The Ubiquitin-Like Protein FAT10 Mediates NF-κB Activation

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ABSTRACT
NF-κB is a central mediator of innate immunity and contributes to the pathogenesis of several renal diseases. FAT10 is a TNF-α-inducible ubiquitin-like protein with a putative role in immune response, but whether FAT10 participates in TNF-α-induced NF-κB activation is unknown. Here, using renal tubular epithelial cells (RTECs) derived from FAT10−/− and FAT10+/+ mice, we observed that FAT10 deficiency abrogated TNF-α-induced NF-κB activation and reduced the induction of NF-κB-regulated genes. Despite normal IkBα degradation and polyubiquitination, FAT10 deficiency impaired TNF-α-induced IkBα degradation and nuclear translocation of p65 in RTECs, suggesting defective proteasomal degradation of polyubiquitinated IkBα. In addition, FAT10 deficiency reduced the expression of the proteasomal subunit low molecular mass polypeptide 2 (LMP2). Transduction of FAT10−/− RTECs with FAT10 restored LMP2 expression, TNF-α-induced IkBα degradation, p65 nuclear translocation, and NF-κB activation. Furthermore, LMP2 transfection restored IkBα degradation in FAT10−/− RTECs. In humans, common types of chronic kidney disease associated with tubulointerstitial upregulation of FAT10. These data suggest that FAT10 mediates NF-κB activation and may promote tubulointerstitial inflammation in chronic kidney diseases.


NF-κB is a ubiquitous transcription factor that controls the expression of genes involved in immune response, apoptosis, and cell-cycle regulation. aberrant regulation of NF-κB may result in inflammatory and autoimmune diseases, impair antiviral immune responses, and contribute to malignant cellular transformation. Activation of NF-κB, with subsequent production of cytokines, chemokines, and adhesion molecules, is an important component of the pathogenesis of several forms of renal disease, including diabetic nephropathy (DN), hypertensive nephrosclerosis (HN), IgA nephropathy (IgAN), membranous glomerulopathy, and HIV-associated nephropathy.

The canonical NF-κB activation pathway can be induced by a variety of stimuli, including TNF-α. TNF-α–induced NF-κB activation is initiated by the engagement of TNF receptor type I at the plasma membrane, with subsequent signal transduction culminating in the activation of the IkB kinase complex, which in turn phosphorylates IkBα, leading to its degradation and subsequent release of nuclear factor κB (NF-κB) dimers.
IκBα. Phosphorylated IκBα is rapidly polyubiquitinated, resulting in degradation of IκBα by the 26S proteasome complex, liberating NF-κB, which then translocates to the nucleus and activates transcription of target genes.13–16

The 26S proteasome, the main protease in eukaryotic cells, recognizes and degrades polyubiquitinated proteins.17,18 The proteolytic core complex of the 26S proteasome is the 20S core particle, which is composed of several subunits. Low molecular mass polypeptide 2 (LMP2) is an IFN-γ-inducible subunit that can replace the constitutive subunit Y (also known as delta or β1) in the 20S particle.19 The presence of LMP2 in the 20S particle results in increased chymotryptic and tryptic activities in vitro and modulates the cleavage site preferences of the proteasome.20,21 Several studies have shown that LMP2 plays an essential role in the degradation of IκBα and subsequent activation of NF-κB.22–24

We previously reported that FAT10 is upregulated in HIV-infected renal tubular epithelial cells (RTECs) in vitro and in kidney specimens from patients with HIV-associated nephropathy and autosomal polycystic kidney disease and that increased expression of FAT10 induces apoptosis in RTECs.25 Knockout of FAT10 causes minimal phenotypic changes in unstressed mice; however, these mice exhibit increased sensitivity to death after LPS injection.26 FAT10 is constitutively expressed in mature dendritic cells and B cells27,28 and is also inducible by the proinflammatory cytokines IFN-γ and TNF-α in cells of various tissue origins29,30; however, the role of FAT10 in the regulation of immune response has not been studied. Our observations that FAT10 is upregulated by HIV-1 infection of RTECs and that the pattern of FAT10 expression was similar to several other NF-κB-regulated genes,31 coupled with the knowledge that NF-κB activation is controlled at multiple levels by the ubiquitin-proteasome system, led us to test the hypothesis that FAT10 participates in the regulation of NF-κB activation.

RESULTS

Characterization of Immortalized Mouse RTECs
Murine RTECs isolated from kidneys of FAT10+/+ and FAT10−/− mice demonstrated typical morphology of RTECs (Figure 1A) and expressed the proximal tubular cell marker alkaline phosphatase (Figure 1B). Cells were also analyzed by quantitative real-time PCR (qPCR) to characterize their expression of other transcripts, including markers specific for epithelial cells (e.g., E-cadherin, cytokeratin), and cells from specific tubular segments, including proximal tubule (CD13), thick ascending limb (Tamm-Horsfall protein), and collecting duct (aquaporin 2). The results demonstrate that the cells expressed RTEC markers but had a mixed phenotype, expressing some genes that are typical of proximal tubular cells and genes of other tubular segments. The expression profile of all analyzed genes was similar in FAT10+/+ and FAT10−/− RTECs, and FAT10 expression was not detected in FAT10−/− RTECs (Figure 1C).

