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Endothelial-specific deletion of CD146 protects against experimental glomerulonephritis in mice

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Running title: Targeting endothelial CD146 protects against GN

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Abstract

CD146 is an endothelial junctional adhesive molecule which expression is increased in human glomerular diseases. However, the pathological significance of this overexpression remains unknown. By using a mouse model of glomerulonephritis, we showed that CD146 expression was highly induced within damaged glomeruli and was associated with renal inflammation and fibrosis. Interestingly, two weeks after glomerulonephritis induction, CD146 knock-out mice showed preserved renal function as proteinuria and blood urea nitrogen levels were significantly lower compared to wild type littermates. Furthermore, renal structure was considerably conserved, since crescents formation, tubular dilation, monocyte and lymphocyte infiltration and interstitial renal fibrosis were highly reduced. Colocalization with markers for different types of glomerular cells showed that CD146 expression was mainly increased within the injured endothelium of the glomerular tuft. Consequently, we generated a new transgenic strain in which CD146 was specifically deleted in the vascular endothelium. Similarly to CD146 knock-out, these mice showed preservation of renal structure and function after the induction of glomerulonephritis compared to wild type animals. These data show that endothelial CD146 plays a major role in glomerulonephritis and may represent a novel therapeutic target to reduce glomerular damage and the progression of renal disease.

Keywords: CD146, experimental GN, inflammation, renal fibrosis

Introduction

Glomerular diseases can lead to renal fibrosis and chronic kidney disease (CKD), either progressively or rapidly. Rapidly progressive or crescentic glomerulonephritis (GN) are a group of rare kidney diseases that may rapidly progress to end stage renal failure as a consequence of pronounced inflammation with glomerular damage and development of crescents¹. Other kidney compartments are also affected, including tubulo-interstitium which shows an inflammation leading to renal interstitial fibrosis. The functional outcome of such structural lesions is a rapid deterioration of renal function². Even though several studies, mostly on preclinical models of experimental GN, provided pathophysiological advances, both on the inflammatory process and in the consequent fibrosis, molecular insights in the regulation and the progression of the disease are still limited and current treatments remain only partially effective.

CD146 is an adhesion molecule detected in all endothelial cells of the vascular tree, regardless of the vessel caliber or anatomical region^{3,4}. It mainly localizes at the intercellular junctions and controls inter-endothelial cell cohesion and paracellular permeability⁵⁻⁷ but also monocyte transmigration⁸ and angiogenesis⁹⁻¹². CD146 exists as different isoforms: a short isoform with a putative PDZ binding motif¹³⁻¹⁶, which may mediate its anchoring to the cytoskeleton, a long isoform with a putative endocytosis motif¹⁷, and a soluble form (sCD146) as the result of metalloprotease-dependent shedding of membrane CD146^{18,19}. sCD146 is detectable in the human serum and its level is modulated in different pathologies such as inflammatory bowel diseases²⁰, abnormal pregnancies²¹ or chronic renal failure¹⁸. Interestingly, we have reported that both membrane expression of CD146 and sCD146 were elevated in patients suffering from different types of renal diseases. Moreover, in human biopsies, high expression of CD146 was associated with renal damage and inflammation²². Furthermore, increased levels of sCD146 in sera of patients were consistent with changes of vascular permeability during chronic renal failure and suggested that CD146 function may be altered in these pathological settings¹⁸.

However, the pathological significance of CD146 overexpression in renal diseases remains unknown.

In this study, to decipher the role of CD146 in renal disease, we used a well-established model of experimental crescentic GN in mice. We demonstrated that the expression of this junctional molecule is highly increased within injured glomerular endothelium leading to severe structural tissue damage thus contributing to the progression of the disease. More importantly, CD146 knock-out (KO) mice or mice in which CD146 was specifically deleted in the vascular endothelium (CD146-EC-del), showed preservation of both renal functional and structural parameters during experimental GN.

Methods

Methods

Mice strains

CD146-floxed and CD146-KO mice were generated as previously described and backcrossed for more than 10 generations into the C57BL/6J background⁸. CD146-floxed mice were first crossed with the B6.Cg-Gt(ROSA)26Sortm6(CAG-ZsGreen1)Hze/J mice (Jackson laboratories) which express the fluorescent protein ZsGreen1 as a reporter for CRE-recombinase activity. Then, mice with endothelial cell-specific deletion of the CD146 gene were generated by further crossing CD146flox-ZsGreen animals with the Cdh5 (PAC)-CreERT2 mouse strain, a gift from Dr Ralf Adams²³. The deletion of the CD146 gene was induced by I.P. injections of tamoxifen diluted in corn oil (10 mg/ml solution, 1 mg tamoxifen/injection) and administered on 3 consecutive days. Primers for genotyping CD146 alleles were 5'-TCACTTGACAGTGTGATGGT-3' (forward primer used to detect CD146 WT, floxed and KO alleles), 5'-CCTTAGAAAGCAGGGATTCA-3' (reverse primer used to detect

CD146 WT and floxed alleles) and 5'-CCCAAATCCTCTGGAAGACA-3' (reverse primer used to detect CD146 KO allele).

CD146 analyses in diverse human glomerulopathies

Human kidney biopsies were collected in a multicentre study (European Renal cDNA Bank—Kröner- Fresenius Biopsy Bank, ERCB) and were obtained from patients after informed consent and with approval of the local ethics committees. Pre-transplant allograft biopsies were used as control kidneys. Total RNA was isolated from microdissected glomeruli, reverse-transcribed, and linearly amplified as previously described²⁴. Fragmentation, hybridization, staining and imaging were performed according to the Affymetrix Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA, USA). A single probe-based analysis tool, Chip- Inspector (Genomatix Software GmbH, Munich, Germany), was used for transcript annotation, total intensity normalization, significance analysis of microarrays²⁵ and transcript identification based on significantly changed probes. Pre-developed TaqMan probes (Applied Biosystems) were used for human CD146 and GAPDH detection by qPCR. mRNA expression was analyzed by standard curve quantification.

