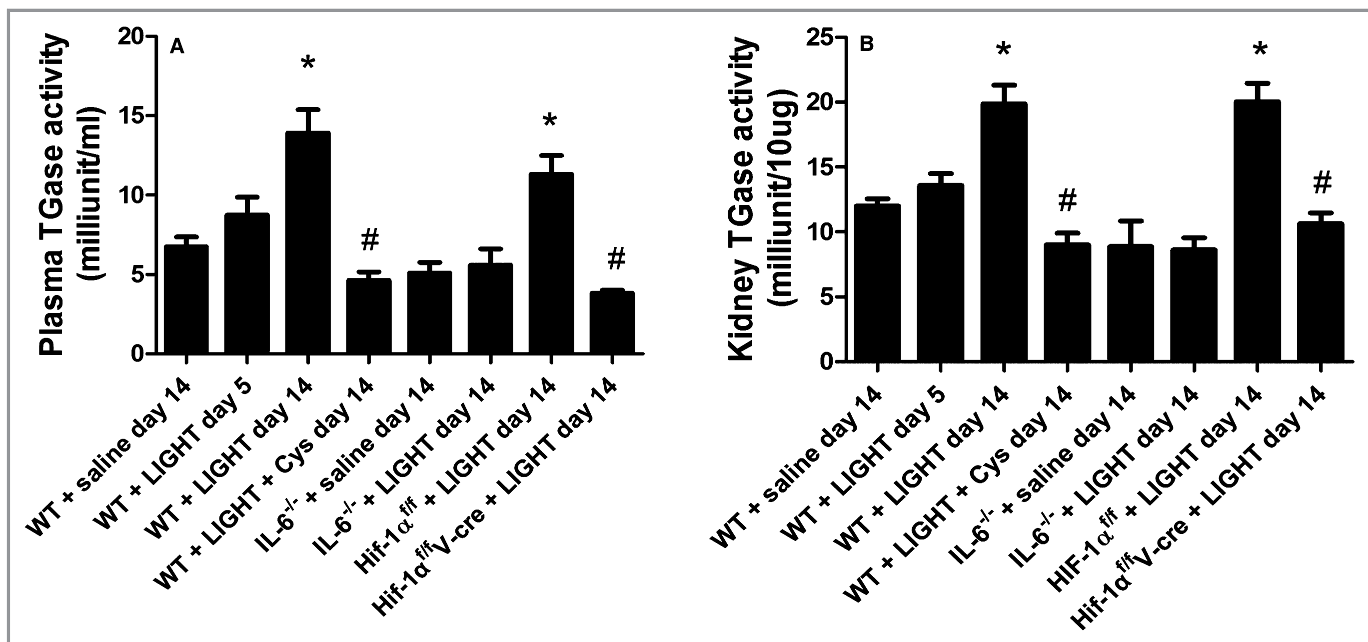

Figure 1. LIGHT (an acronym for homologous to lymphotoxins, exhibits inducible expression, and competes with herpes simplex virus glycoprotein D for herpes virus entry mediator, a receptor expressed by T lymphocytes, also known as TNFSF14) infusion results in an interleukin (IL)-6– and endothelial hypoxia inducible factor (HIF)-1α–dependent increase in plasma and renal transglutaminase activity that is inhibited by cystamine (Cys). Wild-type (WT) or mutant mice were infused with recombinant mouse LIGHT for up to 14 days in the presence or absence of Cys. Plasma transglutaminase (TGase) activity (A) and kidney TGase activity (B) were determined following 5 or 14 days of LIGHT infusion. Data are expressed as mean±SEM; $^*P<0.05$ vs saline-infused mice; $^{\#}P<0.05$ vs LIGHT-infused mice. n=6 for all groups except for WT+saline day 14, WT+LIGHT day 14, where n=8 (V-cre indicates *VE-cadherin-cre*$^+$).

at day 14 (Figure 1B). The increase in plasma and renal TGase activity was prevented with continuous administration of cystamine, a competitive inhibitor of TGase.

To determine if the increased renal TGase was caused by increased transcriptional activity, we prepared kidney RNA from LIGHT-infused animals and saline-infused animals and determined the level of TGase mRNA through quantitative RT-PCR. RNA was probed for the TG2 isoform of TGase, termed tissue TGase, that is the most abundant TGase isoform in the kidney. The results (Figure 2) show an increase in TG2 mRNA evident by day 5 of LIGHT infusion, reaching a significant increase by day 14. These results indicate that LIGHT infusion mediates increased transcription of the *Tgm2* gene encoding TG2.

