Notch3 deletion regulates HIV-1 gene expression and systemic inflammation to ameliorate chronic kidney disease

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Running headline: Notch3 deletion rescues systemic inflammation

Translational statement: Notch3 inhibition has been reported to be an anti-inflammatory strategy for glomerular diseases. Here, using an HIV-1 transgenic mouse model we show that global Notch3 deletion not only improves kidney phenotype but also renal expression of HIV gene, Nef and systemic inflammation which leads to a drastic increase of life span of these mice. Notch3 inhibition can thus play a dual protective role in HIV related chronic diseases.

Abstract

Antiretroviral therapy (ART) has profoundly decreased HIV-1 associated morbidity. However, despite ART, immune cells remain latently infected and slowly release viral proteins, leading to chronic inflammation and HIV associated comorbidities. Thus, new strategies are needed to reduce the inflammatory effects of HIV-1. In previous studies we found that gamma secretase inhibitor (GSIXX) ameliorated renal lesions of HIV-Tg26 mice carrying replication defective HIV-1 PNL4-3 by inhibiting Notch activation. Since gamma secretase inhibition is not a safe strategy in humans, here we examined the specific role of the Notch3 pathway in the pathogenesis of the renal lesions and outcome of HIV-Tg26 mice. We found that Notch3 is activated in podocytes and other renal cells in HIV-Tg26 mice and human biopsies with HIV-1 associated Nephropathy (HIVAN). Knockdown of Notch3 in HIV-Tg26 mice revealed a marked reduction in the mortality rate, improvement in renal injury and function. RNA sequencing and immunolabeling data revealed that Notch3 deletion drastically reduced infiltrating renal macrophages in HIV-Tg-N3KO mice in association with renal reduction of HIV-*nef* mRNA expression levels. In fact, bone marrow derived macrophages from HIV-Tg26 mice showed a significant activation of Notch3 signaling. Further, systemic levels of TNF-alpha and MCP-1 and other inflammatory chemokines and cytokines were reduced in Tg-N3KO mice as compared to HIV-Tg26 mice and this translated to a marked reduction of HIV-induced skin lesions. Taken together, these studies strongly point to a dual inhibitory/therapeutic effect of Notch3 inhibition on HIV-induced systemic, skin and renal lesions independently of ART.

Introduction

Anti-retroviral therapy (ART) has decreased the incidence of HIV-1 related pathologies and dramatically prolonged the lives of people living with HIV-1 (PLWH). However, persistent low viremia exists in PLWH despite ART, which results in continuous immune activation and inflammation[1-4]. These events ultimately result in chronic diseases including chronic kidney disease (CKD), cardiovascular disease, neurological impairment and others[4,5]. One of the main issues leading to these problems is that guidelines for prevention of organ damage remain the same and do not target unique molecular pathways related to disease progression[6]. We have previously reported that renal Notch signaling pathway is activated in patients with HIV-1 associated nephropathy (HIVAN) and in non-replicating HIV-1 transgenic rodent models[7,8].

Notch signaling is important for cell fate decisions in development, homeostasis, and disease. Notch signaling is activated when a Notch receptor (Notch1,2,3 or 4) binds to a Notch ligand (Jagged or Delta) and initiates a series of proteolytic cleavages. The final cleavage is mediated by gamma secretase, which results in release of the Notch intracellular (NIC) domain and its translocation into the nucleus. In the nucleus, NIC binds to RBP-JK (also called CSL (CBF-1, SuH, Lag-1)) transcription factor and converts it into a transcriptional activator of Hes and Hey family genes[9-12]. Notch signaling is essential for nephrogenesis, but its suppression is necessary for terminal differentiation[13-19]. Notch overexpression has been reported in glomerular diseases including HIVAN[19-23]. However, most studies have focused on targeting the gamma secretase. Gamma secretase is an important enzyme complex regulating Notch and other signaling pathways, including Wnt and mTOR [24,25], and thus has yet not been successful in clinical trials. Therefore, an approach to identify and target a specific disease-related Notch member holds promise. The HIV transgenic mouse (HIV-Tg26) and rat models are considered clinically relevant pre-clinical models to study co-morbidities in PLWH on ART as these models, express HIV proteins under LTR promoter (except *gag* and *pol*) but lack viral

replication. These small animals have disease that mimics ART-controlled HIV-infected patients from the continuous stress of viral proteins [26-31]. We previously reported that Notch1, Notch3 and Notch4 pathways are activated in the renal epithelial and interstitial cells of HIV-Tg26 mice[8]. Targeted deletion of Notch1 or Notch2 is embryonic lethal, whereas mice with global deletion of Notch3 or Notch4 develop normally[32-35]. Thus, targeting Notch3 or 4 are relatively safer. In our studies global targeting of the Notch4 pathway in the HIV-Tg26 mouse model led to decreased kidney injury and increased in survival[36]. However, whether Notch3 targeting is also safe and how it compares with Notch4 targeting, remains to studied. In the present study, we took a detailed approach to study if Notch3 axis modulation alone holds a promise in HIV-1.

We evaluated the effects of Notch3 knockout (N3KO) in the HIV-Tg26 mice. Our data show that Notch3 deletion extends the life span of HIV-Tg26 mice and improves renal pathology and function, better than Notch4 deletion. There was marked reduction in the renal infiltrating cells. N3KO not only improved the macrophage-associated inflammation but also decreased HIV-1 gene expression. Complementary to this, bone marrow derived macrophages (BMDMs) from HIV-Tg26 mice have activated Notch3 and increased Notch ligands but not Notch4 activation. Finally, N3KO led to systemic reduction of inflammatory markers, which may be an underlying mechanism of improved disease and prolonged life expectancy. Thus, Notch3 inhibition may serve a dual-protective role in HIV related inflammation.

Material and Methods:

Animal care

The HIV-Tg26 mice (on FVB/N background) were a kind gift from Dr. Paul Klotman (Baylor College of Medicine, Houston, TX)[28]. Heterozygous Tg26 mice were used in the study. Notch3^{d1} [N3KO] mice were obtained from the Jackson Laboratory (JAX:023807). All experiments were

performed under guidelines according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and as approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center (Kansas City, KS, USA). Mice were housed in micro-isolator cages on a high-efficiency particulate air-filtered, ventilated rack. Conditions were aseptic and pathogen free. Animal breeding strategies, renal function evaluation and histological protocols can be found in supplementary methods.

Human tissue

A renal biopsy was taken for the purpose of care for a patient infected with HIV-1 and the diagnosis rendered was HIV-1 associated Nephropathy. The use of this tissue for the study was evaluated and approved for use by the Human Protection Program at the University of Kansas Medical Center.

Antibodies and reagents

The following antibodies were used: anti-β-actin (1:1000; Sigma-Aldrich, St. Louis, MO, USA; A5441); anti-p-NF-κB and anti-Notch3 (1:1000; Cell Signaling Technology, Danvers, MA, USA; 3033s and 2889); anti-MMP-10 (1:1000; My Bio Source, San Diego, CA, USA; MBS2027749); anti-Notch3, DLL4, Jagged1 (1:1000; Abcam, Cambridge, UK; ab23426, ab7280, ab7771 and ab125212). Anti-CD68 for detection in mice (1:100, Ab125212); anti-CD68 for detection in human (1:100; BioCare Medical, CM033A); (Mouse immune Cell phenotyping IHC antibody kit from Cell Signaling Technologies (37495) containing anti-FoxP3, CD11c, CD8 and Granzyme B antibodies; secondary antibodies goat anti-mouse IgG H&L (Alexa Fluor® 594) (ab150116) and goat anti-rabbit IgG H&L (Alexa Fluor® 488) (ab150077), both from Abcam.

RNA-sequencing

RNA-sequencing was carried out in kidneys from 3 months old WT (n=3), HIV-Tg26 (n=7), N3KO (n=4) and HIV-Tg-N3KO (n=6). Total RNA was extraction and RNA-seq were carried out (supplementary methods).

Cell culture, Quantitative PCR, Western Blots and Immunolabeling

Cell culture, Quantitative PCR, Western Blots, Immunohistochemistry and immunofluorescence were performed as previously described[36] (and explained in supplementary methods). For counting CD68, FoxP3, CD8 labelled cells, images were captured from all the areas where these cells were present (n=5-6 per kidney), 6 kidneys per group). Cells positive for Hematoxylin (total cells in the section) and cells with DAB staining (only positive for CD68, CD8 or FoxP3) were counted and percentage of positively labeled cells was calculated per section and averaged. For quantification of immunofluorescence intensity, image J was used, and percent intensity was measured from each image and averaged. For quantification of Notch3 intensity, vascular Notch3 values were subtracted from total intensity to assess glomerular and tubular labelling individually.