Nephrotoxic serum-induced glomerulonephritis

Decomplemented nephrotoxic serum (NTS) was prepared as previously described^{26,27} and 17,5µl of serum per gram of body weight were intravenously injected over two consecutive days (days 1 and 2). For the time course protocol, two-month old C57Bl6/J females were injected with NTS and sacrificed at day 4, 7 and 15 for tissue collection (n=6 mice for each time point). Six mice were injected with PBS and used as controls.

For the CD146-KO protocol, two-month old CD146-KO female mice and their wild type littermates were used. Eight to nine mice from each group were injected with NTS and 6 with

PBS. Body weight and urine samples were taken at days 0, 4, 7 and 15. All mice were sacrificed at day 15 and blood and tissue samples were obtained.

For the CD146-floxed protocol, two-month old female mice were treated with tamoxifen. Eleven of them were injected with NTS and 4 with PBS. CD146-floxed ZsGreen mice bearing no CRE recombinase transgene were treated with tamoxifen and used as controls. Body weight and urine samples were taken at days 0, 4, 8, 12 and 15. Mice were sacrificed at day 15 after obtaining blood samples.

All procedures regarding animal experimentation were in accordance with the European Union Guidelines for the Care and Use of Laboratory Animals and approved by the local ethics committee of the National Institute for Health and Medical Research (Institut National de la Santé et de la Recherche Médicale). Animals were housed at constant temperature with free access to water and food.

Histologic and functional parameters

Half kidneys from each animal were fixed in 4% formalin solution and embedded in paraffin. 4 μ m sections were stained with Masson's trichrome for histological evaluation of renal damage. Tubular dilation was evaluated semi-quantitatively, using the following scale: 0, no tubular damage; 1, damage in 1–25% of the tubules analyzed; 2, damage in 26–50% of the tubules analyzed; 3, damage in 51–75% of the tubules analyzed; 4, damage in >76% the tubules analyzed. Glomerular crescents were measured and expressed as the % number of glomeruli presenting crescents vs total number of glomeruli. Scoring was performed in a masked manner on coded slides by two different investigators. Images were obtained with an Olympus IX83 photonic microscope at X200 magnification. Interstitial fibrosis was assessed semiquantitatively on Sirius red stained paraffin sections at magnification of X200. Fibrosis was then quantified using computer-based morphometric analysis software (Analysis, Olympus) that allowed the formation of a binary image in which the stained area could be

automatically calculated as a percentage of the image area. Proteinuria, expressed as protein g/mmol of creatininuria, was assessed using the Pyrogallol Red method and utilizing a KONELAB automate (Thermo Scientific, Waltman, MA). BUN was assessed in blood plasma obtained on the day of sacrifice. BUN was measured using an enzymatic spectrophotometric method and was expressed as mmol/L.

Immunostainings

Immunohistochemistry was performed on 4µm-thick paraffin-embedded tissue sections. Tissue was deparaffinized and 10mM citric acid, pH6 at 95°C was used for antigen retrieval. Sections were permeabilized with 0.1% triton/PBS. Antibodies against F4/80 (AbD Serotec) and CD3 (Dako), were used. The F4/80 and CD3 positive area was quantified in at least ten photographs at 200X magnification per animal, using publicly available image processing software (ImageJ; Fiji) and expressed as percentage of the total tissue area. Immunofluorescence was performed on 4µm-thick frozen sections fixed in methanol. CD146 (homemade rat anti-mouse), CD31 (Abcam), Claudin-1 (Thermo Scientific), Nestin (BD Pharmingen), Nephrin (Abcam) anti-rabbit and α -SMA (Sigma Aldrich) anti-mouse antibodies were used. Alexa fluor (Invitrogen) secondary antibodies were used for detection. Images were obtained with an Olympus IX83 photonic microscope at X400 magnification.

Quantitative Real-Time PCR

Total RNA was extracted from half kidneys using TRI Reagent (MRC). RNA quality was verified by measuring the OD 260:280 ratio and residual genomic DNA was removed by DNase I treatment (Thermo Fisher Scientific) for 30 minutes at 37°C. A total of 1 mg RNA was transcribed to cDNA using the Maxima First Strand cDNA Synthesis Kit from Thermo Fisher Scientific per manufacturer's instructions. Real-time PCR was performed with the Roche Light Cycler 480 detection system using SYBR Green PCR Master Mix (Roche Diagnostics,

Indianapolis, IN) under the following program: 95°C for 5 minutes, 45 cycles at 95°C for 15 seconds and 60°C for 15 seconds, and 72°C for 15 seconds. For quantitative analysis, experimental genes were normalized to HPRT expression using the DDCT method. Dissociation curves were analyzed to determine that a single product was amplified. Primer sequences are listed in table 1.

Western blot

Proteins were extracted from renal tissue using RIPA lysis buffer (Santa Cruz Biotechnology). Western blot for CD146 (Epitomics) were performed using standard techniques as previously described²⁷. GAPDH (Sigma-Aldrich) was used as loading control.

Statistical Analyses

Values are expressed as mean \pm SEM. Data were analyzed using one-way analysis of variance followed by protected least significant difference Fisher's test of the Statview software package. Error bars represent mean \pm SEM. Results with $P < 0.05$ were considered statistically significant.

IL-1 β	FW: TGTAATGAAAGACGGCACACC RV: TCTTCTTTGGGTATTGCTTGG
TNF- α	FW: TCTTCTCATTCCTGCTTGTGG RV: ATGAGAGGGAGGCCATTTG
ICAM-1	FW: CCCACGCTACCTCTGCTC RV: GATGGATACCTGAGCATCACC
TGF- β 1	FW: TGGAGCAACATGTGGA ACTC RV: GTCAGCAGCCGGTTACCA
Collagen-3	FW: TGGTTTCTTCTCACCCCTTCTC RV: TGCATCCCAATTCATCTACGT

CD146	FW: TCCAGTCATCACAGATTGTCG RV: AAACAGGGACAGTGACCTCCT
VCAM-1	FW: TGGTGAAATGGAATCTGAACC RV: CCCAGATGGTTTCCTT
MCP-1	FW: CATCCACGTGTTGGCTCA RV: GATCATCTTGCTGGTGAATGAGT
Collagen-1	FW: GCAGGTTACCTACTCTGTCTT RV: CTTGCCCATTCATTTGTCT
HPRT	FW: GGAGCGGTAGCACCTCCT RV : CTGGTTCATCATCGCTAATCAC

Table 1 : Primer sequences used for the study.