The *Tgm2* gene is activated by inflammation and hypoxia and specifically includes regulatory elements that respond to IL-6[20] and HIF-1.[30] To determine if IL-6 or HIF-1α is required for LIGHT induction of TG2, experiments were carried out using mice that are globally deficient in IL-6 and mice that have an endothelial specific HIF-1α deficiency (*Hif-1α$^{f/f/}$/VE-cadherin cre^{+}*).[52] The results (Figure 1A and 1B) show that LIGHT induction of plasma and renal TGase activity did not occur in these mice. Likewise, LIGHT-mediated induction of renal TG2 mRNA was significantly inhibited in IL-6–deficient mice or mice with an endothelial specific HIF-1α deficiency (Figure 2). The requirement for IL-6 is consistent with data showing that LIGHT infusion resulted in increased circulating IL-6 (Figure 3). Overall, these results indicate that TG2, encoded by the *Tgm2* gene TG2, is the TGase that is induced by LIGHT. Further, the results from the endothelial HIF-1α–deficient mice suggest that the majority of the TG2 is derived from endothelial cells.

LIGHT-Induced Hypertension Requires TGase and Is Dependent on IL-6 and Endothelial HIF-1α

To determine if elevated TGase is required for LIGHT-induced hypertension, mice were continuously infused with LIGHT for 14 days in the presence or absence of cystamine. Blood pressure was monitored periodically from the day of injection through day 14 through the use of tail-cuff plethysmography. We found (Figure 4A) that systolic blood pressure increased significantly by 3 days of LIGHT infusion and continued to increase throughout the 14-day experiment. Blood pressure in the saline-infused mice remained constant. The results

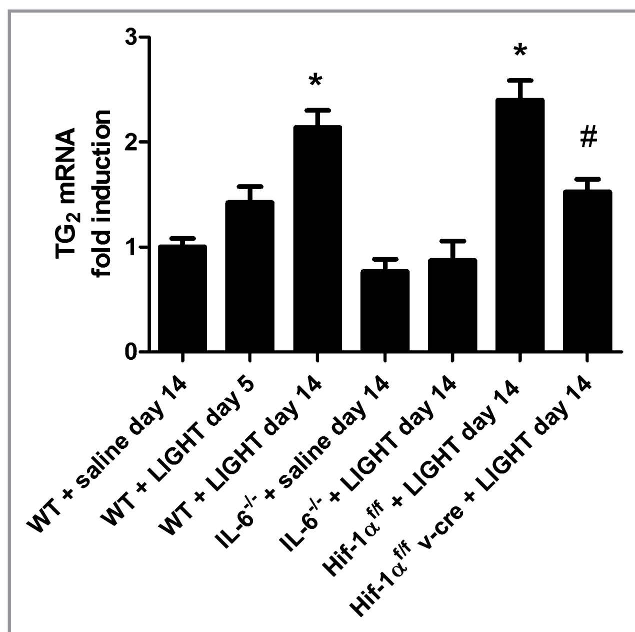

Figure 2. LIGHT (an acronym for homologous to lymphotoxins, exhibits inducible expression, and competes with herpes simplex virus glycoprotein D for herpes virus entry mediator, a receptor expressed by T lymphocytes, also known as TNFSF14)-induced TG2 mRNA in kidneys. Wild-type mice were infused with recombinant mouse LIGHT for either 5 or 14 days. Kidneys were obtained, and RNA was extracted and analyzed by quantitative RT-PCR for TG2 mRNA. Data are expressed as mean±SEM; *P<0.05 vs saline-infused mice; #P<0.05 vs hypoxia inducible factor (HIF)-1α$^{f/f}$+LIGHT-infused mice. n=6 for all groups except for WT+saline day 14, WT+LIGHT day 14, where n=8.