Enzyme linked immunoassay (ELISA) and chemokine/cytokine profiling

Serum was assessed for presence of TNFα and MCP-1 using mouse ELISA kits (R&D SYSTEMS, USA) according to manufacturer's instructions. For analysis of chemokines and cytokines in serum of Tg26 and Tg-N3KO mice, serum samples from 3 male mice (200ul each) were pooled from each group. From each group 150 μl serum was used for chemokine array using the chemokine Proteome Profiler Array kit (Cat No# ARY020, R&D SYSTEMS) and 200ul was used from each group for cytokine array using the cytokine proteome profiler array kit (Cat No# ARY028, R&D SYSTEMS). Manufacturer's instructions were followed.

Statistics

Mean± standard error (SE). is used to express the data. Unpaired Student's *t*-test was used to measure statistical significance, a comparison between control and test groups was done. To compare more than two groups, a one-way ANOVA was performed, followed by Tukey's multiple comparison test using graph pad. $P<0.05$ was considered statistically significant.

Results

HIV-1 induces Notch 3 activation in HIV-1 kidneys

Tg26 mice express seven of the nine HIV-1 genes[28]. The renal expression of Notch3 was evaluated in HIV-Tg26 mice and compared to that of age matched wildtype (WT) mice. Notch3 is normally expressed in the vascular smooth muscle cells and was seen expressed in both WT and HIV-Tg26 renal sections (asterisks, Fig:1a and b). Compared to WT sections, Notch3 was brightly labeled in the cells lining the Bowmans capsule (parietal epithelial cells) and glomerular cells (arrow, Fig:1b) of HIV-Tg26 kidney sections. In tubular regions, Notch3 was elevated in tubular epithelial and interstitial cells (arrow, Fig:1c and d). Quantitative analysis revealed a significant increase of Notch3 in both glomerular and tubular regions of HIV-Tg26 mice as opposed to WT mice (Fig:1e and 1f). Further, in patient diagnosed with HIVAN, we found that Notch3 is expressed and elevated in glomerular, tubular and interstitial cells (arrows, Fig: 1g and 1h). To determine whether Notch3 expression is induced by HIV, we infected a human podocyte cell line with either pseudotyped HIV-1 (PNL-4) lentiviral vector or its corresponding control vector. Western blots of lysates from these cells demonstrated a significant upregulation of Notch3 intracellular domain (N3IC) (Fig: 1i and 1j), indicating that HIV-1 induces Notch3 activation.

Notch3 inhibition improves renal function and increases life span of the Tg26 mice

Notch3 expression was not restricted to one cell type, thus we globally deleted Notch3 in Tg26 mice to determine the downstream function of Notch3 activation. We bred the HIV-Tg26 mice with genetically deleted Notch3 knockout (N3KO) mice to generate HIV-Tg26 mice with Notch3 deletion (HIVTg-N3KO). HIV-Tg-N3KO mice were born in the normal Mendelian fashion and were active and fertile. We found that by 6 months 80% of HIV-Tg26 mice (n=28) died whereas 75% of HIV-Tg-N3KO mice (n=26) survived (Fig: 2a). HIV-Tg26 mice present with skin papillomata and ulcers by the age of 3 months; these abnormalities were strikingly low in HIV-Tg-N3KO mice (Fig: 2b). Further HIV-Tg-N3KO mice had a significant improvement in proteinuria (Fig: 2c-d) and renal function, as measured by blood urea nitrogen (BUN) (Fig. 2g) compared to HIV-Tg26 mice. The difference in proteinuria was found to be largely the result of effects in female HIV-Tg26 mice, as the difference in males did not quite reach statistical significance (Fig: 2f).

Notch 3 deletion ameliorates renal injury in HIV-Tg26 mice.

To detect histological changes in kidneys conferred by N3KO in HIV-Tg26 mice, tubulointerstitial injury, glomerular injury, and infiltrating immune cells were quantified. The N3KO mice did not show any phenotypic or histological abnormalities and were comparable to WT mice. In contrast, HIV-Tg26 mice showed significant tubular injury, which was reduced in HIVTg26-N3KO mice, although these changes did not reach statistical significance probably due to the disease variability (Fig. 3a and 3d). However, when male and female mice were separated, a significant reduction of tubular injury was observed in males compared to female mice (supplementary Fig.S1a and S1b). Glomerular injury in HIV-Tg-N3KO mice was significantly reduced regardless of sex (Fig. 3b and e). This effect was most prominent in male N3KO-Tg mice, where glomerular injury was minimal (Supplementary Fig.S1c and S1d). The immune cell infiltrate was also drastically reduced in HIV-Tg-N3KO mice compared to the HIV-Tg26 mice (Supplementary Fig. S1e and S1f). Importantly, all the male HIV-Tg-N3KO-Tg mice

(n=6) showed negligible inflammatory infiltration compared to male HIV-Tg26 mice (Supplementary Fig:S1f). In addition, fibrotic lesions as assessed by Mason trichrome staining were highly positive in HIV-Tg26 kidneys compared to HIV-Tg-N3KO kidneys. As expected, kidneys from both WT and N3KO mice showed no fibrotic lesions (Supplementary figure S2).

Notch3 deletion inhibits inflammatory markers and HIV-1 genes

To determine downstream targets affected by Notch3, bulk RNA sequencing (RNA-seq) was conducted in kidneys from WT, N3KO, HIV-Tg26, and HIV-Tg-N3KO mice. Consistent with our findings, transcriptome analysis revealed inflammatory marker genes amongst the topmost upregulated genes in HIV-Tg26 mice. Gene expression viewed in a heatmap shows many of these inflammatory genes that were increased in HIV-Tg26 mouse kidneys (red) trend towards normalization in HIV-Tg-N3KO mice, similar to WT and N3KO mice (Fig:4a). Volcano plots indicated that differentially expressed genes in HIV-Tg26 verses WT kidneys such as *chil3*, *retnlg*, *ccl2* and *mmp10* skewed towards highly upregulated (Log_2 (fold enrichment)) in HIV-Tg26 kidneys (Fig: 4b). These genes were undifferentiated between Tg-N3KO and WT mouse kidneys (Fig: 4c). A list of genes comparing HIV-Tg26 versus WT has been shown by us before[37]. Genes that were most significantly downregulated in HIV-Tg-N3KO versus HIV-Tg26 kidneys are shown in figure 4d. Raw data has been submitted to NIH: sequence read archive (SRA) website and can be availed by using accession numbers, PRJNA578136 (WT and HIV-Tg26), PRJNA680191 (N3KO), PRJNA1010236 (HIV-Tg-N3KO) and PRJNA580295 (N4KO and HIV-Tg-N4KO).

Previously, we reported that N4KO in HIV-Tg26 mice also ameliorates disease progression by reducing inflammation[36]. Thus, to identify differences between Notch3 and Notch4 KO in HIV-Tg26 mice, we compared the transcriptomic data from HIV-Tg-N3KO and HIV-Tg-N4KO mice (Fig.4 d). Compared to HIV-Tg-N4KO mice, HIV-Tg-N3KO mice were more

protected against disease as indicated by the robust decrease in the expression of certain inflammatory genes such as *mmp10* (matrix metallopeptidase 10), *il7r* (interleukin 7 receptor) and *ccl2 (*chemokine (C-C motif) ligand 2)[38-42].We confirmed the upregulation of MMP10 via immunohistochemistry, where it was not only found expressed in infiltrating cells, but also in tubular and glomerular cells (Supplementary Fig: S3 a-d). Nuclear factor kappa (phospho-p65), another major contributor of HIV pathology, showed a similar expression pattern as that seen for MMP-10 (Supplementary Fig: S3 e-h). Further we validated upregulation of *ccl2* and other inflammatory genes known to play a major role in HIV pathogenesis (*tnfa*, *s100a9*, *Itgam*) by quantitative PCR (Fig:4 e, f, g and h). Interestingly, the HIV genes *nef* and *env* were significantly downregulated in both HIV-Tg-N3KO and HIV-Tg-N4KO kidneys (Fig:4d). Consistent with that, qPCR showed a significant reduction *of nef* in HIV-Tg-N3KO and HIV-Tg-N4KO mice compared to HIV-Tg26 mice (Fig: 4i). Together these data indicate that N3KO not only protects the HIV-Tg26 mice from renal inflammation but reduces HIV gene expression.