Results

CD146 is induced in damaged glomeruli

To assess the expression of CD146 during the progression of glomerular disease, we induced a passive nephrotoxic serum glomerulonephritis (NTS-GN) in wild type mice. Animals were sacrificed at 4, 7 and 15 days post NTS administration. Immunofluorescence showed a weak expression of CD146 in glomeruli of healthy animals (**Fig1A**). CD146 expression progressively increased from day 4 to day 15 in peritubular capillaries and mainly within damaged glomeruli. This upregulation was confirmed by western blotting of renal tissues (**Fig1B and 1C**). The specificity of the CD146 antibodies has been checked by using renal tissues from CD146 KO mice (**FigS1**). Thus, CD146 expression was highly and continuously increased with the progression of NTS-GN.

CD146 knock-out mice are protected from nephrotoxic serum-induced glomerulonephritis

To assess the role of CD146 in GN, we injected NTS in both CD146 WT and KO mice. Mice were sacrificed 15 days post NTS administration. NTS-GN resulted, as expected, in a significant body weight increase in WT animals (**Fig2A**). In accordance, renal function was altered since both proteinuria and blood urea nitrogen (BUN) were highly increased (**Fig2B and 2C**). In contrast, in CD146 KO mice, body weight and BUN increase were blunted. Furthermore, elevation of proteinuria was significantly lower in these mice. Quantification of histological lesions by Masson's Trichrome coloration showed that NTS injection induced less damages in CD146 KO mice (**Fig2D**). Indeed, a significant structural protection in CD146 KO animals was demonstrated by a significant decrease of both crescent formation and tubular dilation compared to WT ones (**Fig2E and 2F**). Thus, CD146 KO animals were protected from glomerular and tubular lesions induced by NTS-GN, which preserved renal function and blunted proteinuria.

CD146 KO mice show reduced inflammation and renal fibrosis after NTS injection

Evaluation of inflammatory markers in kidneys by qPCR showed that the induction of IL-1 β , TNF- α and ICAM-1 mRNAs was blunted in CD146 KO mice, 15 days after NTS injection (**Fig3A, 3B and 3C**). Accordingly, F4/80 immunostaining showed less macrophage infiltration in the renal cortex of CD146 KO mice (**Fig3D and 3E**). Furthermore, these animals displayed reduced CD3 cell infiltrates on day 15 (**Fig3F and 3G**). Consequently, renal fibrosis was limited in mice lacking CD146. Indeed, mRNA expression of fibrotic markers such as TGF- β and collagen I was blunted in CD146 KO mice (**Fig3H and 3I**). Quantification of Sirius Red coloration confirmed a reduction of renal interstitial fibrosis in these animals (**Fig3J**).

Altogether, these data demonstrate that lack of CD146 markedly restricted renal inflammation and renal interstitial fibrosis in NTS-GN.

CD146 is increased in the glomerular endothelium during the progression of NTS-GN

To identify the cell type overexpressing CD146 within damaged glomeruli after aggression, we performed colocalization experiments using appropriate markers of the glomerular components, at day 7 post NTS administration. CD146 was slightly expressed at basal conditions in the vascular tuft (**Fig1A**). After induction of NTS-GN, CD146 overexpression colocalized mainly with the endothelial marker CD31/PECAM (**Fig4A**). In contrast, CD146 was not detected in podocytes, as there was no colocalization with nestin (**Fig4B**). It was neither detected in parietal cells, as we did not detect any colocalization of CD146 with Claudin-1 (**Fig4C**), nor in mesangial cells (**Fig4D**). Thus, our data confirmed that CD146 was highly overexpressed mainly within injured glomerular endothelial cells after the induction of the disease.

CD146 endothelial-specific deletion protects mice against NTS-GN

To estimate the impact of the endothelial CD146 overexpression in the progression of NTS-GN, we generated the CD146-EC-del mouse strain. CD146 was specifically deleted in the vascular endothelium by interbreeding mice harboring a tamoxifen-inducible VE-cadherin Cre-recombinase²³ with CD146flox-ZsGreen mice (**Fig5A**). The specificity of CD146 deletion in endothelial cells was verified by immunofluorescence, after tamoxifen injection and 15 days post NTS administration (**Fig5B**) and further confirmed by qPCR (**Fig5C**). CD146-EC-del mice showed limited increase of body weight, proteinuria and BUN (**Fig6A, 6B and C**). This functional protection was accompanied by an overall preservation of renal structure (**Fig6D**), as number of crescents (**Fig6E**) and tubular damage (**Fig6F**) were both dramatically reduced compared to respective controls 15 days after the induction of NTS-GN. Furthermore, F4/80

immunostaining showed reduced monocyte infiltration in the renal cortex of CD146-EC-del mice (**Fig7A**). In accordance, qPCR experiments showed that upregulation of both VCAM-1 and MCP1 were fully abolished (**Fig7B and 7C**). Subsequently, TGF- β 1 and Collagen I mRNAs increased expression was blunted in CD146-EC-del mice compared to control kidneys (**Fig7D and 7E**). These data demonstrate that endothelial overexpression of CD146 promotes the progression of experimental GN in mice.

CD146 transcript numbers are increased in human glomerulopathies

CD146 mRNA expression was analyzed in microdissected glomeruli from renal biopsies of patients suffering from diabetic nephropathy (DN, n=7), minimal change disease (MCD, n=5), IgA nephropathy (IgA, n=27), focal segmental glomerulosclerosis (FSGS, n=10), membranous glomerulonephritis (MGN, n=21), lupus nephritis (SLE, n=32) and rapidly progressive glomerulonephritis (RPGN, n=23). Compared to control biopsies (n=6), damaged glomeruli showed a pronounced transcriptional upregulation of CD146 transcripts (**Fig8**).