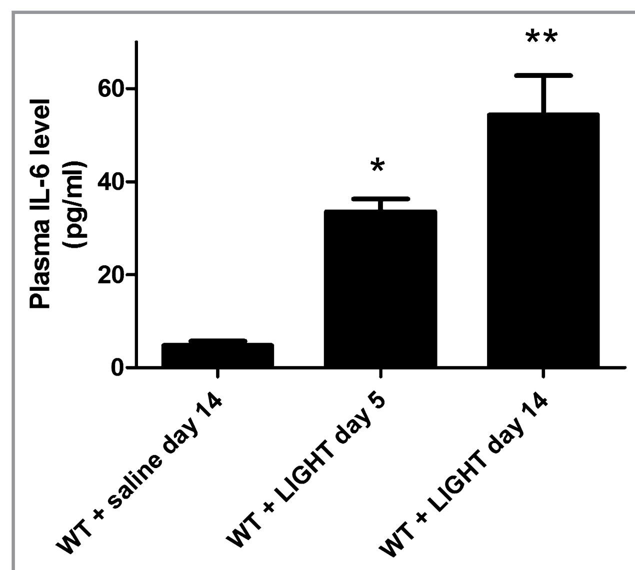

Figure 3. LIGHT (an acronym for homologous to lymphotoxins, exhibits inducible expression, and competes with herpes simplex virus glycoprotein D for herpes virus entry mediator, a receptor expressed by T lymphocytes, also known as TNFSF14)-induced interleukin (IL)-6. Wild-type mice were infused with recombinant mouse LIGHT for either 5 or 14 days. Plasma was obtained, and the concentration of IL-6 was determined by ELISA. Data are expressed as mean±SEM; *P<0.05 vs saline-infused mice; **P<0.01 vs saline-infused mice. n=6 for WT + LIGHT day 5 and n=8 for WT+saline day 14 and WT+LIGHT day 14.