Notch3 deletion inhibits invasion of inflammatory cells in HIV-kidneys

Increased Notch3 expression has been linked to increased macrophage associated kidney injury previously[23]. Infiltration of CD68+ macrophages have been shown to be associated with adverse outcomes[43]. We next labeled a kidney biopsy from HIVAN patient for CD68. CD68+ macrophages were in the glomeruli, outside the Bowman's capsule and in the tubulointerstitial areas (Fig: 5 a). Interestingly, these areas (arrows) in a serial section also showed bright green Notch3 labeling (Fig: 5 b), indicating a co-labelling. Next, we asked if N3KO leads to overall reduction in the number of CD68+ macrophages. CD68+ macrophages were found in clumps in HIV-Tg26 kidneys (Fig:5c, arrow). Quantification of these cells revealed a 50% reduction in these cells in HIV-Tg-N3KO kidneys compared to HIV-Tg26 kidneys (Fig: 5 f). In contrast WT and N3KO kidney sections were negative for CD68 (Fig:5c). To assess other infiltrating cells that Notch3 deletion may affect, we conducted staining for FoxP3 (a marker for T Regulatory

cells), CD8 (T lymphocytes cells), Granzyme B (found in NK cells and cytotoxic T cells) and CD11c (a marker for dendritic cells). Interestingly there was a marked increase in the FOXP3 (Fig:5d and g) and CD8+ (Fig:5e and h) cells, however Granzyme or CD11c+ cells were not found in kidneys (supplementary S4). These data indicated that N3KO reduced the invasion of many inflammatory cell types in the HIV-Tg26 kidneys.

Bone marrow derived macrophages in HIV-Tg26 mice exhibit Notch3 activation

Since macrophage markers and infiltrating cells were reduced in HIV-Tg-N3KO kidneys, we reasoned that recruitment of mononuclear cells/macrophages from the bone marrow play a role in the pathogenesis of renal and systemic lesions in HIV-Tg26 mice. To determine whether bone marrow derived cells from HIV-Tg26 mice have active Notch signaling compared to those derived from WT mice, we isolated bone marrow derived macrophages (BMDMs) from both. Cells stained positive for F4/80 marker at differentiation (Fig 6a and b). Western blots revealed that renal Notch 3IC, Delta like 4 (Dll4) and Jagged 1 were significantly increased in HIV-Tg26 mice compared to WT controls (Fig: 6c, d, and e). These data reflect that Notch pathway activation in the bone marrow cells itself may be responsible for inflammation in the kidneys and other organs of these mice.

N3KO reduces circulating TNFα, MCP1 and other inflammatory molecules in HIV-Tg26 mice leading to overall disease amelioration

Notch3 activation in macrophages suggest that these cells may contribute to release of inflammatory cytokines that affect the outcome of HIV-Tg26 mice and that these changes are reduced upon N3KO. ELISA assays showed that HIV-Tg-N3KO mice had a significant reduction in the levels of serum TNFα and MCP1 compared to HIV-Tg26 mice (Fig. 7a, b) . In addition, dot blot analysis showed reduction in the levels of various cytokines and chemokines in the

serum of HIV-Tg-N3KO mice when compared with HIV-Tg26 mice (Fig: 7c and d) (supplementary tables 1 and 2). Collectively, the data suggest defects in the Notch signaling of bone marrow cells in HIV-Tg26 mice, which translate into systemic inflammation and thus infiltration of immune cells in various tissues. Notch3 deletion ameliorated these defects and the overall phenotype of the HIV-Tg-N3KO mice was improved when compared with HIV-Tg26 mice including a drastic improvement in skin lesions.

Discussion

Our study describes a novel mechanism through which activation of the Notch3 pathway affects clinical outcome and survival of HIV-Tg26 mice. Furthermore, to the best of our knowledge, this study demonstrates for the first time that inhibition of Notch3 signaling can specifically improve outcome of HIV-chronic kidney disease, uncovering a potential new target to prevent their progression in people living with HIV-1.

ART is a clinically successful cocktail of antiviral drugs (albeit with side effects) that target various stages of HIV replication cycle. HIV-Tg26 mice carry a 7.4kb proviral HIV-1 DNA construct containing a deletion encompassing most of the *gag* and *pol* genes, and thus HIV-1 cannot replicate in these mice. *Gag* encodes the capsid proteins necessary for the assembly of viral particles, while *pol* encodes the HIV-1 reverse transcriptase, protease, and integrase, which are essential for HIV-1 replication and therefore are specific targets of ART. Regardless of the absence of *gag* and *pol*, the HIV-LTR can induce the expression of other HIV genes (e.g. *nef*, *vpr*) in the HIV-Tg26 mice and cause several lesions, including skin papilloma's, cataracts and CKD[26,30,44].

Using the HIV-Tg26 mouse model and HIV-Tg rat model, we have previously shown that Notch ligands and receptors are activated in a variety of renal cells[7,8]. We took a genetic approach to globally delete Notch4 intracellular domain in the HIV-Tg26 mice. Notch4 deletion in Tg26 mice resulted in a marked improvement in renal function, an increase in the life span

and reduction in renal inflammatory infiltrates in HIV-Tg26 mice. In previous studies, Notch3 expression pattern was not clear as glomerular cells showed intense Notch3 labeling in nuclei but the number of glomerular cells positive for Notch3 expression remained same between WT and HIV-Tg26 mice. However, Western blots of the kidney lysates obtained from HIV-Tg26 mice compared to WT mice showed activation of Notch3[8]. Thus, using validated antibodies, we first investigated Notch3 expression pattern in HIVAN. Compared to WT mice where Notch3 was only expressed in vascular smooth muscle cells (VSMC), Notch3 was expressed in VSMC and both glomerular and extraglomerular regions in HIV-Tg26 mice. Notch3 was also activated in biopsy samples from HIVAN patients. Similar to studies on Notch4, N3KO resulted in amelioration of renal disease progression and an increased life span in HIV-Tg26 mice. However, compared to Notch4 deletion, N3KO resulted in better survival rate of Tg26 mice by 6 months (75% compared to 65%). To further evaluate differential effects of Notch3 and Notch4, RNA sequencing was conducted in kidneys. This showed macrophage associated inflammatory genes to be upregulated in HIV-Tg26 kidneys, which normalized upon N3KO as compared to N4KO. Matrix-metalloproteinase (MMP) family genes were upregulated in Tg26 kidneys; however, *mmp10* and *ccl2* were uniquely downregulated by N3KO. The MMP proteins either promote or restrict macrophage flux into the tissues[45]. In diabetic kidneys MMP10 contributed to the inflammatory macrophage response which was ameliorated by MMP-10 knockout[46]. In fact, Notch target protein Hey2 has been reported to regulate MMP10[47]. We show that protein expression of MMP10, similar to NFκB (p65), was reduced by N3KO.

The role of Notch3 in inflammation has been studied in non-HIV kidney diseases. Notch3 was activated in lupus nephritis and extracapillary glomerulonephropathy. In nephrotoxic sheep serum (NTS) induced kidney injury and ischemia reperfusion injury N3KO mice exhibited disease protection compared to WT mice. Renal macrophages derived from N3KO mice failed to activate inflammatory cytokines[23]. In vitro, overexpression of active Notch3 in podocytes led to reorganized cytoskeleton leading to a migratory, proliferative and

proinflammatory phenotype[22]. We show that Notch3IC can be induced in response to HIV-1 proteins and can remain activated in CKD such as that induced by HIV-1. Since there were less inflammatory infiltration in HIV-Tg-N3KO mice compared to Tg26 mice, we reasoned that it may be due to less infiltrating cells from extrarenal sources. The RNA seq supported our reasoning showing a drastic decrease in the macrophage associated markers in the HIV-Tg-N3KO kidneys as opposed to HIV-Tg26 kidneys. Thus, we focused on the BMDMs from these mice. To our surprise, we found activation of Notch3 signaling and upregulation of Notch ligands in the BMDMs from HIV-Tg26 mice. Similar to previous studies,[23] we speculate that Notch3 activation in glomerular cells and perhaps BMDMs can activate the migratory, proliferative and infiltrating properties of cells. We also speculate that intraperitoneal macrophages or cells of other myeloid lineage such as Tregs (Fox4 positive) may also have Notch3 activation in the HIV-Tg26 mice. Furthermore, our studies suggest that HIV-1 genes may change the fate of BMDMs by activating the Notch3 signaling pathway. Taken together, these changes may be responsible for the renal and systemic inflammatory responses and thus N3KO led to decreased systemic TNF alpha and MCP1, as well as the recruitment of inflammatory in the kidney and likely in skin lesions, therefore increasing the life span of these mice.