Discussion

A variety of chronic inflammatory diseases is associated with disruptions of the inter-endothelial function and junction integrity²⁸. Defects in the organization of endothelial junctions occur also during the progression of chronic renal diseases. Indeed, in biopsies from patients with nephropathies of several etiologies, we have previously reported that high expression of endothelial CD146 was associated with endocapillary proliferation, proteinuria and inflammation²². Moreover, increased levels of CD146 were consistent with changes in renal morphometry during the progression of chronic renal failure suggesting that the junctional function supported by CD146 may be altered in these pathological settings. However the pathological significance of this overexpression remained unclear. Interestingly in our study,

increased expression of CD146 seemed to promote renal disease since its deletion protected kidney from immune aggression and prevent severe renal dysfunction. Thus CD146 may represent a novel therapeutic target against the initiation and the progression of glomerulonephritis.

In our study, CD146 was overexpressed in renal microcirculation, mainly within injured glomeruli and already since the early stages of the disease. CD146 was also found to be overexpressed within proximal tubules at later time points (**Fig1A**). This is consistent with previous observations that reported CD146 expression in renal tubular cells *in vivo* and *in vitro* under pathological conditions^{22,29}. In our experimental model, where the starting point of the disease is the glomerular compartment, CD146 KO mice were protected against the development of renal inflammation, proteinuria and weight gain. CD146 was also reported to be expressed in myeloid cells in mice³⁰, but we showed that CD146 was mainly overexpressed within injured endothelium thanks to colocalization experiments. Thus, we generated an endothelial specific KO of CD146 to discern the specific role of endothelial CD146 in NTS-GN. Similar to the global CD146 KO, CD146-EC-del mice were also preserved after the induction of GN. This clearly demonstrates a major role of the endothelial cells in controlling glomerular damage but also interstitial inflammation. These results are in accordance with our previous study demonstrating that endothelial CD146 is involved in the regulation of monocyte transendothelial migration *in vitro*⁸. Indeed, upon tumor necrosis factor- α stimulation, CD146 expression was increased at both cellular junctions and apical membrane of HUVEC and further contributed to monocyte transmigration through the endothelial monolayer. Similar results were more recently reported in a mouse model of multiple sclerosis, in which targeting endothelial CD146 decreased neuroinflammation by limiting lymphocyte extravasation on the blood brain barrier³¹. Considering these data, it would be of major interest to further delineate

the role of CD146 in glomerular and interstitial inflammation in the pathogenesis of renal diseases.

Another interesting finding of our study is that renal fibrosis was blunted in both CD146 KO and CD146-EC-del mice. Even if this observation is reported in a model of crescentic GN, which is a rare disease in humans, this is of particular interest as renal fibrosis is the common evolution of all kidney diseases and is highly predictive of the progression of CKD³². The following hypotheses can be proposed to explain the reduction of fibrosis burden. First, the diminution of fibrosis could be a consequence of reduced inflammation in the injured kidney. Second, endothelial CD146 could play a key role in the control of the fibrotic process in response to the activation of endothelial cells. Indeed, endothelial to mesenchymal transition (Endo-MT) has been proposed to contribute to the accumulation of activated fibroblasts and myofibroblasts in fibrotic kidneys, in different models of experimental nephropathy in mice³³. In this line, the reduction of fibrosis could be partially explained by a reduction in the Endo-MT when CD146 is down-expressed. However, more recent studies demonstrated that the contribution of the Endo-MT program to the emergence of renal fibrosis was moderate³⁴. Another hypothesis could be the role of CD146 to induce cell activation as it has been described in the epithelial to mesenchymal transition phenomenon in other tissues, a major event in the metastatic dissemination^{35,36}. Indeed, as CD146 can be shed at the cell membrane to generate soluble CD146, sCD146 released by endothelial cells, could stimulate fibroblast proliferation and transition to a myofibroblast phenotype, leading to renal extracellular matrix deposition and fibrosis. Consequently, another important question to explore will be to determine the CD146 isoform involved in the observed effects. We have described two different isoforms, long and short, which differ by their intracellular part. Whereas the long isoform is present at the junction of endothelial cells, and plays a major role in monocyte transmigration, the short isoform is rather present at the apical membrane and could be shed to

generate the soluble form of CD146 that will bind and stimulate monocytes^{16,18}. Whether one or both isoforms are modulated in NTS-induced GN and whether they are able to cooperate in the phenomenon will have to be explored.

GN is recognized as the second most frequent cause of end-stage renal disease (ESRD) worldwide³⁷. Despite aggressive immunosuppressive therapies, rapidly progressive GN can lead to irreversible renal damage with CKD and frequently ESRD. Considering the need for therapeutic targets to withhold glomerular damage and tubulo-interstitial inflammation and fibrosis in GN, we used a powerful experimental tool sharing some features with human glomerulopathies, to investigate the role of CD146 in renal inflammatory injury. Indeed, injection of sheep serum rich in immunoglobulins against glomerular antigens induces an immediate inflammatory response characterized by renal infiltration of cells of the immune system, followed by severe glomerular damage and rapid loss of renal function^{27,38,39}. This is a robust model of rapidly progressive renal disease as mice are dying the latest 4 weeks after the induction of the disease^{27,40}. Thus, this model of GN can be used to delineate the pathogenic processes leading to immune nephritis, over a quick time frame and allowed to identify several factors that potentially dictate disease severity. This has important clinical implications, both from the perspective of genetic susceptibility as well as clinical therapeutics⁴¹. Indeed, several mediators such as cytokines, chemokines, adhesion molecules, surface receptors and extracellular matrix proteins have been shown to play a crucial role in mediating the inflammatory response in CKD⁴²⁻⁵². Thus, a major finding of our study is that CD146 deletion is beneficial in this model of GN since in both CD146 KO and CD146-EC-del mice the progression of the disease was blunted. Consequently, it is of interest to develop specific inhibitory devices against CD146. This is reinforced by the exaggerated expression of this junctional molecule within glomeruli in biopsies of patients suffering from different type of GN in comparison with healthy controls (**Fig8**). Interestingly, our data are also in accordance with

publically available data obtained by the NephroSeq consortium showing overexpression of CD146 in glomeruli from patients with various glomerular diseases (<https://www.nephroseq.org/resource/login.htm>). Further experiments using additional and non-glomerular experimental models of renal disease, will be necessary to investigate whether the beneficial effects of CD146 deletion are model-dependant, or can be considered as a more common protective approach against renal inflammation and fibrosis.