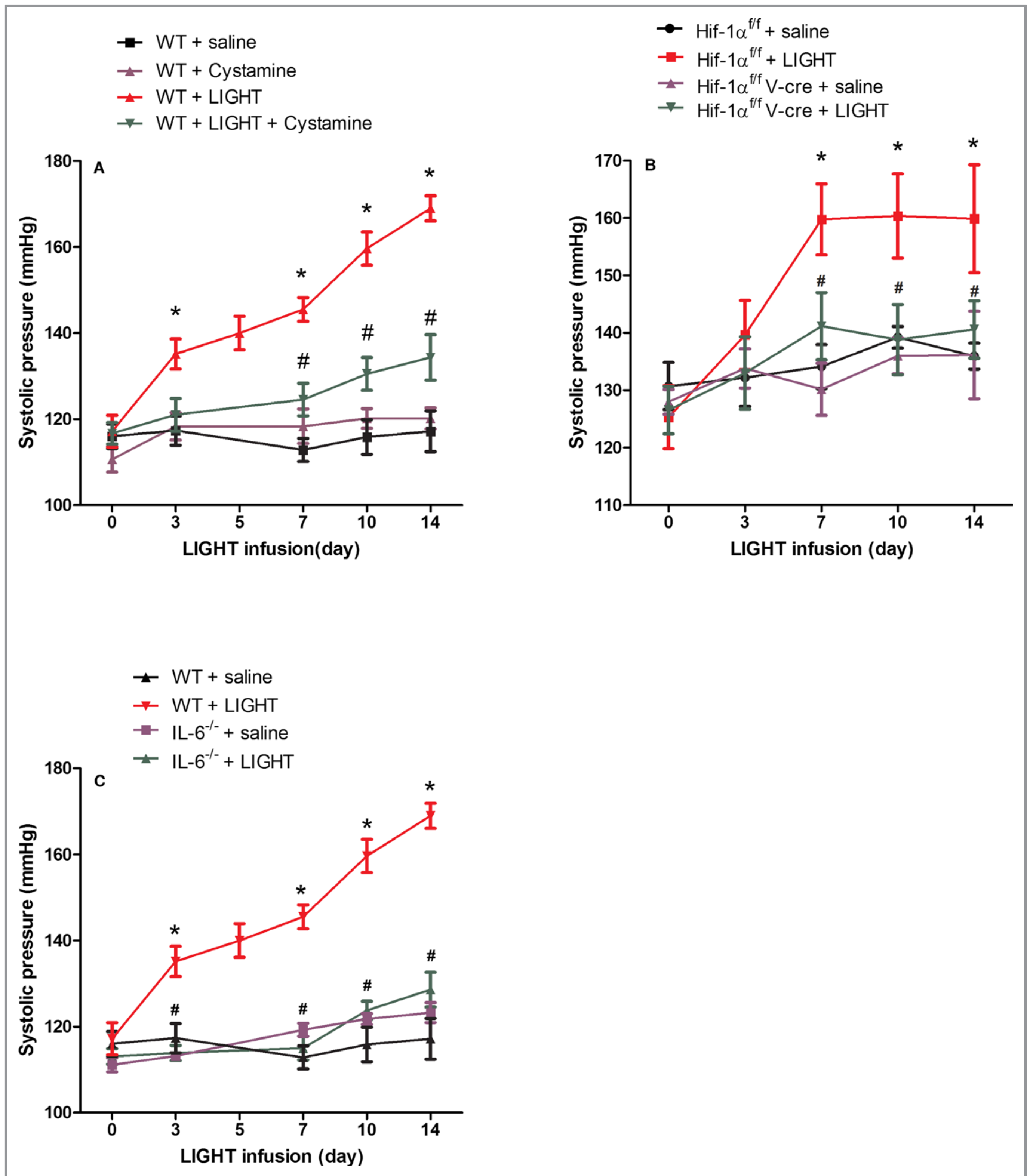

Figure 4. LIGHT (an acronym for homologous to lymphotoxins, exhibits inducible expression, and competes with herpes simplex virus glycoprotein D for herpes virus entry mediator, a receptor expressed by T lymphocytes, also known as TNFSF14)-induced hypertension is inhibited by cystamine and is dependent on interleukin (IL)-6 and endothelial hypoxia inducible factor (HIF)-1α. Wild-type (WT) or mutant mice were infused with recombinant mouse LIGHT for up to 14 days in the presence or absence of cystamine (Cys). Systolic blood pressure was measured at periodic intervals by tail-cuff plethysmography. Data are expressed as mean±SEM; $^{*}P<0.05$ vs saline-infused mice, $^{\#}P<0.05$ vs C57BL6+LIGHT (A and C) or HIF-1$\alpha^{f/f}$+LIGHT (B). n=6 for all groups except for WT+saline day 14, WT+LIGHT day 14, where n=8 (V-cre indicates *VE-cadherin-cre*$^{+}$).

(Figure 4A) also show that the presence of cystamine significantly inhibited LIGHT-induced hypertension.

We also conducted LIGHT infusion experiments using IL-6–deficient mice and mice with an endothelial cell–specific deficiency in HIF-1α (*Hif-1α$^{f/f}$/VE-cadherin cre^{+}*). LIGHT-mediated induction of hypertension did not occur in mice with an endothelial cell–specific deficiency in HIF-1α (Figure 4B) or a global deficiency in IL-6 (Figure 4C). As shown in Figure 1, LIGHT-mediated induction of plasma and renal TGase did not occur in these mice. These results show that LIGHT infusion induces a robust increase in blood pressure that requires TGase and is dependent on IL-6 and endothelial HIF-1α.

LIGHT-Induced Renal Impairment Requires TGase and Is Dependent on IL-6 and Endothelial HIF-1α

To determine if renal impairment was associated with LIGHT-induced hypertension and the induction of TGase, we collected urine in metabolic cages on days 3, 5, and 14 for determination of proteinuria and urine osmolality. Saline-infused animals were used as controls, and cystamine-treated animals served to determine the contribution of TGase to any observed changes in proteinuria or urine osmolality. The results show that proteinuria (Figure 5A) began to increase by 3 and 5 days of LIGHT infusion, although the increase did not achieve statistical significance. However, by day 14, there was a significant LIGHT-induced elevation in urinary protein (Figure 5A). We also observed a significant reduction in urine osmolality after 14 days of LIGHT infusion (Figure 5B). These LIGHT-induced changes in renal physiology were prevented by cystamine treatment. Additionally, these changes did not occur in IL-6–deficient mice or mice with an endothelial HIF-1α deficiency. Overall, these results indicate that LIGHT-induced renal impairment is dependent on IL-6– and endothelial HIF-1α–mediated induction of TG2.

IL-6– and Endothelial HIF-1α–Dependent Induction of TGase Is Required for Production of AT_1-AAs in LIGHT-Infused Mice

To determine if LIGHT-induced hypertension and renal impairment were accompanied by the production of AT_1-AAs and to determine if TGase is required for cytokine-induced AT_1-AA production, we carried out LIGHT infusion experiments in the presence or absence of cystamine, as described here