While Notch3 inhibition appears to be an excellent strategy to reduce inflammation, based on our previous studies, we stress that a combined approach to decrease the activity of both Notch 3 and Notch4 may have better outcomes. However, since age related vascular lesions have been detected in Notch3 mice [48,49], long term inhibition of these pathways may have negative consequences in humans. Further studies are needed to identify the most relevant downstream Notch3 signaling pathways that are involved in the pathogenesis of these lesions to determine whether they can be targeted with more specific and safer therapies.

Since the Notch pathway is activated in glomerular diseases with or without HIV-1, it remains to be determined whether HIV-1 can induce the activation of Notch3 directly. Using a pseudotyped HIV-1 replication defective construct, we found that HIV-1 can activate Notch3 directly in podocytes. In addition, N3KO in HIV-Tg26 mice resulted in a significant drop in the expression of *nef* and *env*. In support of these findings, the HIV-Tat protein can interact with the extracellular domain of the Notch receptors and was suggested as a non-canonical ligand for Notch activation[50-53]. In addition, previous studies in HIV-Tg26 mice and human podocytes showed that HIV-1 can precipitate the development of HIVAN and affect the cytoskeletal structure of podocytes cultured from children with HIVAN[54,55]. Moreover, there are two RBP-JK binding sites adjacent to the NFκB binding sites on the HIV-LTR promoter. RBP-JK binds the LTR and promotes recruitment of histone deacetylate (HDACs) and polycomb repressive complexes (PRC) in CD4+ T cells. Knock down of RBP-JK or PRC was shown to result in proviral activation[56-58]. RBP-JK is a known transcriptional repressor which changes into a transcriptional activator upon NIC binding. Thus, we speculate that NIC binding to RBP-JK in HIV-Tg26 leads to activation of Notch downstream pathways in HIV-Tg26 mice. This is supported by reduction in *nef* and *env* upon N3KO in HIV-Tg26 mice. Further studies will be required to clarify this mechanism, since, in HIV-Tg26 mice, the LTR promoter is primarily bound by the active p50/p65 heterodimer [59].

We have found that the Notch3 pathway is activated in BMDMs and several kidney cell types from HIV-Tg26 mice. Activation of the Notch3 pathway leads to significant systemic and renal inflammatory changes that precipitate the development of chronic kidney disease and affect the survival of these mice. Our data also suggest that HIV-1 genes can directly activate the Notch3 pathway in immune cells and human podocytes. It is also tempting to speculate that the Notch3 pathway may interact with other cells of the innate immune system as well as major risk factors that predispose to the development of CKDs in people of African descent, including

the APOL1 risk variants. Nonetheless, regardless of the APOL1 risk variants, it appears that new therapies that target the Notch3 pathway, may have a key role preventing the development of HIV-CKDs by decreasing the chronic systemic and renal inflammatory changes induced by HIV-1 despite ART.

Author contributions

M.T., N.S., M.M., J.D., S.Y., A.C., A.R., H.E., T.F and M.S. performed experiments. S.G. and P.C and performed RNA seq analysis and data sharing, respectively. J.T., provided expertise in BMDM isolation, P.R. and N.D performed inflammatory marker assessment. P.S. provided resources. H.E. N.S. and M.S. wrote the manuscript. T.F., P.S., P.R. N.D. and M.S. critically revised the manuscript.

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Figure Legends

Fig.1. HIV-1 induces Notch3 activation in kidneys. (A-B) immunolabeling was performed for presence of Notch3 in renal paraffin sections. Notch3 (green) labeling in kidney sections of 3months old wildtype (A and C) and Tg26 mice (B and D). Arrows indicate glomerular and tubular interstitial cells highly positive for Notch3 expression. Asterisks indicate blood vessels where Notch3 is normally expressed. (E-F) Quantification of Notch3 expression (intensity, green) as assessed using image J. (G and H) Normal versus HIVAN patient kidney biopsy (representative from n=3 in each group), nuclear expression indicating activation. (E-F) Differentiated immortal human podocytes were infected with empty vector or HIV-1 construct (PNL4) followed by protein blots for the presence of Notch3 intracellular (IC) domain (~92 kD). Protein blots were quantified for Notch3 using β-actin (as loading control), to which the values were normalized (n=4). Unpaired Student's t-test was used, and data represented as fold change (****P<0.0001). (Scale bar: 50μm).

Fig. 2. Notch deletion improves disease progression and lifespan in Tg26 mice. (A) Kaplan Meier curve showing 6 months mortality rate in Tg26 (n=26) and Tg-N3KO (n=28) mice. (B) Phenotypic appearance of WT, N3KO, Tg26 and Tg-N3KO mice, note skin papillomata on the forehead of Tg26 mouse whereas Tg-N3KO mouse appears normal. (C) Urine was collected in metabolic cages overnight from WT, N3KO, Tg26 and Tg-N3KO mice at 3 months of age before euthanasia. Proteinuria was assessed in 2ul urine by SDS-PAGE electrophoresis followed by Coomassie staining of the gels. "C" represents positive control BSA (bovine serum albumin) as a standard and "L" is the protein ladder. (D-F) Albumin and creatinine ratio in urine was measured using ELISA. Panel D shows albumin/creatinine ratio in both males and females combined (n=4, WT) (n=4, N3KO) (n=10, Tg26) (n=10,Tg-N3KO). Panel E and F show albumin

and creatinine for males and females separately. (G) Renal function was assessed in both males and females (n=4-8) as assessed by blood urea nitrogen levels (*P<0.05).

Fig. 3. Notch3 deletion ameliorates kidney injury in Tg26 mice

(A, B, C) Kidney sections from 3 months old WT, N3KO, Tg26 and Tg-N3KO mice were stained with Periodic acid Schiff (PAS) to determine kidney injury. PAS staining showing tubulointerstitial injury (a), glomerular tubular injury (b) and percentage inflammation (c). Note severe kidney injury in Tg26 kidneys (n=9) as compared to Tg-N3KO (n=12) kidneys. (D, E, F) Blind quantitation of percent tubulointerstitial injury, percent glomerular injury and percentage infiltration (*P<0.05), scale bar 50μm.

Fig. 4. Notch3 targets macrophage associated inflammatory genes in HIV kidney

(A) Heat map showing differential expression of genes obtained from mRNA sequencing of kidneys from WT, N3KO, Tg26, Tg-N3KO. (B) Volcano plot comparing gene expression in kidneys from Tg vs WT and Tg-N3KO vs WT mice (C) Volcano plot showing reduced gene expression in the upper right quadrant (Tg-N3KO vs WT) when compared to the upper right quadrant from figure 4b. (D) Macrophage associated inflammatory genes and HIV genes downregulated more in kidneys from Tg-N3KO mice as compared to kidney from Tg-N4KO mice. (E, F, G and H) Quantitative PCR validating the upregulated expression of *ccl2*, *tnfa*, *itgam* and *s100a9*, genes related to macrophage associated inflammation in Tg26 mice that almost normalized in Tg-N3KO kidneys. **(I)** Quantitative PCR validating that *nef* is reduced in Tg-N3KO kidneys as compared to kidneys from Tg26 mice (n=3 per condition). (*P<0.05,**P<0.01, ***P<0.001).

Fig. 5. Notch3 deletion reduces CD68 positive macrophage invasion

(A and B) Serial sessions from HIVAN patient biopsy immunostained for the presence of CD68 and Notch3. Note the presence of CD68 positive cells in and around glomerulus, the same areas were also labelled bright for the presence of Notch3 (arrows, Fig:5B). (C, D and E) Paraffin sections from kidneys of WT, N3KO, Tg26, Tg-N3KO mice, labeled for immune cell markers: CD68, FoxP3 and CD8, respectively). (F, G and H) Quantification of CD68, FoxP3 and CD8 positive cells from Tg26 and Tg-N3KO mice, blindly assessed in Tg26 (n=5) and Tg-N3KO (n=5) kidneys. Each dot represents percent positive cells from the entire kidney where inflammatory invasions were prominent (*P<0.05), (Scale bar: 50μm).