Nevertheless, our results support a major role of the endothelium in kidney to control the aggression towards all the compartments, glomeruli but also tubules. We demonstrated that endothelial CD146 plays a major role in the progression of GN. Thus, targeting this junctional molecule may represent a good therapeutic strategy to prevent the progression of GN.

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Competing financial interests

The authors have declared that no conflict of interest exists.

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Novelty and Significance

What is new?

- The expression of CD146/MCAM is highly increased in humans and rodents in glomerular diseases.
- CD146 KO mice showed preservation of renal structure and function after the induction of experimental glomerulonephritis, compared to wild type animals.
- Similar protective effects were observed when CD146 was deleted specifically in the vascular endothelium, as both functional and structural renal parameters were close to healthy animals.

What is relevant?

While defects in the organization of endothelial junctions have been reported to participate to chronic renal disease, this study provides evidence that the expression CD146/MCAM is highly increased within injured glomerular endothelium, leading to severe structural tissue damage promoting thus the progression of renal disease.

Summary

CD146/MCAM can promote renal inflammation and further fibrosis during the progression of glomerular diseases. Because this protein expression is elevated during the progression of glomerulonephritis in rodents and in humans, our findings may have therapeutic relevance in chronic renal disease.

Figure Legends

Figure 1. CD146 is induced in experimental GN in mice

CD146 (in green) is highly upregulated within damaged kidneys progressively from day 4 to day 15 post NTS administration in mice and mainly localized within the injured glomerulus (A). Renal cryosections were counterstained with Evans blue (red). This upregulation was confirmed by western blot (B, C). *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$. (* NTS versus CTL, Scale bar $50\mu\text{m}$).

Figure 2. CD146 knock-out mice are protected against NTS-induced glomerulonephritis.

Body weight increase in mice after the induction of the disease (A). Renal function was evaluated by proteinuria, expressed as grams of protein/mmol of creatinine (B) and BUN (C). All three parameters reveal a slower progression of the disease in CD146 knock-out animals. Mason trichrome staining of kidney slices (D) shows that renal structure was preserved in CD146 knock-out since they developed less glomerular crescents (E) and tubular dilation (F). Crescents are expressed as the percentage (%) of glomeruli presenting cellular crescents. *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$. (* NTS versus CTL, # KO NTS versus NTS, Scale bars $50\mu\text{m}$ for large microphotograph and $20\mu\text{m}$ for zoomed-in area).

Figure 3. CD146 knock-out mice develop less renal inflammation and fibrosis.

QPCR for $\text{Il-1}\beta$ (A), $\text{TNF-}\alpha$ (B) and ICAM-1 (C) showed restricted inflammatory response in kidneys of CD146 KO mice 15 days after the induction of NTS-GN. Immunohistochemistry for F4/80 (D, E) and CD3 (F, G), confirmed respectively limited monocyte and lymphocyte adhesion in CD146 KO mice. At the same time point QPCR for $\text{TGF}\beta$ (H), collagen III (I) and quantification of Sirius red staining (J), showed restriction of the fibrotic response in CD146

KO mice.*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (* NTS versus CTL, # KO NTS versus NTS, Scale bar 50 μ m).

Figure 4. CD146 is strongly induced in glomerular endothelial cells after NTS administration.

Double immunofluorescence in renal cortical slices for CD146 and various markers of glomerular components such as endothelial cells (CD31/PECAM1) (A), podocytes (Nestin) (B), parietal (Claudin-1) (C) and mesangial cells (α -SMA) (D), showed that CD146 expression was induced mainly in glomerular endothelial cells, 7 days post NTS-GN. (Scale bar 20 μ m).

Figure 5. Generation of CD146-EC-del mice.

CD146-floxed mice were first crossed with the B6.Cg-Gt(ROSA)26Sortm6(CAG-ZsGreen1)Hze/J mice (Jackson laboratories) which express the fluorescent protein ZsGreen1 as a reporter for CRE-recombinase activity. Then, mice with endothelial cell-specific deletion of the CD146 gene were generated by further crossing CD146 flox-Zs Green animals with the Cdh5 (PAC)-CreERT2 mouse strain established by Ralf Adams²³ (A). The deletion of the CD146 gene was induced by I.P. injections of tamoxifen diluted in corn oil (10 mg/ml solution, 1 mg tamoxifen/injection) and administered on 3 consecutive days.

Immunofluorescence for CD146 confirmed its deletion from the vascular endothelium (B). In accordance, CD146 mRNA expression was also decreased in the kidneys of the CD146-EC-del mice (C). ***, $P < 0.01$; #, $P < 0.05$ (* NTS versus CTL, # KO NTS versus NTS, Scale bar 20 μ m).

Figure 6. Endothelial-specific deletion of CD146 protected renal function and structure after the induction of NTS-GN

In CD146-EC-del mice the NTS-induced increase of body weight (A), proteinuria (B) and BUN (C) were all blunted. Furthermore, Masson's trichrome staining in renal cortical slices (D) reveals that glomerular crescents (E) and tubular dilation (F) were highly reduced 15 days

post NTS injection. *, $P < 0.05$; **, $P < 0.01$ (NTS versus CD146-EC-del); #, $P < 0.05$; ##, $P < 0.01$ (NTS versus CTL-PBS), (Scale bar 50 μ m).

Figure 7. CD146 endothelial-specific deletion protected mice against both inflammation and renal interstitial fibrosis

F4/80 staining showed that monocyte infiltration was blunted in CD146-EC-del mice 15 days post NTS injection (A). In accordance, QPCR for VCAM-1 (B) and MCP-1 (C) showed that upregulation of those inflammatory markers was blunted. Furthermore, QPCR for TGF- β 1 (D) and Colla1 (E) showed similar results. *, $P < 0.05$; **, $P < 0.01$, #, $P < 0.05$; ##, $P < 0.01$. (* NTS versus CTL, # NTS versus CD146-EC-del, Scale bar 50 μ m).

Figure 8. Expression of CD146 transcripts in glomerular diseases in humans

Gene expression data obtained from isolated glomeruli of patients with diabetic nephropathies (DN), minimal change disease (MCD), IgA nephropathy (IgA), focal segmental glomerulosclerosis (FSGS), membranous glomerulonephritis (MGN), lupus nephritis (SLE), rapidly progressive glomerulonephritis (RPGN) and controls (pre-transplant allograft biopsies). CD146 expression was significantly and differentially regulated in glomerulopathies compared with controls.