TNF-α–induced Expression of CXCL2 and MCP-1 Is Diminished in FAT10−/− RTECs and in Kidneys and Liver from FAT10−/− Mice
FAT10−/− and FAT10+/+ RTECs were incubated with TNF-α (10 ng/ml; Invitrogen) for 24 h. After incubation, cells were collected and expression of CXCL2 and MCP-1 was analyzed by qPCR. TNF-α increased expression of CXCL2 and MCP-1 by 29.2-fold and 22.3-fold in FAT10+/+ RTECs compared with control, respectively. In FAT10−/− RTECs, however, the response to TNF-α was greatly diminished, with MCP-1 and CXCL2 expression increasing by
only 2.4- and 5.8-fold compared with control, respectively (Figure 2, A and B).

\( \text{FAT10}^{+/+} \) and \( \text{FAT10}^{-/-} \) mice wereadministered an injection of 250 ng of TNF-\( \alpha \). Kidneys and liver were harvested 6 h later, and levels of MCP-1 and CXCL2 mRNA were analyzed by qPCR. Basal expression of MCP-1 in kidneys from \( \text{FAT10}^{-/-} \) mice was lower than in \( \text{FAT10}^{+/+} \) mice \((P < 0.05; \text{Figure 2, C and D})\). Intraperitoneal injection of TNF-\( \alpha \) induced a 2.2-fold increase in MCP-1 expression in kidneys of \( \text{FAT10}^{+/+} \) mice \((P < 0.05)\) but had no effect on MCP-1 expression in kidneys from \( \text{FAT10}^{-/-} \) mice (Figure 2C). Renal CXCL2 expression increased after TNF-\( \alpha \) injection in \( \text{FAT10}^{+/+} \) and \( \text{FAT10}^{-/-} \) mice (Figure 2D); however, the increase in CXCL2 expression was significantly blunted in \( \text{FAT10}^{-/-} \) mice (2.2-fold increase) compared with \( \text{FAT10}^{+/+} \) mice (4.2-fold increase; \( P < 0.01 \)). Basal expression of MCP-1 and CXCL2 in livers of \( \text{FAT10}^{-/-} \) mice was higher than in \( \text{FAT10}^{+/+} \) mice \((P < 0.05; \text{Figure 2, E and F})\). MCP-1 and CXCL2 expression both increased significantly (6.2- and 6.6-fold increase, respectively; \( P < 0.01 \)) in liver of \( \text{FAT10}^{-/-} \) mice after TNF-\( \alpha \) administration (Figure 2, E and F), but TNF-\( \alpha \) had no effect on hepatic MCP-1 and CXCL2 expression in \( \text{FAT10}^{-/-} \) mice. Splenic MCP-1 expression increased nonsignificantly in TNF-\( \alpha \)-injected \( \text{FAT10}^{+/+} \) and \( \text{FAT10}^{-/-} \) mice (Figure 2E), whereas that of CXCL2 expression increased modestly in spleen of \( \text{FAT10}^{-/-} \) (Figure 2F).

\textbf{Figure 2.} TNF-\( \alpha \)-induced CXCL2 and MCP-1 expression is significantly reduced in \( \text{FAT10}^{-/-} \) RTECs and in kidneys and liver of \( \text{FAT10}^{-/-} \) mice. \( \text{FAT10}^{+/+} \) and \( \text{FAT10}^{-/-} \) RTECs were grown in six-well plates for 24 h. (A and B) After addition of TNF-\( \alpha \) (10 ng/ml) to the culture media, cells were further incubated for 16 h. (C through F) Female \( \text{FAT10}^{+/+} \) and \( \text{FAT10}^{-/-} \) mice (22 to 25 g) were administered an intraperitoneal injection of TNF-\( \alpha \) (250 ng/ per mouse) or saline for 6 h. Total RNA was isolated, and MCP-1 and CXCL2 expression in kidneys (C and D) or liver and spleen (E and F) was analyzed by qPCR. Results are normalized to cyclophilin A, and values for fold difference are in comparison with untreated \( \text{FAT10}^{+/+} \) RTECs or mice that were administered saline. Data are means \( \pm \) SD \((n = 3)\). *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \) versus corresponding untreated RTECs or mice that were administered saline; ###\( P < 0.001 \), ####\( P < 0.001 \) versus \( \text{FAT10}^{-/-} \) RTECs or mice treated with TNF-\( \alpha \).