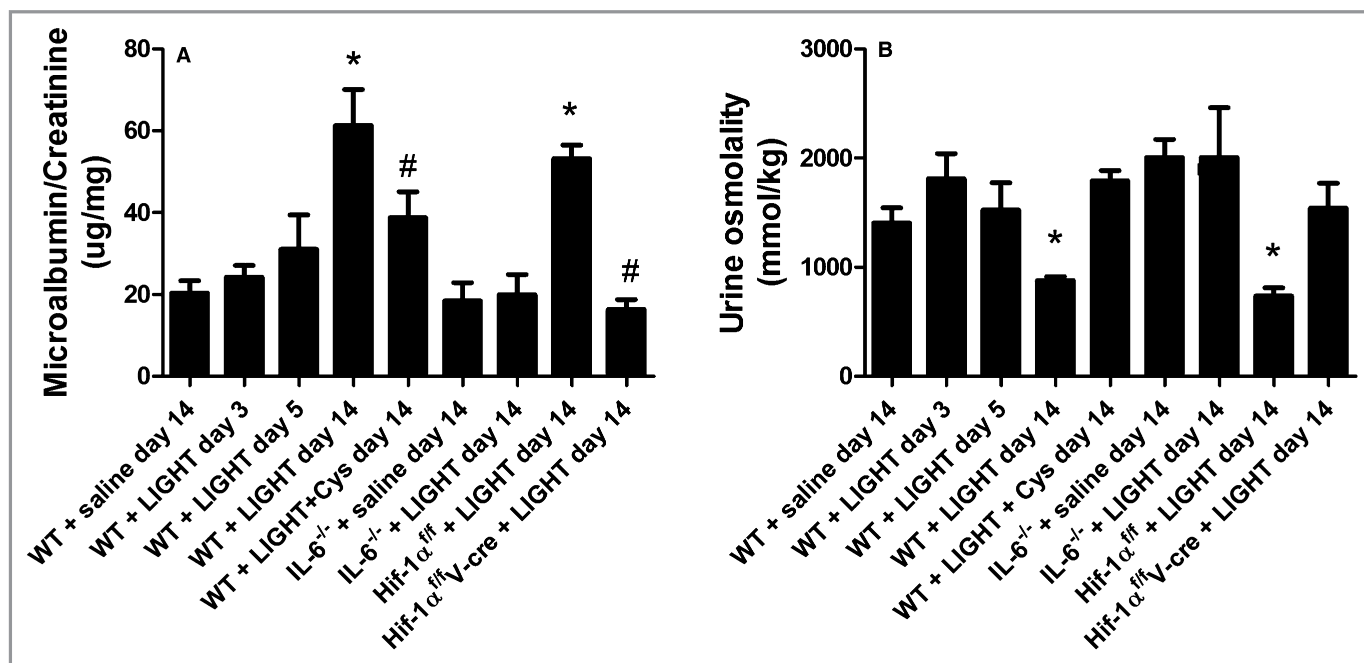

Figure 5. LIGHT (an acronym for homologous to lymphotoxins, exhibits inducible expression, and competes with herpes simplex virus glycoprotein D for herpes virus entry mediator, a receptor expressed by T lymphocytes, also known as TNFSF14)-induced proteinuria and urine osmolality is inhibited by cystamine and is dependent on interleukin (IL)-6 and endothelial hypoxia inducible factor (HIF)-1α. Wild-type (WT) or mutant mice were infused with recombinant mouse LIGHT for up to 14 days in the presence or absence of cystamine (Cys). Twenty-four-hour urine specimens were collected at the times indicated, and the ratio of albumin to creatinine (A) and urine osmolality (B) was determined. Data are expressed as mean±SEM; $^{*}P<0.05$ vs saline-infused mice; $^{\#}P<0.05$ vs LIGHT-infused mice. n=6 for all groups except for WT+saline day 14, WT+LIGHT day 14, where n=8 (V-cre indicates *VE-cadherin-cre*$^{+}$).