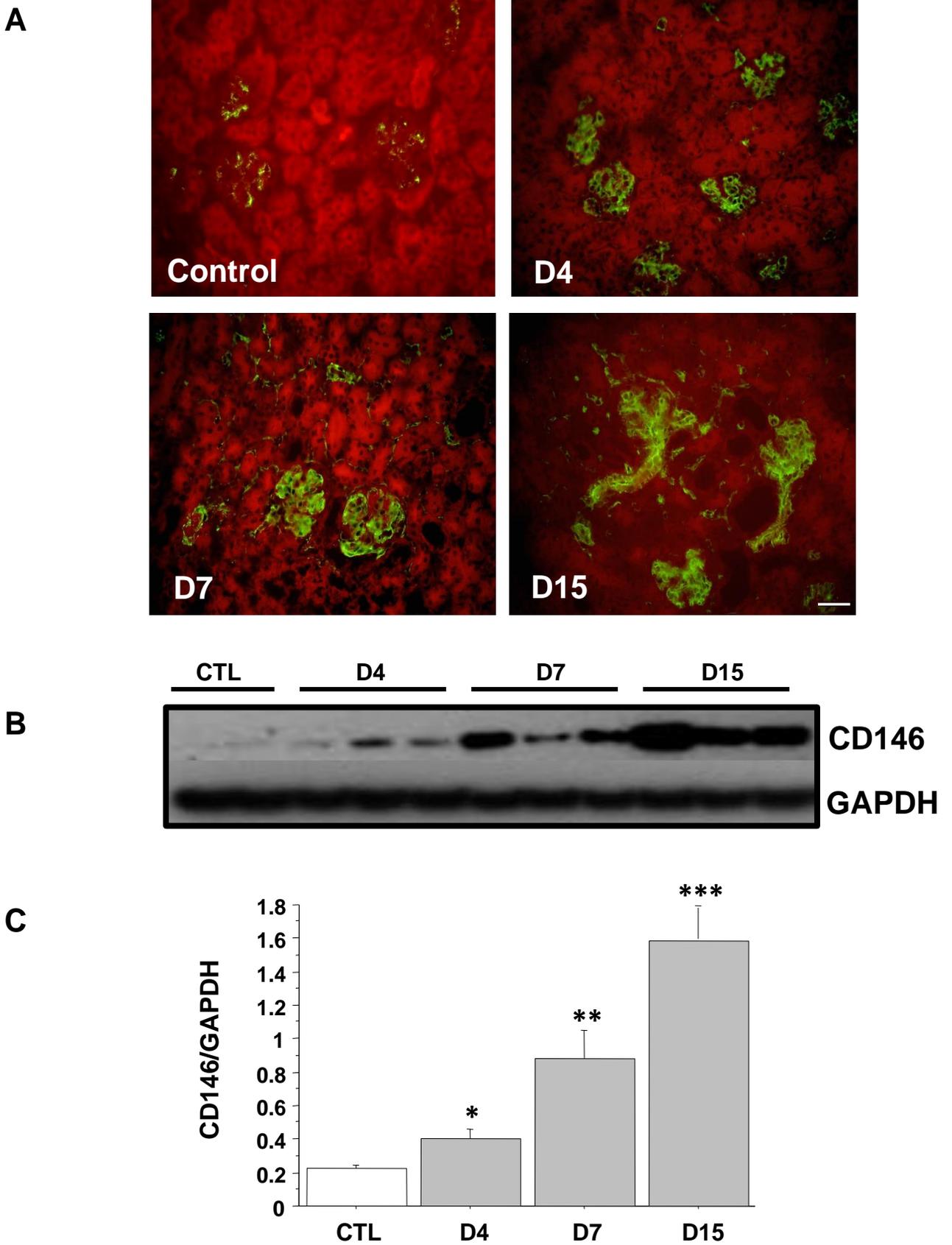


Figure 1

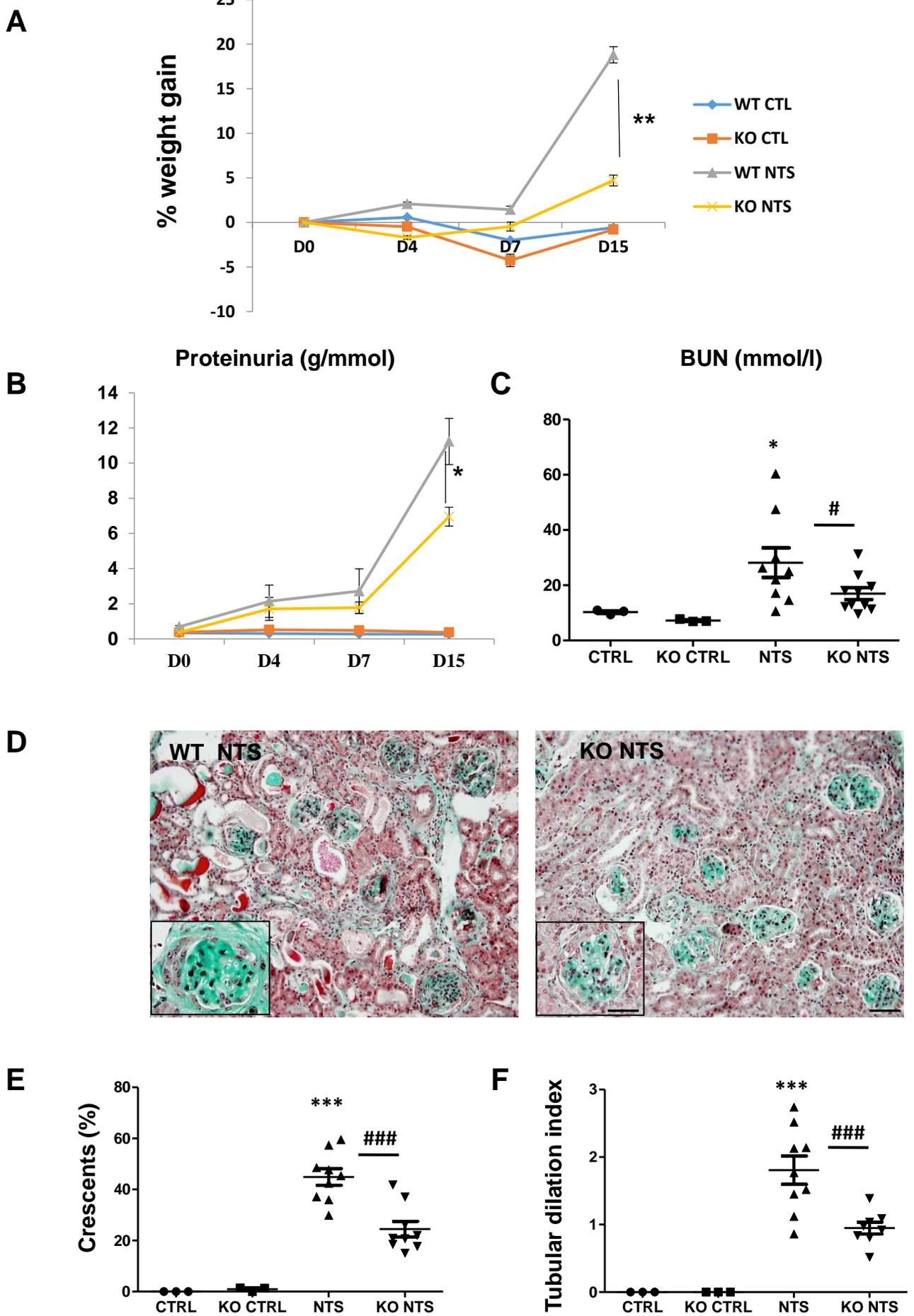