Fig. 6. Abnormal Notch expression in macrophages of TG26 mice

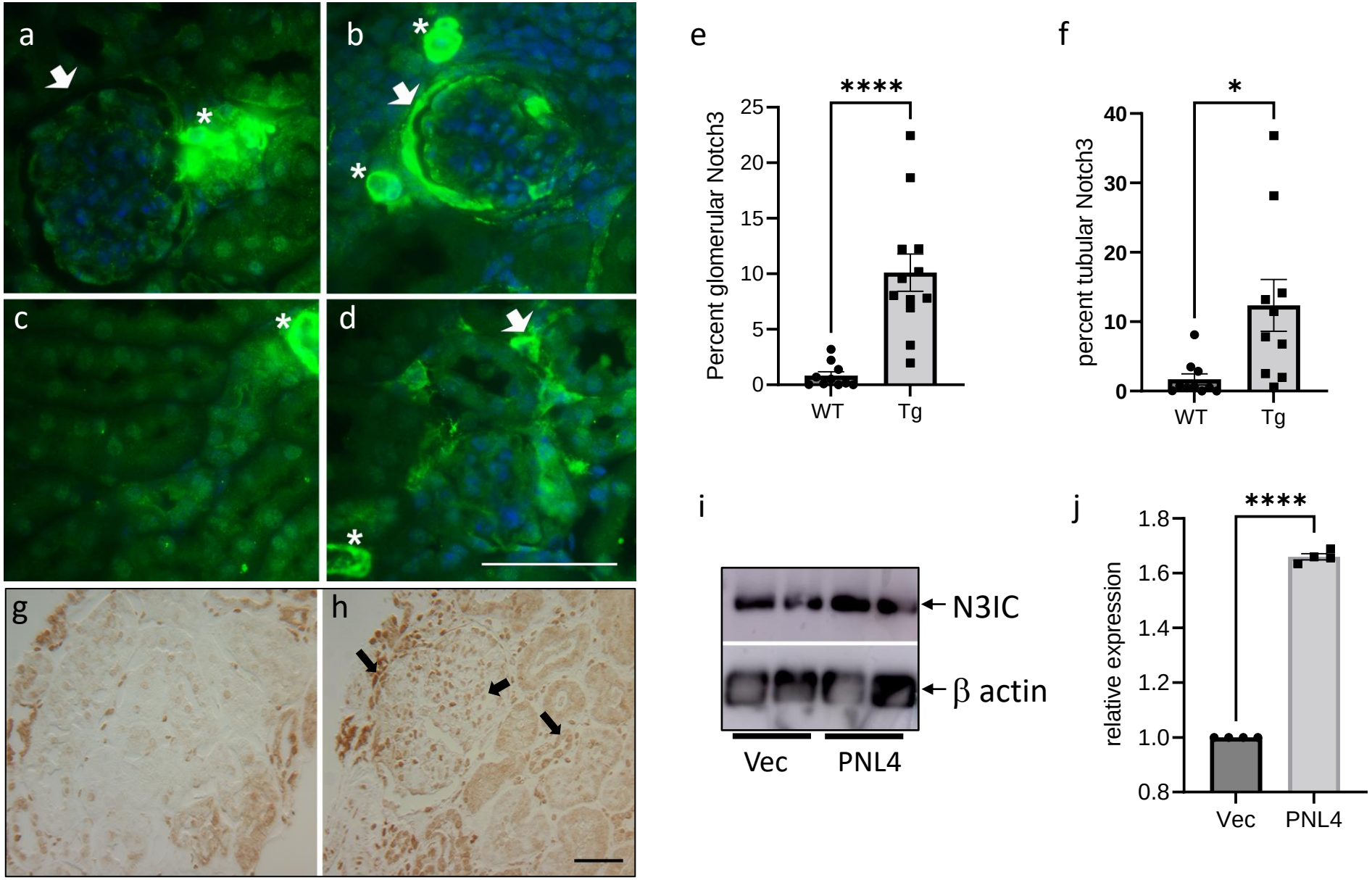
(a) Schematic showing macrophage isolation from bone marrow in mice. **(b)** Labeling of differentiated bone marrow cells expressing macrophage marker F4/F80 in fixed cells.

(c) Lysates obtained from differentiated macrophages from the basement membrane cells of WT and Tg mice were subjected to Western blot analysis for N3IC, Dll4, and Jagged1. CD68 was used as a loading control. **(d-f)** Quantification of bone marrow cells obtained from 3 mice in each group (*P<0.05,*** P<0.001).

Fig. 7. Notch3 ameliorates systemic inflammation

(A-B) ELISA assays were performed for the presence of TNFα and MCP-1 in serum obtained from WT (n=6-10), N3KO (n=6-10), Tg26 (n=10-12) and Tg-N3KO (n=10-12) mice, both males and females. Data is expressed as pg/ml (**P<0.01, ***P<0.001, ****P<0.0001). (C and D) Dot blots comparing the expression of various cytokines (C) and chemokines (D) and in the pooled serum from Tg26 (n=3) and Tg-N3KO (n=3) mice. Dots in duplicates represent one analyte. Dots having ≥ 1.5-fold differences in mean pixel density between Tg26 and N3-TgKO are labelled.

Figure:1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b
c
d
g
h
e
Percent glomerular Notch3
****
25
20
15
10
5
0
WT
Tg
f
percent tubular Notch3
*
40
30
20
10
0
WT
Tg
i
N3IC
β actin
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relative expression
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1.6
1.4
1.2
1.0
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Vec
PNL4