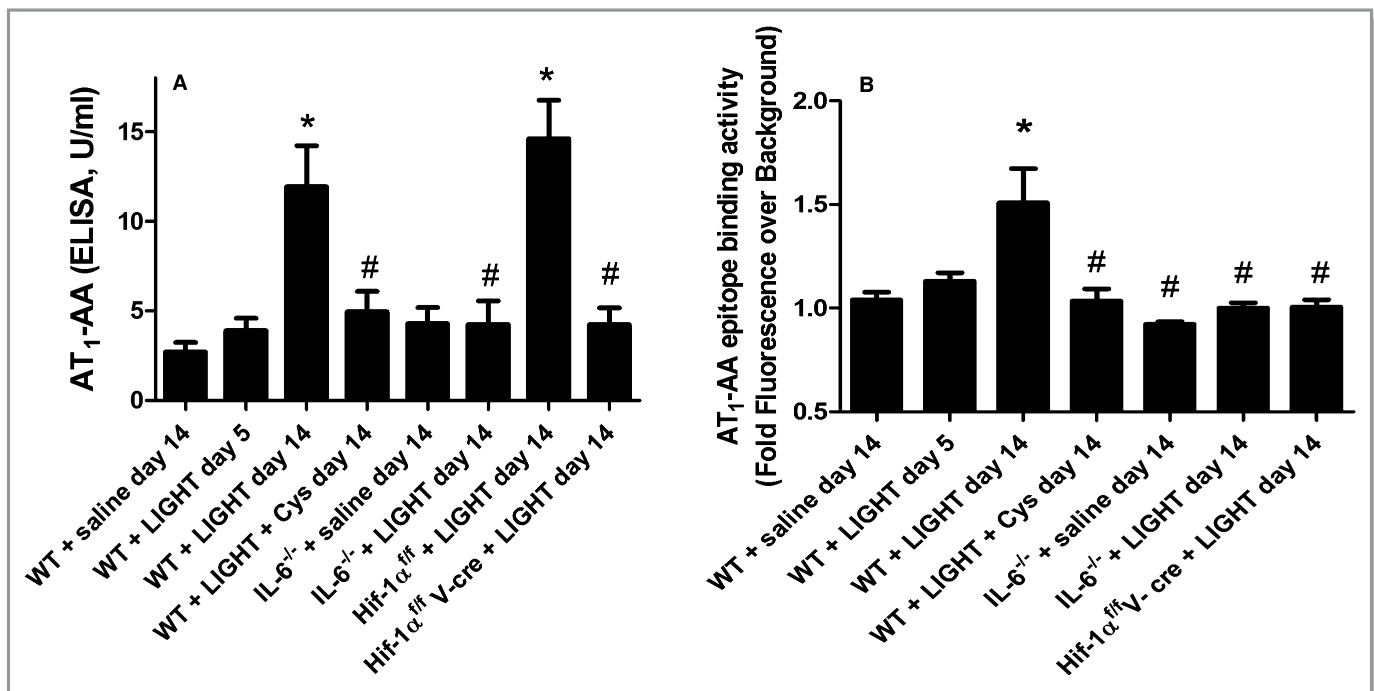

Figure 6. LIGHT (an acronym for homologous to lymphotoxins, exhibits inducible expression, and competes with herpes simplex virus glycoprotein D for herpes virus entry mediator, a receptor expressed by T lymphocytes, also known as TNFSF14)-induced agonistic autoantibodies to the AT_1 angiotensin receptor (AT_1-AAs) production is inhibited by cystamine (Cys) and is dependent on interleukin (IL)-6 and endothelial hypoxia inducible factor (HIF)-1α. Wild-type (WT) or mutant mice were infused with recombinant mouse LIGHT for up to 14 days in the presence or absence of Cys. AT_1-AA levels were determined by using a cell-based ELISA (A) or a bacterial epitope peptide display assay (B). Data are expressed as mean±SEM; *P<0.05 vs saline-infused WT mice; $^{\#}P$<0.05 vs LIGHT-infused WT mice. n=6 for all groups except for WT+saline day 14, WT+LIGHT day 14, where n=8 (A). n=4 for WT+saline day 14, n=5 for WT+LIGHT day 5, n=8 for WT+LIGHT day 14, n=5 for WT+LIGHT+cys day 14, n=3 for IL-$6^{-/-}$+saline day 14 and IL-$6^{-/-}$+LIGHT day 14, n=6 for HIF-1$\alpha^{f/f}$V-cre+LIGHT day 14 (B) (V-cre indicates VE-cadherin-cre^{+}).

earlier. Some mice were killed after 5 days of LIGHT infusion, while others were killed after 14 days of infusion. Blood was collected for determination of AT_1-AA titer. Autoantibody titers were determined by using a cell-based ELISA, and a peptide display assay in which the epitope peptide recognized by human AT_1-AA (AFHYESQ) is displayed on the cell wall of specially engineered bacteria. The results from each assay (Figure 6A and 6B) are in good agreement and show that AT_1-AAs were slightly (though not significantly) elevated at day 5 of infusion and that autoantibody titers were significantly elevated following 14 days of LIGHT infusion. The results of the epitope peptide display assay indicates that the antibodies produced following LIGHT infusion bind to the same 7–amino acid epitope on the second extracellular loop of the AT_1 receptor that is recognized by autoantibodies in women with preeclampsia. Additionally, the induction is blocked by the presence of cystamine, indicating that TGase activity is required for LIGHT-induced autoantibody production. Mutant mice lacking IL-6 or endothelial HIF-1α also failed to show LIGHT-induced autoantibody production (Figure 6A and 6B). Together, these results show that AT_1-AAs are produced in LIGHT-infused mice by a process that depends on IL-6– and endothelial HIF-1α–dependent induction of TGase.

Discussion

Research reported here was undertaken to identify factors that contribute to LIGHT-induced hypertension.[9] Using a combination of pharmacological and genetic approaches, we showed that elevated TGase activity was required for LIGHT-induced hypertension and renal impairment. Pharmacologically, we showed that LIGHT-induced hypertension and renal impairment did not occur in the presence of cystamine, a well-known competitive inhibitor of TGase activity. Genetically, we showed that LIGHT-induced hypertension, renal impairment and elevated TGase were dependent on IL-6 and endothelial HIF-1α. These results extend earlier observations that TGase inhibitors or siRNA to tissue TGase (TG2) can lower blood pressure in animal models of hypertensive disease.[22,23] We also show here that LIGHT induced the production of AT_1-AAs and that elevated TGase is required for autoantibody production. LIGHT-induced AT_1-AA production also requires IL-6 and endothelial HIF-1α. Thus, our results show that LIGHT-induced

TGase, hypertension, renal impairment, and the production of AT_1-AAs are inhibited by cystamine and require IL-6 and endothelial HIF-1α.