Figure 2

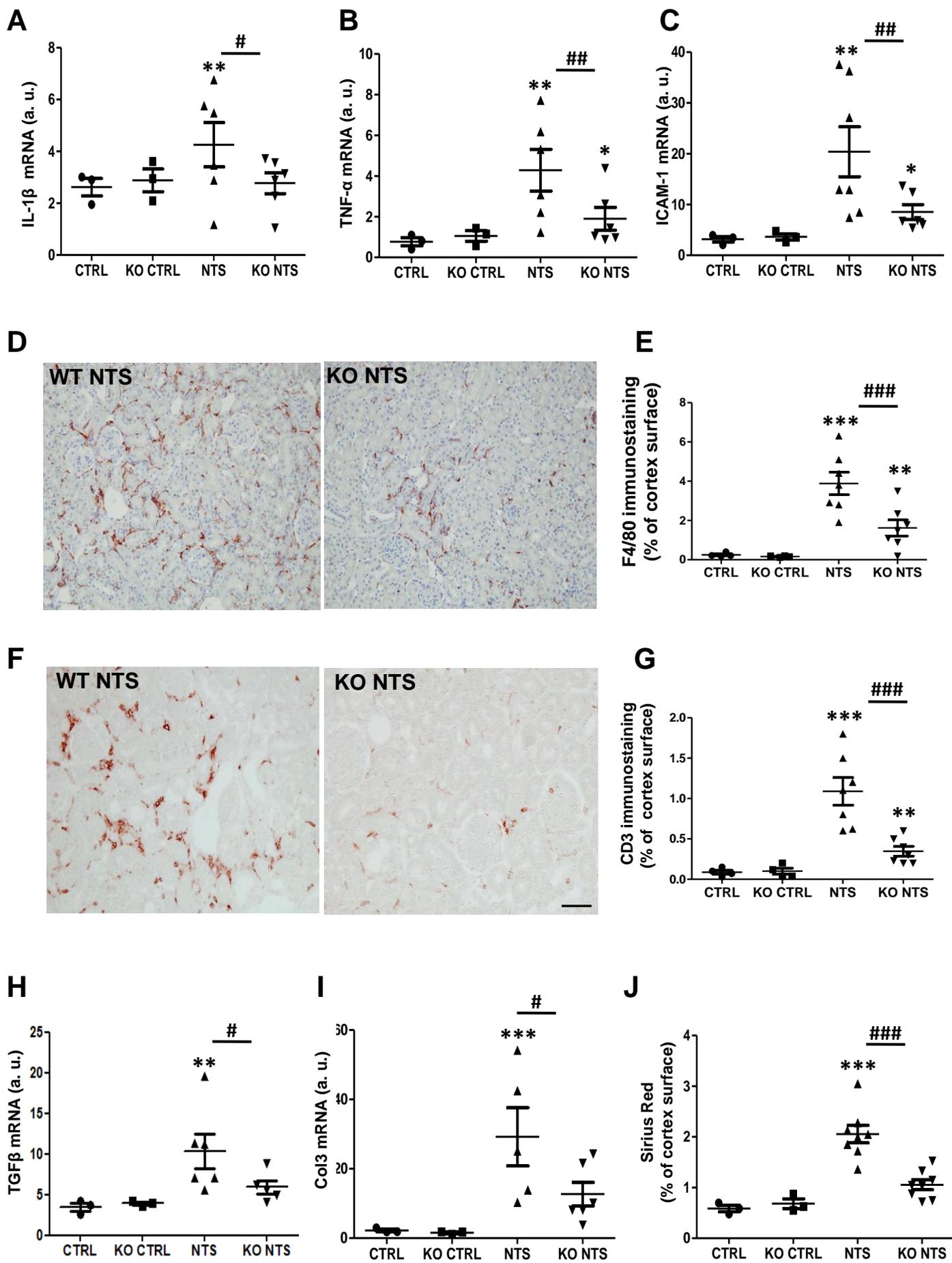


Figure 3

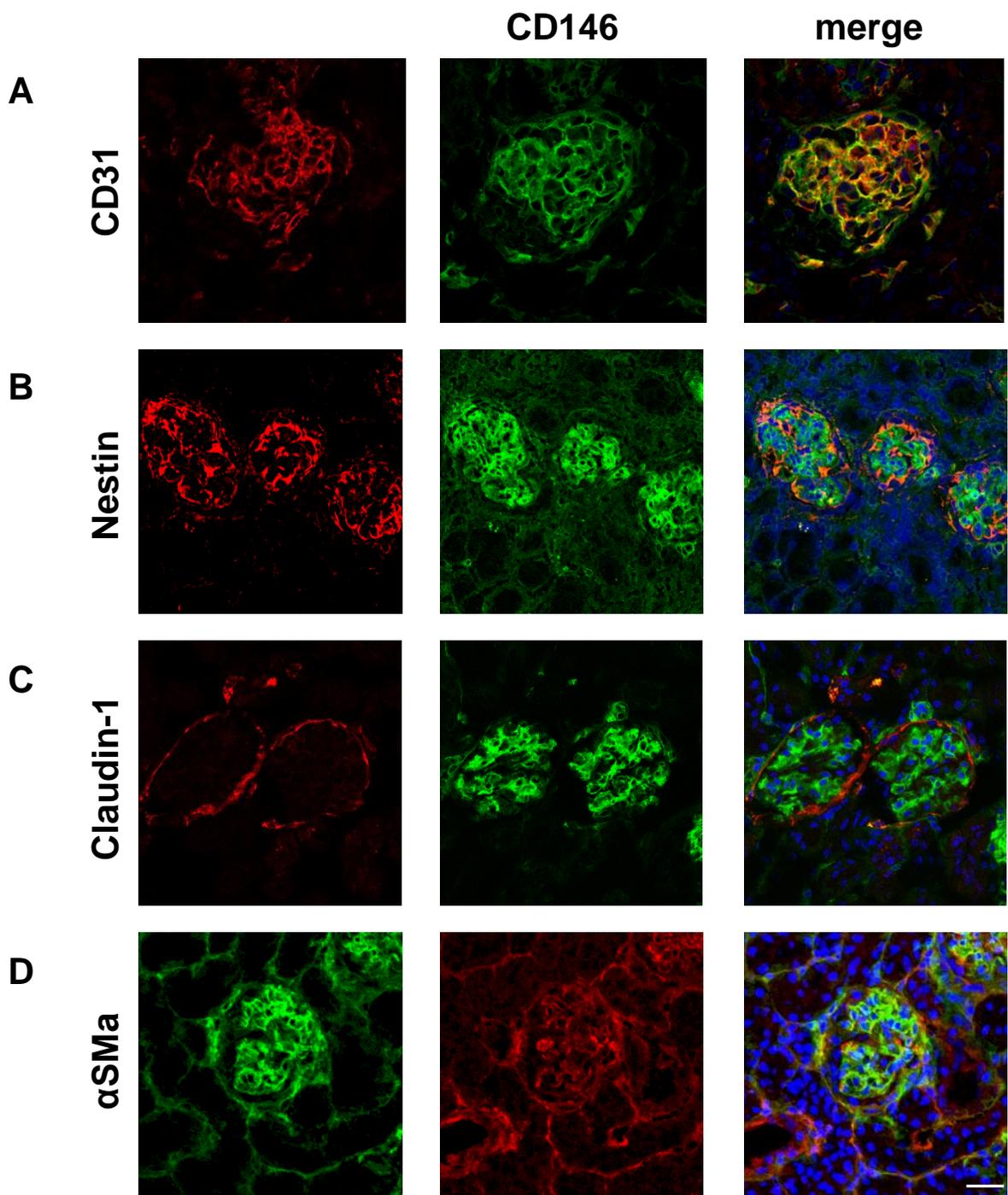