\textbf{TNF-\( \alpha \) and LPS-Induced NF-\( \kappa B \) Activation in RTECs Is Dependent on FAT10}\n
Because CXCL2 and MCP-1 expression are regulated by NF-\( \kappa B \),\textsuperscript{32} we studied whether TNF-\( \alpha \)- or LPS-induced NF-\( \kappa B \) activation is impaired in RTECs from \( \text{FAT10}^{-/-} \) mice. An NF-\( \kappa B \)-reporter plasmid (pGL2/NF-\( \kappa B \) luciferase) was transfected into \( \text{FAT10}^{+/+} \) and \( \text{FAT10}^{-/-} \) RTECs for 24 h, and cells were assayed for luciferase activity after additional incubation with TNF-\( \alpha \) or LPS (Invitrogen) for 16 h. A plasmid encoding renilla luciferase was co-transfected as an internal control for transfection efficiency. Basal activity of pGL2/NF-\( \kappa B \) luciferase was not significantly different in \( \text{FAT10}^{+/+} \) versus \( \text{FAT10}^{-/-} \) RTECs. Both TNF-\( \alpha \) and LPS treatment increased pGL2/NF-\( \kappa B \) luciferase activity in \( \text{FAT10}^{-/-} \) RTECs (2.8- and 3.0-fold, respectively; \( P < 0.001 \)); however, TNF-\( \alpha \)- and LPS-induced NK-\( \kappa B \) activation was almost completely abolished in \( \text{FAT10}^{-/-} \) RTECs (Figure 3A).

To determine whether defective TNF-\( \alpha \)- and LPS-induced NF-\( \kappa B \) activation in \( \text{FAT10}^{-/-} \) RTECs was caused specifically by lack of FAT10 expression, we co-transfected \( \text{FAT10}^{-/-} \) RTECs with an NF-\( \kappa B \)-reporter plasmid (pGL2/NF-\( \kappa B \) luciferase), a plasmid encoding renilla luciferase (internal control), and either FAT10-expressing plasmid (pHR-FAT10-ires-EGFP) or empty vector control (pHR-ires-EGFP). After 24 h, cells were further incubated with TNF-\( \alpha \) (10 ng/ml) or vehicle for 16 h. As shown in Figure 3B, TNF-\( \alpha \) and LPS treatment increased pGL2/NF-\( \kappa B \)-mediated luciferase expression 1.8- and 2.7-fold, respectively \((P < 0.01)\) in \( \text{FAT10}^{-/-} \) RTECs co-transfected with plasmid pHR-FAT10-ires-EGFP but luciferase was not induced in \( \text{FAT10}^{-/-} \) RTECs co-transfected with the control vector pHR-ires-EGFP. These results demonstrate that expression of FAT10 restored TNF-\( \alpha \)- and LPS-induced...
activation of the NF-κB reporter construct pGL2/NF-κB in RTECs from FAT10+/− mice.

FAT10 Is Necessary for TNF-α–Induced Nuclear Translocation in RTECs

Before TNF-α treatment, p65 was located predominantly in the cytoplasm of FAT10+/+ and FAT10−/− RTECs (Figure 4A). Fifteen and 60 min after treatment with TNF-α (20 ng/ml), most p65 was detected in the nuclei of FAT10+/+ RTECs but remained in the cytoplasm of FAT10−/− RTECs, suggesting that TNF-α–induced nuclear translocation of NF-κB is defective in FAT10−/− RTECs. Similar results were obtained using freshly isolated primary nonimmortalized RTECs from FAT10+/+ and FAT10−/− mice (Figure 4B), demonstrating that the observed defect in p65 translocation was not a result of immortalization with T antigen. We also studied whether TNF-α–induced p65 translocation was impaired in peritoneal macrophages isolated from mice. There was no difference in TNF-α–induced nuclear translocation of p65 in FAT10+/+ macrophages as compared with FAT10−/− macrophages (Supplemental Figure 1).

FAT10−/− RTECs were infected with either HR-FAT10-IREs-EGFP or HR-IREs-EGFP lentivirus. Three days later, cells were incubated with TNF-α (20 ng/ml) or vehicle for 15 and 60 min. As shown in Figure 5, before TNF-α incubation, p65 protein was located in the cytoplasm of FAT10−/− RTECs infected with either virus; however, after TNF-α treatment (15 and 60 min), p65 was detected primarily in the cytoplasm of FAT10−/− RTECs infected with control lentivirus, whereas p65 was detected predominantly in nuclei in FAT10−/− RTECs infected with HR-FAT10-IREs-EGFP. These results demonstrate that transduction of FAT10−/− RTECs with a FAT10-expressing vector is sufficient to restore TNF-α–induced NF-κB nuclear translocation.