Considerable evidence from human studies shows a strong association between hypertension and elevated inflammatory cytokines, including the inflammatory marker C-reactive protein.[61–65] These results have led some to hypothesize that hypertension is in part an inflammatory disorder. This hypothesis is supported by experimental studies in mice showing that TNF-α, IL-6, and IL-17 are required for angiotensin II–induced hypertension in mice.[66–70] Additional support comes from experiments showing that the introduction of IL-17 or LIGHT, a TNF superfamily member, into mice results in hypertension.[9,50] Animal models, such as the one we describe here, that recapitulate the hypertensive consequences of elevated inflammatory cytokines provide critically important experimental opportunities to determine the mechanism of cytokine-induced hypertension, a knowledge gap we have addressed by research reported here. Using our experimental model, we present data indicating that endothelial TG2 is required for LIGHT-induced hypertension. Our research provides original evidence that TG2 is a previously unrecognized factor linking inflammation, autoimmunity, and hypertension.

Because AT_1-AAs are likely to be significant contributors to hypertension,[32] it is important to understand the pathophysiological conditions and molecular mechanisms that initiate their production. Evidence linking inflammation with AT_1-AA production is provided by experiments showing that proinflammatory cytokines (TNF, IL-6, IL-17, and LIGHT) induce AT_1-AA production in pregnant animals.[46–48] In these experiments, antibody was detected within 5 days of cytokine infusion or injection. However, proinflammatory cytokines also cause hypertension in nonpregnant animals,[9,50] but the possibility of pathogenic autoantibody production in these cases has not been thoroughly examined until now. Earlier studies failed to detect AT_1-AA production in nonpregnant rats within 5 days of initial infusion of proinflammatory cytokines TNF, IL-6, and IL-17.[46–48] However, these investigators did not infuse nonpregnant animals for longer times and thus may have missed AT_1-AA production occurring after 5 days of cytokine infusion. We, too, did not observe a significant increase in AT_1-AA production following 5 days of cytokine infusion (Figure 6). However, by 14 days of LIGHT infusion, AT_1-AA production was readily apparent (Figure 6). Thus, AT_1-AAs appear within 5 days of cytokine injection in pregnant mice but require a longer time to appear in LIGHT-injected nonpregnant mice. The cytokine infusion models of AT_1-AA production represent well-defined and experimentally pliable model systems for identifying the molecular mechanisms that underlie autoantibody production.

A commonly considered mechanism for an autoimmune response is posttranslational modification, resulting in the creation of a neoantigen that is recognized as nonself by the immune system.[71,72] A large percentage of proteins in the body are posttranslationally modified, and it is now clear that some of these modifications create neoantigens that stimulate an autoimmune response.[73,74] One of the best examples of this is the posttranslational modification of arginine residues to citrulline, a process termed "citrullination" that contributes to autoimmune diseases such as rheumatoid arthritis and multiple sclerosis.[72] Elevated TGase is commonly associated with inflammation and may contribute to autoimmunity by posttranslational modification of proteins, possibly by the addition of serotonin.[75] We have recently reported that circulating TGase activity is elevated in women with preeclampsia and in an experimental mouse model of preeclampsia.[23] Further, we have shown that TG2 modifies AT_1 receptors in placentas of women with preeclampsia and in the placentas of a mouse model of preeclampsia by the addition of a small-molecular-weight molecule.[23] We have shown here that LIGHT-induced hypertension in nonpregnant