Figure:2

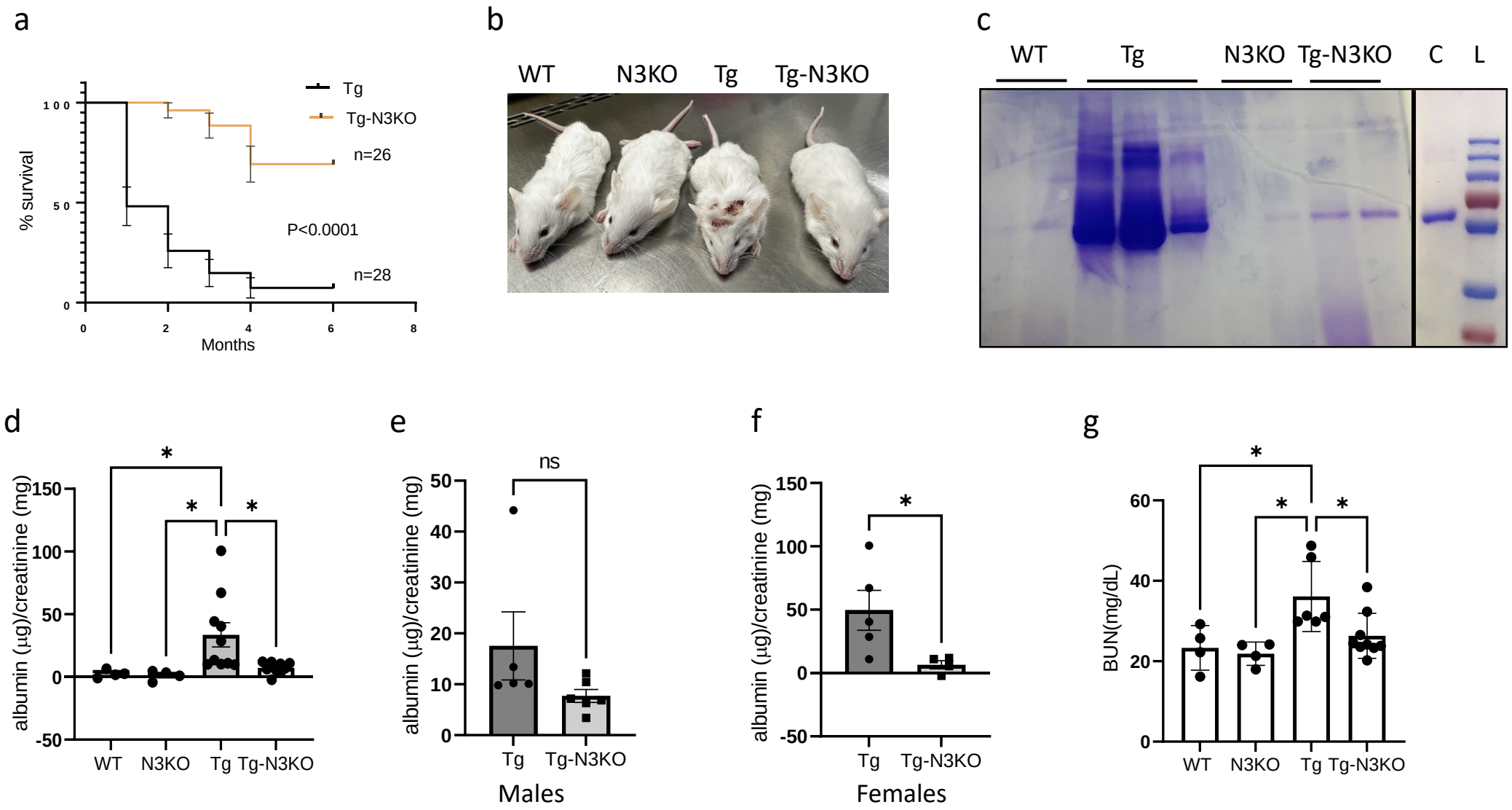

a
Tg
Tg-N3KO
n=26
P<0.0001
n=28
% survival
Months
b
WT
N3KO
Tg
Tg-N3KO
c
WT
Tg
N3KO
Tg-N3KO
C
L
d
albumin (µg)/creatinine (mg)
WT
N3KO
Tg
Tg-N3KO
e
ns
albumin (µg)/creatinine (mg)
Tg
Tg-N3KO
Males
f
albumin (µg)/creatinine (mg)
Tg
Tg-N3KO
Females
g
BUN(mg/dL)
WT
N3KO
Tg
Tg-N3KO

Figure:3

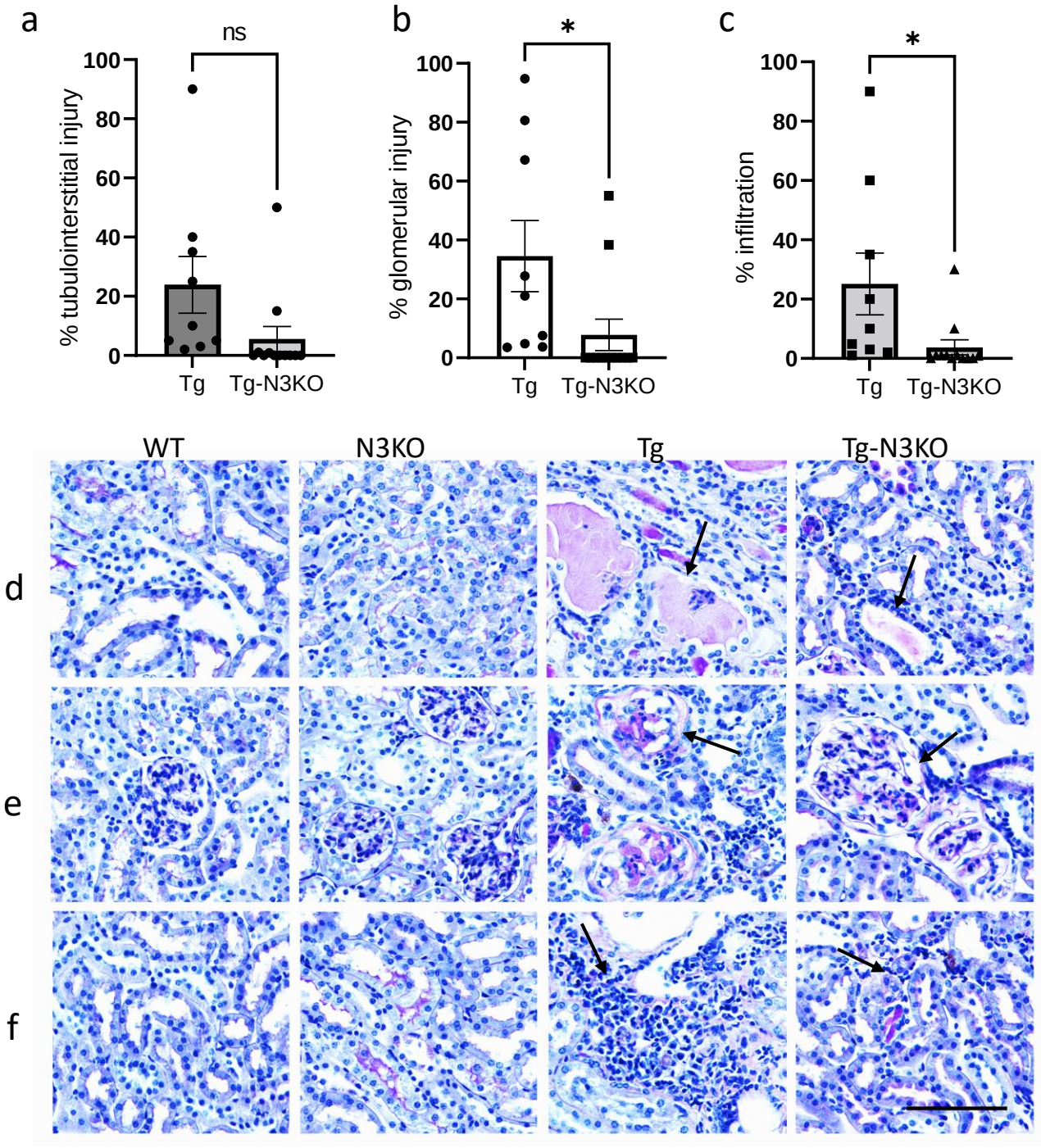
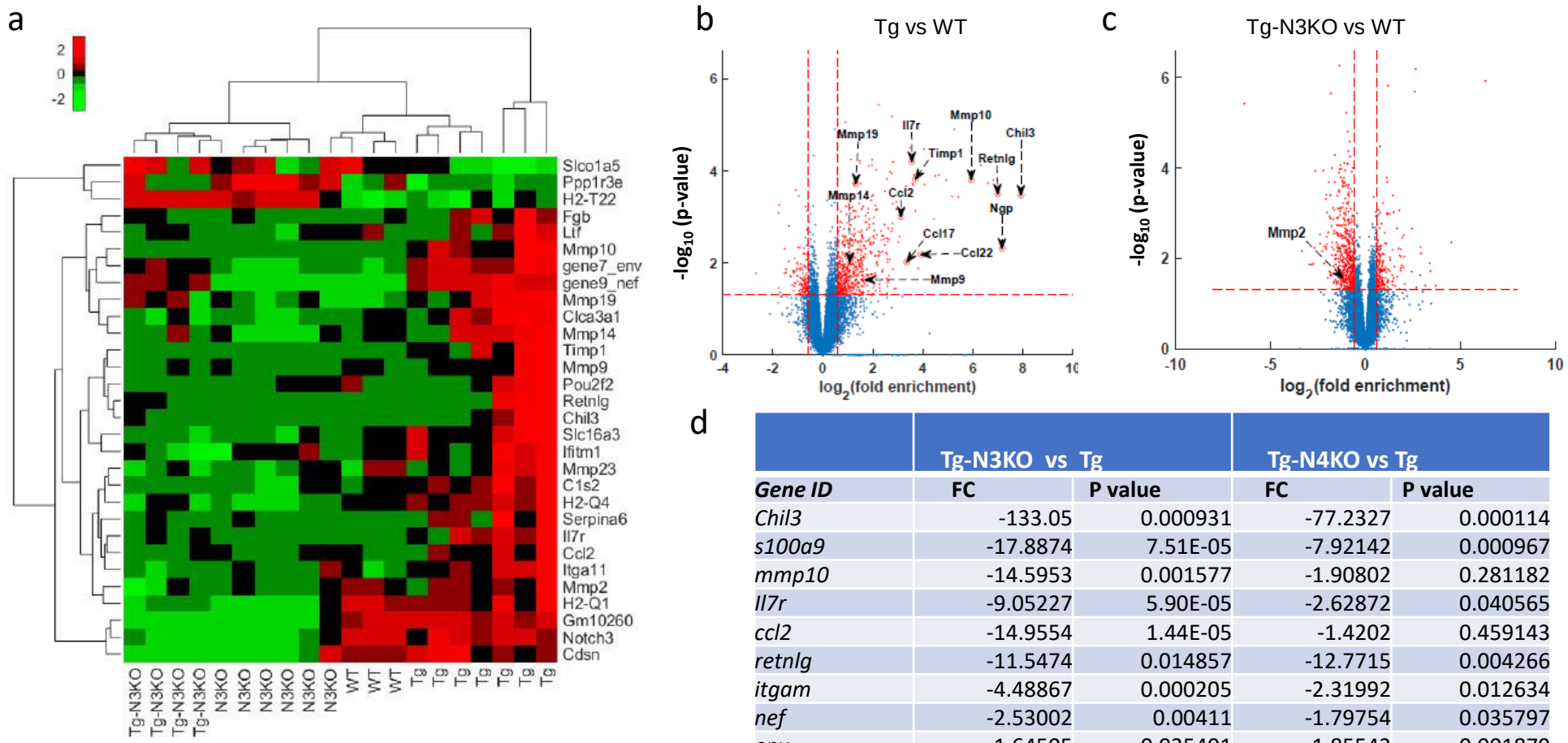
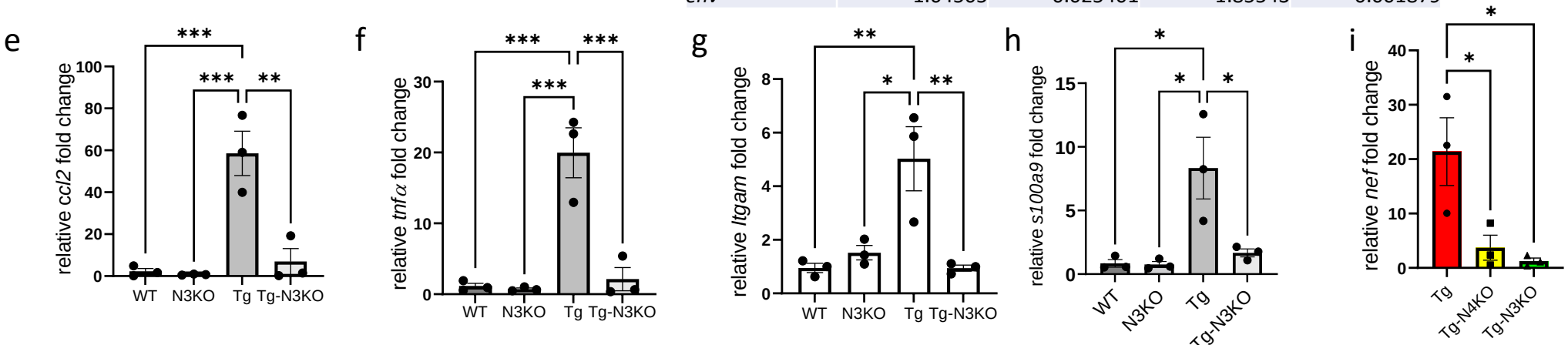