Figure 3. FAT10 is necessary for TNF-α– and LPS-induced activation of the NF-κB reporter vector pGL2/NF-κB luciferase in FAT10+/− RTECs. (A) FAT10+/+ and FAT10−/− RTECs were co-transfected with pGL2/NF-κB luciferase and renilla luciferase plasmids. After 24 h, TNF-α (10 ng/ml) or LPS (1 μg/ml) was added to the media and the cells were further incubated for 16 h. (B) FAT10−/− RTECs were co-transfected with pGL2/NF-κB luciferase, renilla, and either pHr/FAT10-IRES-EGFP or pHr-IRES-EGFP. After 24 h, TNF-α (10 ng/ml) or LPS (1 μg/ml) was added and cells were further incubated for 16 h. Luciferase activity was measured using a luminometer. Results are normalized to renilla luciferase and expressed as fold induction relative to TNF-α–induced nuclear translocation of p65 in FAT10+/+ cells but not FAT10−/− cells. (A) Primary RTECs were freshly isolated from FAT10+/+ and FAT10−/− mice. Six days later, cells were incubated with TNF-α (20 ng/ml) for 20 min and subsequently immunostained with p65 antibody. TNF-α–induced nuclear translocation of p65 occurred only in FAT10+/+ cells.
were incubated with TNF-α virus (HR-IRES-EGFP). Three days after transduction, cells infected with lentivirus (HR-FAT10-IRES-EGFP) or empty control lentivirus were grown on coverslips for 24 h and then infected with HR-ires-EGFP or HR-FAT10-IRES-EGFP lentivirus. Three days after infection, cells were treated with TNF-α (20 ng/ml) or vehicle for 10 min. TNF-α-induced IκBα protein levels decreased significantly after TNF-α treatment and reached a nadir at 10 min, and levels pretreatment levels by 20 min after TNF-α treatment, but pretreatment levels decreased by 5 min after TNF-α treatment in FAT10−/− RTECs (Figure 9A). To confirm whether FAT10 affects LMP2 expression, we transduced FAT10−/− RTECs with the FAT10-expressing lentivirus HR-FAT10-IRES-EGFP or control vector. FAT10 transduction significantly increased LMP2 expression (3.0 ± 0.5-fold; p < 0.01) in FAT10−/− RTECs, whereas the level of subunit Y did not change (Figure 9B). LMP2 mRNA was also reduced in FAT10−/− RTECs compared with FAT10+/+ RTECs, and transduction with HR-FAT10-IRES-EGFP significantly increased LMP2 mRNA levels (Supplemental Figure 2).

Expression of LMP2 in FAT10−/− RTECs Restores TNF-α–induced IκBα Degradation

After TNF-α stimulation, IκBα protein levels rapidly decreased (66 ± 5% at 5 min and 58 ± 6% at 20 min compared with baseline levels; p < 0.05) in FAT10−/− RTECs transfected with pCMV-SPORT6/LMP2 but not in FAT10−/− RTECs transfected with the control vector pCMV-SPORT6 (89 ± 10% at 5 min and 105 ± 15% at 20 min compared with baseline

FAT10 Does not Affect the Ubiquitination of Phosphorylated IκBα and Does Not Interact with p-IκBα

After treatment with TNF-α and MG132 (to block proteasomal degradation of polyubiquitinated proteins), p-IκBα was immunoprecipitated and ubiquitinated p-IκBα was detected by Western blotting using anti-ubiquitin. The ubiquitinated p-IκBα levels were similar in FAT10+/+ and FAT10−/− RTECs (Figure 8A), suggesting that the inability of FAT10−/− RTECs to activate NF-κB is not a result of defective ubiquitination of p-IκBα.

Because FAT10 conjugation to substrate proteins can target them for degradation by the 26S proteasome, we investigated whether FAT10 forms conjugates with IκBα, thereby facilitating its proteasomal degradation; however, when we immunoprecipitated protein from FAT10+/+ cells using anti–p-IκBα after treatment with TNF-α, we were unable to detect FAT10 in the immunoprecipitate (Figure 8A). Furthermore, when a FLAG-FAT10 fusion protein was expressed in the FAT10−/− RTECs and subsequently immunoprecipitated using an anti-FLAG antibody, we were unable to detect p-IκBα or FAT10–p-IκBα conjugates in the immunoprecipitates (Figure 8B).

FAT10 Affects Expression of Proteasome Subunit LMP2

LMP2 protein levels in FAT10−/− RTECs was 24.5 ± 5.2% of that observed in FAT10+/+ RTECs (p < 0.001), whereas the constitutive proteasome subunit Y was unchanged (Figure 9A). To confirm whether FAT10 affects LMP2 expression, we transduced FAT10−/− RTECs with the FAT10-expressing lentivirus HR-FAT10-IRES-EGFP or control vector. FAT10 transduction significantly increased LMP2 expression (3.0 ± 0.5-fold; p < 0.01) in FAT10−/− RTECs, whereas the level of subunit Y did not change (Figure 9B). LMP2 mRNA was also reduced in FAT10−/− RTECs compared with FAT10+/+ RTECs, and transduction with HR-FAT10-IRES-EGFP significantly increased LMP2 mRNA levels (Supplemental Figure 2).