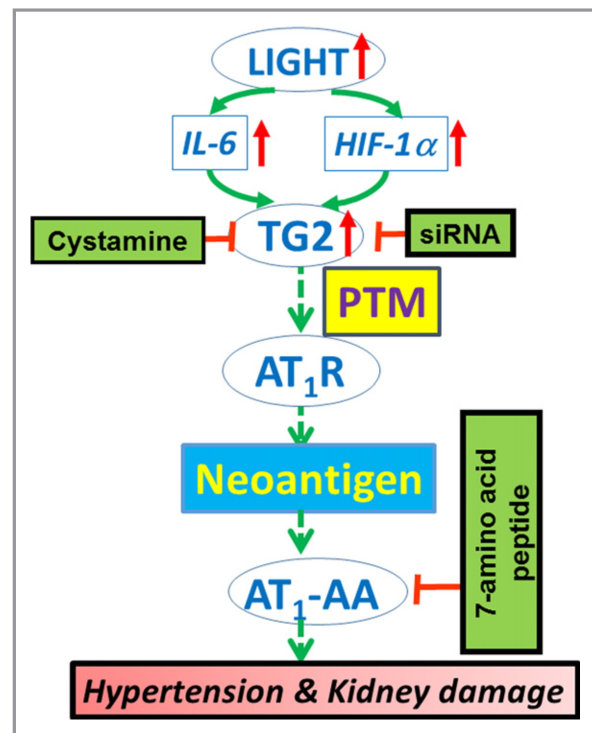

Figure 7. Hypothesis concerning cytokine-induced hypertension. A model of hypertension involving LIGHT (an acronym for homologous to lymphotoxins, exhibits Inducible expression, and competes with herpes simplex virus glycoprotein D for herpes virus entry mediator, a receptor expressed by T lymphocytes, also known as TNFSF14) induced tissue transglutaminase (TG2) and the posttranslational modification (PTM) of AT_1 angiotensin receptor (AT_1Rs) resulting in the creation of a neoantigen that underlies autoimmune production of agonistic autoantibodies to the AT_1 angiotensin receptor (AT_1-AAs). The resulting autoantibodies contribute to hypertension by activation of AT_1Rs.

mice is accompanied by the production of AT_1-AAs and that the production of these pathogenic autoantibodies is blocked by cystamine, a competitive inhibitor of TGase. An interesting feature of these autoantibodies is that they uniformly recognize the same epitope (AFHYESQ) located on the second extracellular loop of AT_1 receptors. It is noteworthy that the epitope peptide contains a glutamine (**Q**) (1 of only 5 in the entire protein), a potential target of posttranslational modification by TGase. Thus, cytokine-induced TG2 production in endothelial cells may result in posttranslational modification of AT_1 receptors and autoimmune recognition of the TG2-modified receptor. Our findings raise the interesting possibility that TG2-mediated PTM of AT_1 receptors may contribute to autoimmune recognition and autoantibody production (Figure 7), a possibility to be tested by future research.

The mammalian TGase family includes 8 enzymatically active homologues (TG1-7 and factor XIIIa) that catalyze the posttranslational modification of selected glutamine residues on proteins.[76] Factor XIIIa is well known for its role in blood clotting and is located primarily in platelets. TG1 and TG3 are produced in keratinocytes of skin, and TG5 is produced in hair follicles. TG4 is predominantly found in the prostate. The expression patterns of TG6 and TG7 are not well characterized. TG2 (also called tissue TGase or endothelial TGase) is the most widely distributed member of the TGase family because of its presence in endothelial cells, smooth muscle cells, and fibroblasts. It is encoded by the *Tgm2* gene and is the most studied.[15] Previous studies have shown that TNF-α and IL-6 induce *Tgm2* gene expression in cultured hepatocytes.[19,20] Recent studies have shown that *Tgm2* expression is enhanced synergistically by interferon-γ and TNF-α in human small intestinal cells.[18] We show here that LIGHT-induced renal TGase and TG2 mRNA is prevented in IL-6–deficient mice and mice with an endothelial specific deletion of HIF-1α. IL-6 and HIF-1 are key transcriptional regulators of *Tgm2* gene expression.[15,30,77] Our results suggest that the majority of plasma and renal TGase activity induced by LIGHT is TG2 derived from endothelial cells. Further, the effect of the endothelial specific HIF-1α knockout on LIGHT-induced hypertension suggests that endothelial TG2 is a major contributor to cytokine-induced hypertension. A role for TGase in blood pressure control was originally indicated by studies showing that cystamine treatment reduced blood pressure in the spontaneously hypertensive rat.[22] In a different setting, work from our laboratory showed that the increase in blood pressure in a mouse model of preeclampsia (a hypertensive condition of pregnancy) was prevented by cystamine or by siRNA knockdown of tissue TGase (TG2) mRNA.[23] Thus, TG2 is an attractive target for antihypertensive therapy. Because TG2-deficient mice have no apparent developmental or physiological defects,[78,79] it is likely that TG2-specific inhibitors will have no adverse side effects because of the inhibition of TG2.[80,81]

Our results suggest that transglutaminase is an essential link between inflammation, autoimmunity, and hypertension. Genetic approaches presented here indicate that IL-6 and endothelial HIF-1α are required for LIGHT-induced TGase gene expression and that transglutaminase is required for LIGHT-induced hypertension, renal impairment, and AT_1-AA production. The results from endothelial HIF-1α–deficient mice suggest that the LIGHT-induced TGase is primarily from endothelial cells. Future research is required to determine the molecular mechanisms by which LIGHT induces *Tgm2* gene expression, the role of IL-6 in this process, and the mechanisms by which TG2 contributes to hypertension. Our results suggest that potent and specific TG2 inhibitors may be useful drugs for blood pressure control in some individuals.

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Disclosures

None.

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