a
ns
% tubulointerstitial injury
100
80
60
40
20
0
Tg
Tg-N3KO
b
*
% glomerular injury
100
80
60
40
20
0
Tg
Tg-N3KO
c
*
% infiltration
100
80
60
40
20
0
Tg
Tg-N3KO
WT
N3KO
Tg
Tg-N3KO
d
e
f

Figure:4



d

	Tg-N3KO vs Tg		Tg-N4KO vs Tg	
Gene ID	**FC**	**P value**	**FC**	**P value**
Chil3	-133.05	0.000931	-77.2327	0.000114
s100a9	-17.8874	7.51E-05	-7.92142	0.000967
mmp10	-14.5953	0.001577	-1.90802	0.281182
Il7r	-9.05227	5.90E-05	-2.62872	0.040565
ccl2	-14.9554	1.44E-05	-1.4202	0.459143
retnlg	-11.5474	0.014857	-12.7715	0.004266
itgam	-4.48867	0.000205	-2.31992	0.012634
nef	-2.53002	0.00411	-1.79754	0.035797
env	-1.64505	0.025401	-1.85543	0.001879



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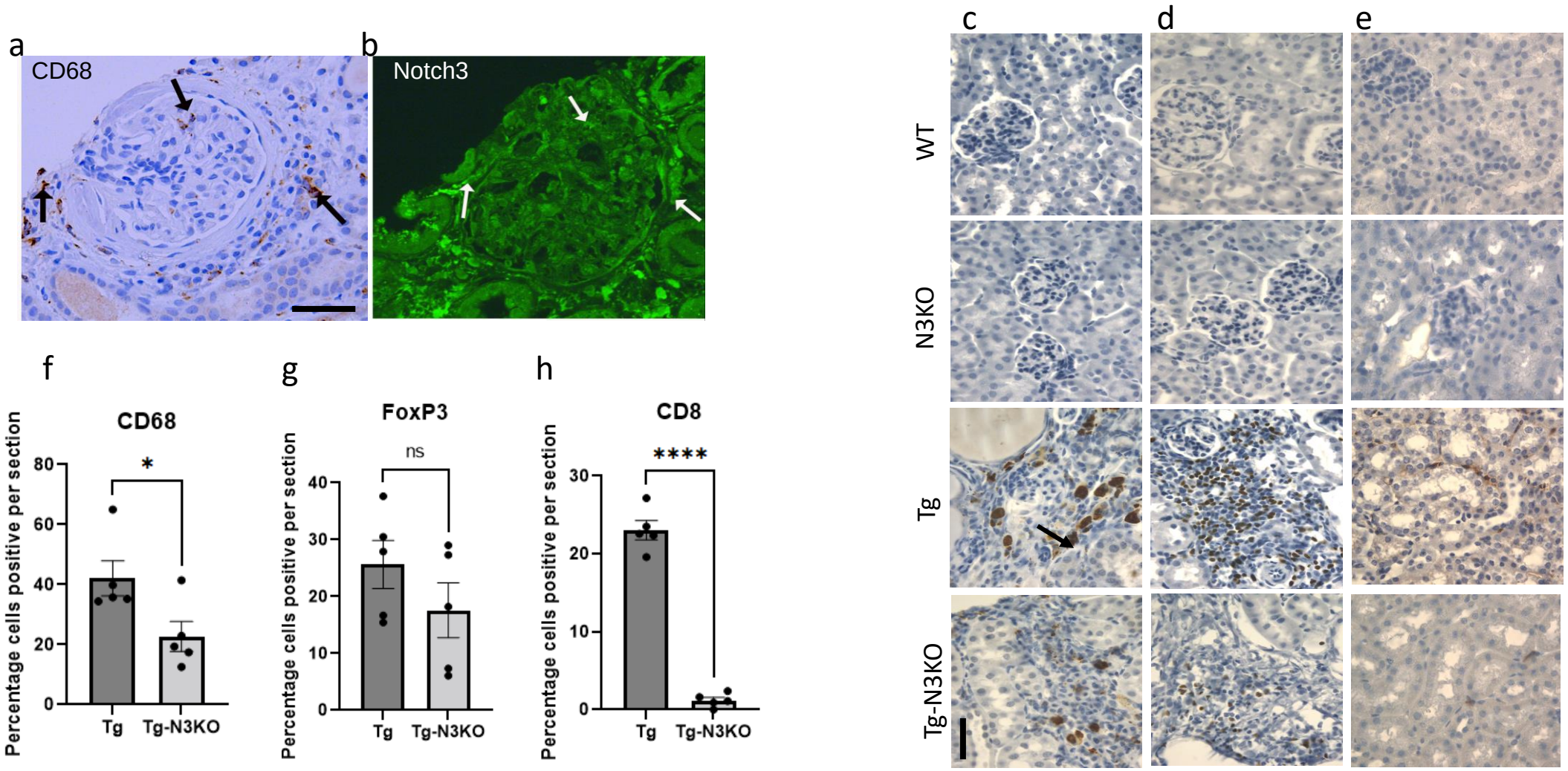
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CD68
b
Notch3
c
d
e
WT
N3KO
Tg
Tg-N3KO
f
CD68
Percentage cells positive per section
80
60
40
20
0
*
Tg
Tg-N3KO
g
FoxP3
ns
Percentage cells positive per section
40
30
20
10
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Tg
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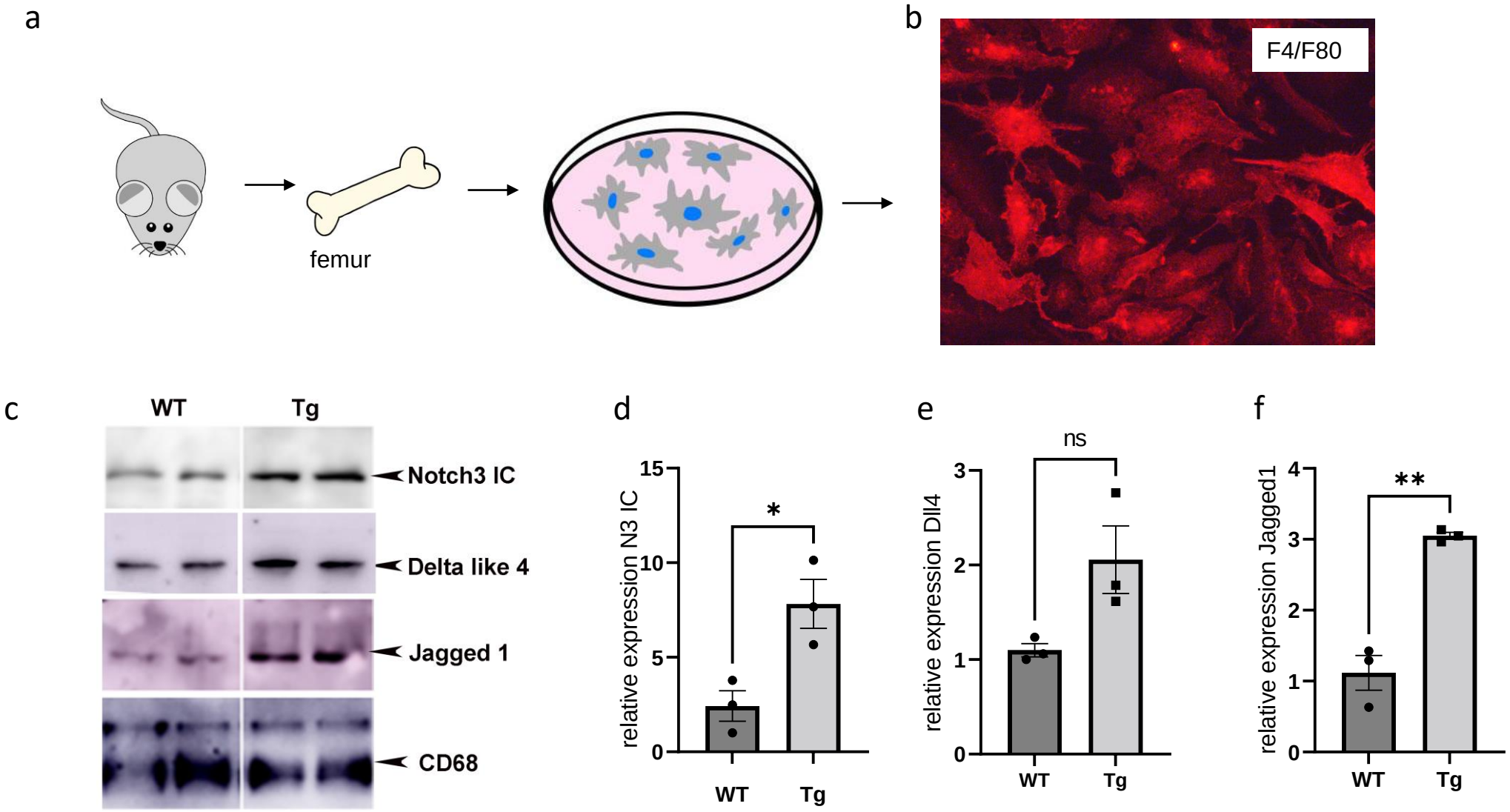
h
CD8
****
Percentage cells positive per section
30
20
10
0
Tg
Tg-N3KO