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FAT10 Is Necessary for TNF-α–induced IκBα Degradation in RTECs

Analysis of total IκBα in FAT10+/+ RTECs by Western blotting demonstrated that protein levels decreased by 5 min after TNF-α treatment and reached a nadir at 10 min, and levels remained decreased from baseline through 60 min (Figure 6, A and B). In contrast, levels of total IκBα protein in FAT10−/− RTECs did not decrease after TNF-α exposure, suggesting that TNF-α–induced IκBα degradation is deficient in FAT10−/− RTECs (Figure 6, A and B). There was no significant difference in IκBα mRNA expression between FAT10+/+ and FAT10−/− RTECs before or after TNF-α exposure (Figure 6C). FAT10−/− RTECs were transduced with a FAT10-expressing lentivirus (HR-FAT10-IRES-EGFP) or empty control lentivirus (HR-ires-EGFP). Three days after transduction, cells were incubated with TNF-α (20 ng/ml) or vehicle for 10 min. IκBα protein levels decreased significantly after TNF-α treatment in FAT10−/− RTECs infected with HR-FAT10-IRES-EGFP but not in cells infected with the control virus (Figure 6D); therefore, expression of FAT10 in FAT10−/− RTECs was sufficient to restore TNF-α–induced IκBα degradation.

TNF-α–induced IκBα Phosphorylation Is Not Affected in FAT10−/− RTECs

In both FAT10+/+ and FAT10−/− RTECs, levels of p-IκBα increased rapidly after TNF-α treatment, reaching maximal levels at 5 min. TNF-α–induced IκBα phosphorylation in
levels; \( P > 0.05 \); Figure 9C). LMP2 transfection also decreased levels of p-IkB\( \alpha \) in \( FAT10^{+/+} \)-RTECs after TNF-\( \alpha \) treatment in RTECs (0.2 ± 0.1 of baseline levels after 20 min; \( P < 0.01 \)) but not in control-transfected RTECs (11.35 ± 2.12-fold higher compared with baseline levels after 20 min; \( P < 0.001 \); Figure 9C). Transfection of pCMV-SPORT6/LMP2 increased LMP2 levels in \( FAT10^{+/+} \)-RTECs by 14.2 ± 0.4-fold (\( P < 0.01 \)). Baseline (before TNF-\( \alpha \) stimulation) p-IkB\( \alpha \) levels were also increased 9.58 ± 2.12-fold (\( P < 0.01 \)) compared with control-transfected cells.

**FAT10 Is Upregulated in RTECs in Common Renal Diseases**

\( FAT10 \) mRNA was upregulated in DN, IgAN, and HN compared with normal kidney biopsy specimens taken from living kidney transplant donors by 2.50-, 2.02-, and 1.92-fold, respectively (Figure 10A). The differences in \( FAT10 \) expression for DN and IgAN compared with controls were statistically significant (\( q < 0.05 \)), whereas the increase in HN specimens did not reach statistical significance (\( q = 0.20 \)). Because GFR and proteinuria are important predictors of renal outcome in most kidney diseases, we analyzed whether tubulointerstitial \( FAT10 \) expression correlated with estimated GFR (eGFR) or proteinuria at the time of biopsy in patients with DN and IgAN. \( FAT10 \) expression was significantly correlated with proteinuria (\( R = 0.45, P = 0.02 \)) and eGFR (\( R = -0.40, P = 0.02 \)) in DN and IgAN.

To determine whether \( FAT10 \) protein is upregulated in DN, HN, and IgA, we performed immunohistochemistry for \( FAT10 \) in renal biopsy specimens from patients with these diseases (Figure 10). We detected \( FAT10 \) in RTECs and in some glomeruli in DN specimens. \( FAT10 \) was also detected in RTECs in HN biopsy specimens. In IgAN, \( FAT10 \) expression was more diffuse with protein detected in most RTECs and some glomerular epithelial cells. \( FAT10 \) protein was not detected in normal kidney specimens.