Figure:6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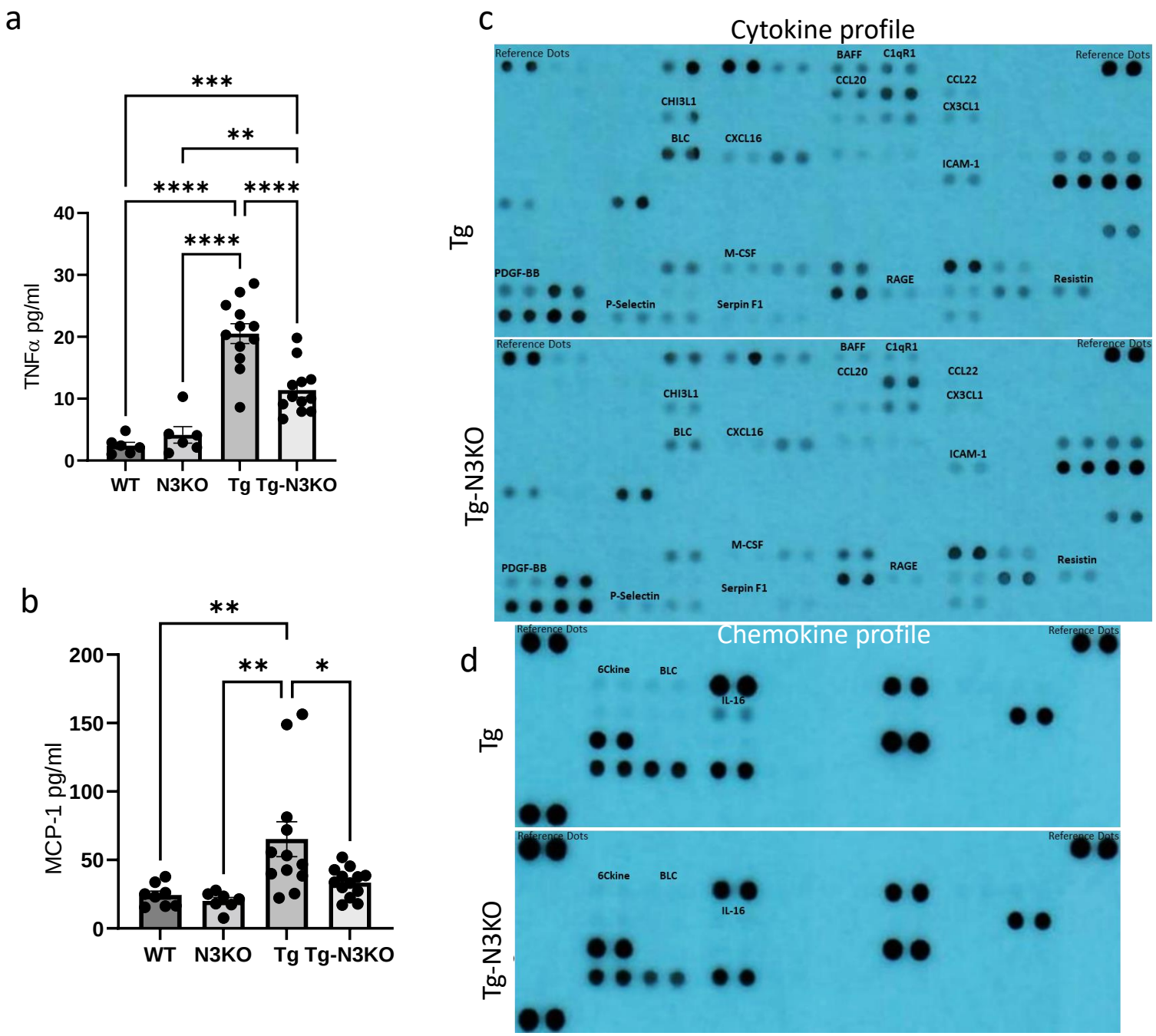
femur
b
F4/F80
c
WT
Tg
Notch3 IC
Delta like 4
Jagged 1
CD68
d
relative expression N3 IC
15
10
5
0
*
WT
Tg
e
ns
relative expression Dll4
3
2
1
0
WT
Tg
f
relative expression Jagged1
4
3
2
1
0
**
WT
Tg

Figure:7


a
***
**
****
****
****
TNFα pg/ml
40
30
20
10
0
WT
N3KO
Tg
Tg-N3KO
b
**
**
*
MCP-1 pg/ml
200
150
100
50
0
WT
N3KO
Tg
Tg-N3KO
c
Cytokine profile
Tg
Tg-N3KO
Reference Dots
BAFF
C1qR1
CCL20
CCL22
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Resistin
P-Selectin
Serpin F1
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Reference Dots
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BLC
IL-16