**DISCUSSION**

NF-\( \kappa B \) activation increases transcription of a large number of genes with myriad effects on inflammation, cellular survival, and cell-cycle regulation.\(^1\)–\(^3\) Increased production of proinflammatory mediators by RTECs has been demonstrated in many forms of acute and chronic kidney disease and contributes to the pathogenesis of these diseases.\(^4\)–\(^5\),\(^34\)–\(^36\) In previous studies investigating the mechanisms by which HIV-1 induces renal disease, we infected human RTECs with HIV-1 to determine the genes that are differentially expressed in RTECs after HIV-1 infection.\(^31\) The predominant response of these cells was increased production of cytokines, chemokines, and adhesion molecules. Hierarchical cluster analysis of the differentially expressed genes demonstrated that \( FAT10 \) was regulated similarly to several chemokines, including MCP-1 and CXCL2, which are regulated by NF-\( \kappa B \).\(^32\),\(^37\) Lukasiak et al.\(^38\) recently demonstrated that \( FAT10 \) is upregulated in hepatocellular carcinoma specimens in which other inflammatory genes are expressed, including LMP2. The investigators concluded from those studies that \( FAT10 \) is a marker of immune activation; however, no studies of the role of \( FAT10 \) in mediating immune activation have been published.

Canaan et al.\(^26\) analyzed the murine and human \( FAT10 \) promoter 5’ untranslated region and intron using software to predict
transcription factor binding sites. They found several predicted binding sites for NF-κB in the promoter and 5′ untranslated region. Moreover, the investigators found that \( \text{FAT10}^{-/-} \) mice demonstrate increased susceptibility to lethal LPS administration. In many rodent models, increased LPS susceptibility is mediated via increased production of cytokines (so-called “cytokine storm”), leading to cardiovascular collapse and resulting in death.

We therefore initially hypothesized that FAT10 is a negative regulator of NF-κB activation and that knockout mice would have higher levels of renal chemokine and cytokine production after TNF-α stimulation. To our surprise, RTECs from \( \text{FAT10}^{-/-} \) mice demonstrated diminished inducible NF-κB reporter activity and impairment of TNF-α–induced IkBα degradation and p65 nuclear translocation. Moreover, TNF-α–induced expression of \( \text{CXCL2} \) and \( \text{MCP-1} \) were significantly diminished in kidneys from \( \text{FAT10}^{-/-} \) mice.

These studies are the first to establish a role for FAT10 in the activation of NF-κB and subsequent expression of proinflammatory genes. We believe that this role for FAT10 is specific to particular cell types. That \( \text{FAT10}^{-/-} \) mice develop normally and un-ressed mice exhibit minimal phenotypic differences strongly suggests that NF-κB–mediated signaling is intact in many or most cells. Moreover, unpublished data from our laboratory demonstrate that whereas HIV-1 strongly upregulates FAT10 in RTECs, FAT10 is not expressed at appreciable levels in CD4 cells before or after infection with HIV-1 and that not all cell types increase expression of FAT10 in response to TNF-α. Further studies are needed to determine the role of FAT10 in NF-κB regulation in nonrenal cells.

In addition to TNF-α, a variety of extracellular and intracellular signals can activate NF-κB. Although several distinct and overlapping signaling pathways can lead to NF-κB activation, most converge at the step of activation of the IkB kinase signalosome, which phosphorylates IkBα. Our data demonstrate that phosphorylation and subsequent polyubiquitination of IkBα is normal in RTECs from \( \text{FAT10}^{-/-} \) mice. Furthermore, we have demonstrated that the decreased ability of \( \text{FAT10}^{-/-} \) RTECs to degrade ubiquitinated IkBα is associated with decreased expression of the inducible LMP2 proteasome subunit.

Upon IFN-γ stimulation, LMP2 is induced and replaces the constitutive proteasome 20S subunit Y. Previous studies showed that LMP2 plays a key role in the degradation of phosphorylated IkBα and subsequent activation of NF-κB.32–34 We found that transduction of \( \text{FAT10}^{-/-} \) RTECs with \( \text{FAT10} \) induced LMP2 expression and that transfection of LMP2 into RTECs partially restored TNF-α–induced IkBα degradation. These data strongly suggest that altered proteasomal subunit expression in \( \text{FAT10}^{-/-} \) cells causes impaired TNF-α–induced IkBα degradation. It is possible that transfection of LMP2 into RTECs only partially restored TNF-α–induced IkBα degradation because the other inducible 26S proteasomal subunits (LMP7 and/or LMP10) may also be required for full NF-κB activation in RTECs or because of insufficient transfection efficiency. Because FAT10 conjugation to substrate proteins can target them for degradation by the 26S proteasome,35 it is also possible that FAT10 could directly form conjugates with IkBα or noncovalently associate with IkBα and facilitate its proteasomal degradation; however, our immunoprecipitation studies failed to detect an interaction between FAT10 and phosphorylated IkBα.

In this study, we found that levels of LMP2 protein in \( \text{FAT10}^{-/-} \) RTECs are much lower than in \( \text{FAT10}^{+/+} \) RTECs. Transduction of \( \text{FAT10}^{-/-} \) RTECs with \( \text{FAT10} \)-encoding lentivirus increased LMP2 protein expression and restored degradation of IkBα, suggesting that the LMP2 deficiency in \( \text{FAT10}^{-/-} \) RTECs accounts at least in part for the inability of NF-κB activation by TNF-α in RTECs; however, the mechanism whereby FAT10 influences LMP2 expression requires further study.

These studies demonstrate that FAT10 is significantly upregulated in DN and IgAN and that expression of FAT10 correlates positively with proteinuria and negatively with eGFR. Although the level of FAT10 mRNA upregulation was modest in biopsy samples, they are similar to the level of FAT10 upregulation in HIV-infected RTECs in our previous studies.31

In conclusion, these studies elucidate a novel role for FAT10 in TNF-α–induced NF-κB activation and demonstrate that FAT10 is necessary for normal expression of LMP2 and degradation of IkBα in RTECs. Moreover, we have demonstrated that FAT10 expression is increased in the tubulointerstitium and expression levels are correlated with proteinuria and eGFR in the most common forms of chronic progressive renal dis-
Ease. Because NF-κB–induced production of inflammatory mediators contributes to the pathogenesis of these diseases, our studies establish FAT10 as a potential therapeutic target in the prevention and/or treatment of renal disease.

**CONCISE METHODS**

**Animals**
C57BL6 FAT10−/− mice have been previously characterized. Wild-type C57BL6 mice were purchased from Charles River Laboratories. Mice were housed in a facility approved by the American Association for Accreditation of Laboratory Animal Care. All studies were approved by the Mount Sinai Institutional Animal Care and Use Committee.

**Generation of Murine RTEC Lines**
RTECs were grown from FAT10−/− or FAT10−/− mouse kidneys by plating minced kidney onto collagen-coated culture plates in REGM SingleQuots media (Lonza) selective for growth of RTECs according to previously published methods. A subpopulation of these cells was conditionally immortalized by infection with VSV-HR−tsTag-IRES-Hygro. For all subsequent studies, RTECs were expanded at 33°C until they reached 80% confluence and subsequently cultured at 37°C for 14 d to induce T antigen degradation and cellular differentiation.

**Transfection and Lentivirus Transduction**

pGL2/NF-κB luciferase was constructed by subcloning the NF-κB promoter from pNF-κB/SEAP (Clontech) into pGL2 luciferase (Promega). pH-R-FAT10-IRES-EGFP and pflag/FAT10 were constructed by subcloning the FAT10 coding sequence from pcDNA4-FAT10 into pH-2-IRES-EGFP and pCMVlag6C (Invitrogen), respectively. pHMV-SPORT6/LMP2 was purchased from Open Biosystems. Plasmids were transfected using Lipofectamine 2000 reagent, and the transfection efficiency was 21.16 ± 4.18%. pH-FAT10-IRES-EGFP or pH-2-IRES-EGFP (EGFP control) was used to generate VSV-G– pseudotyped lentivirus for transduction as described previously.

**Alkaline Phosphatase and Luciferase Assays**
Alkaline phosphatase activity was detected by incubating cells with Immuno-BCIP/NBT liquid substrate plus (MP Biomedicals) at 25°C for 1 h. Luciferase activity was determined by Dual-Luciferase reporter assay kit (Promega) and MiniLumat LB9506 (Berthold) using a luminometer.

**Immunofluorescence and Immunohistochemistry**
RTECs grown on glass coverslips were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, incubated with anti-human p65 (1:200; Santa Cruz Biotechnology) and Cy3-goat anti-rabbit IgG antibody (1:200; Invitrogen), and imaged by fluorescence microscopy. Paraffin -embedded specimens from patients with DN, HN, and IgAN (n = 5 for each) and normal human kidney were stained with affinity-purified anti-FAT10.

**Immunoprecipitation**
Cells were washed in cold PBS and resuspended in lysis buffer (50 mM HEPES [pH 7.5], 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, and protein inhibitor cocktail [Pierce]) and sonicated. Cell lysates were centrifuged, and supernatants were used for immunoprecipitation using anti-p-IκB (1:50; Cell Signaling) and protein G–agarose (Roche) or anti-FLAG beads (Sigma) according to the manufacturers’ protocol.

**Western Blotting**
Whole-cell lysates were prepared as described previously. Whole-cell lysates (100 μg) were loaded on a 4 to 20% denaturing polyacrylamide gel (Invitrogen) and electroblotted onto polyvinylidine difluoride membranes. Western blotting was performed using the following primary antibodies: Polyclonal anti-human IκBα (1:4000); monoclonal anti-human p-IκBα (1:4000) antibody (Cell Signaling); polyclonal anti-human FAT10 (1:4000; Biomol); anti-human LMP2 (1:4000) and anti-human proteasome Y (1:4000; ABR-Affinity BioReagents); and anti–human β-actin (1:5000; Santa Cruz Biotechnology). Secondary antibodies were horseradish peroxidase–conjugated goat anti-mouse IgG.
Figure 9. FAT10 increases expression of LMP2 protein in RTECs. (A) LMP2 protein level is decreased in FAT10<sup>−/−</sup> RTECs. FAT10<sup>+/+</sup> and FAT10<sup>−/−</sup> RTECs were cultured in six-well plates (2 × 10<sup>6</sup> cells/well) overnight. LMP2, Y, and β-actin were analyzed by Western blotting, and LMP2 and Y were quantified by densitometry and normalized to β-actin. Data are means ± SD (n = 3). **P < 0.01 versus FAT10<sup>+/+</sup> RTECs. (B) Expressing FAT10 in FAT10<sup>−/−</sup> RTECs increases the protein level of LMP2. FAT10<sup>+/−</sup> RTECs were cultured in six-well plates (2 × 10<sup>6</sup> cells/well) overnight and then infected with HR-FAT10-RES-EGFP or its control virus HR-RES-EGFP. Three days after infection, cells were collected; the protein level of LMP2, Y, and β-actin was determined by Western blotting; and the amount of LMP2 and Y was quantified by densitometry and normalized to β-actin. Data are means ± SD (n = 3). **P < 0.01 versus FAT10<sup>−/−</sup> RTECs infected with control virus HR-RES-EGFP. (C) LMP2 transfection restores TNF-α–induced IκBα degradation in FAT10<sup>−/−</sup> RTECs. FAT10<sup>−/−</sup> RTECs were cultured in six-well plates (2 × 10<sup>6</sup> cells/well) overnight before transient transfection with pCMV-SPORT6/LMP2 or control vector pCMV-SPORT6. Twenty hours after transfection, cells were incubated with or without TNF-α (20 ng/ml) for 5 and 20 min. The protein level of LMP2, IκBα, p-IκBα, and β-actin was determined by Western blotting, and LMP2, IκBα, and p-IκBα protein was quantified by densitometry and normalized to β-actin. Data are means ± SD (n = 3). *P < 0.05, **P < 0.01 versus corresponding RTECs at baseline (0 min); #P < 0.01 versus FAT10<sup>−/−</sup> RTECs transfected with the plasmid control vector pCMV-SPORT6 at baseline.

mRNA Expression Profiling of Human Renal Biopsies

The study was performed as outlined previously. Briefly, human renal biopsy specimens were procured in an international study, the European Renal eDNA Bank-Kroener-Fresenius biopsy bank (participating centers in appendix). Biopsies were obtained from patients after informed consent and with approval of local ethics committees. Patient characteristics are shown in Supplemental Table 1. Living-donor kidney samples were obtained during the cold ischemia period.

Total RNA was extracted from microdissected tubulointerstitium and glomeruli obtained from living donors (n = 7) and patients with IgAN (n = 25), DN (n = 7), and HN (n = 20). After one round of amplification of 300 to 800 ng of RNA, RNA quality and quantity were analyzed (Agilent Technologies). Fragmentation, hybridization, staining, and imaging were performed according to the manufacturer’s guidelines (Affymetrix). A detailed description of the protocol and statistical analysis has been previously reported.

Statistical Analysis

For qPCR and protein densitometry data, t test for unpaired data was used to evaluate the differences between groups, and a two-sided P < 0.05 was used to define significant differences between groups.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health/National Institute of Diabetes and Digestive and Kidney Diseases grants R01 DK078510 (M.J.R.) and P30 DK081943-01 (M.K.).

The members of the ERCB-KFB at the time of the study included C.D. Cohen, H. Schmid, M. Fischeder, L. Weber, M. Kretzler, D.
Figure 10. FAT10 is upregulated in common human renal diseases. (A) FAT10 mRNA expression was significantly higher in tubulointerstitial specimens from patients with IgAN (2.02-fold; \( q = 0.03 \)), and DN (2.50-fold; \( q < 0.01 \)) than from normal kidney specimens. The increase in FAT10 expression in HN (1.92-fold; \( q = 0.2 \)) did not reach statistical significance. **\( q < 0.05 \). (B) Pooled expression data for DN and IgAN revealed significant correlation between \( \text{eGFR} \) and \( \text{FAT10 expression} \). (C) FAT10 protein localization in kidney biopsies from patients with DN, IgAN, and HN. FAT10 is expressed in tubular epithelial cells and in glomeruli in DN. FAT10 is also expressed in tubular epithelial cells in HN. In IgAN, FAT10 expression is more diffuse with protein detected in most tubular epithelial cells and in some glomerular cells. Nonimmune rabbit IgG controls performed on serial sections (bottom) were negative. Magnification: \( \times 400 \) for hypertensive nephrosclerosis, \( \times 200 \) for all others.

REFERENCES


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