Figure 4

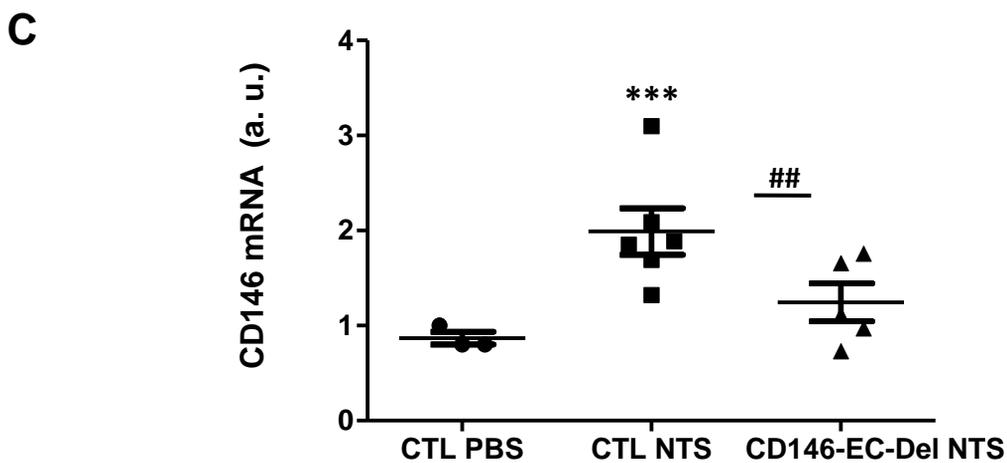
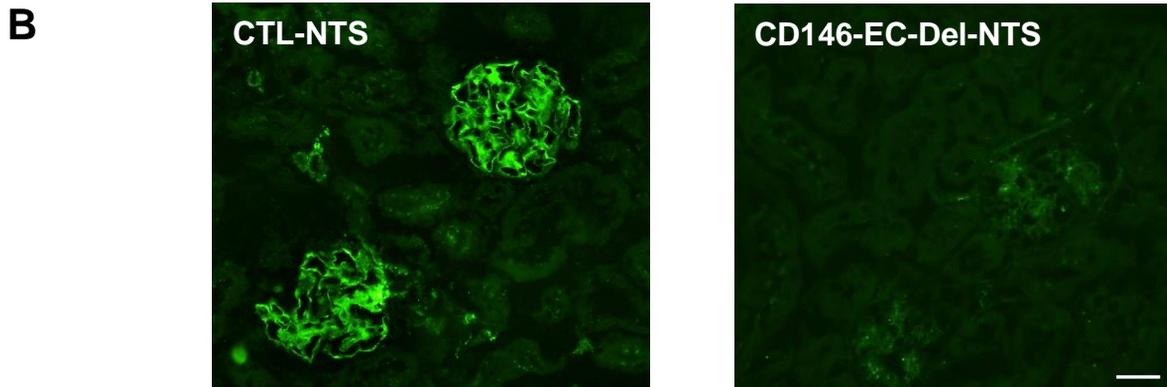
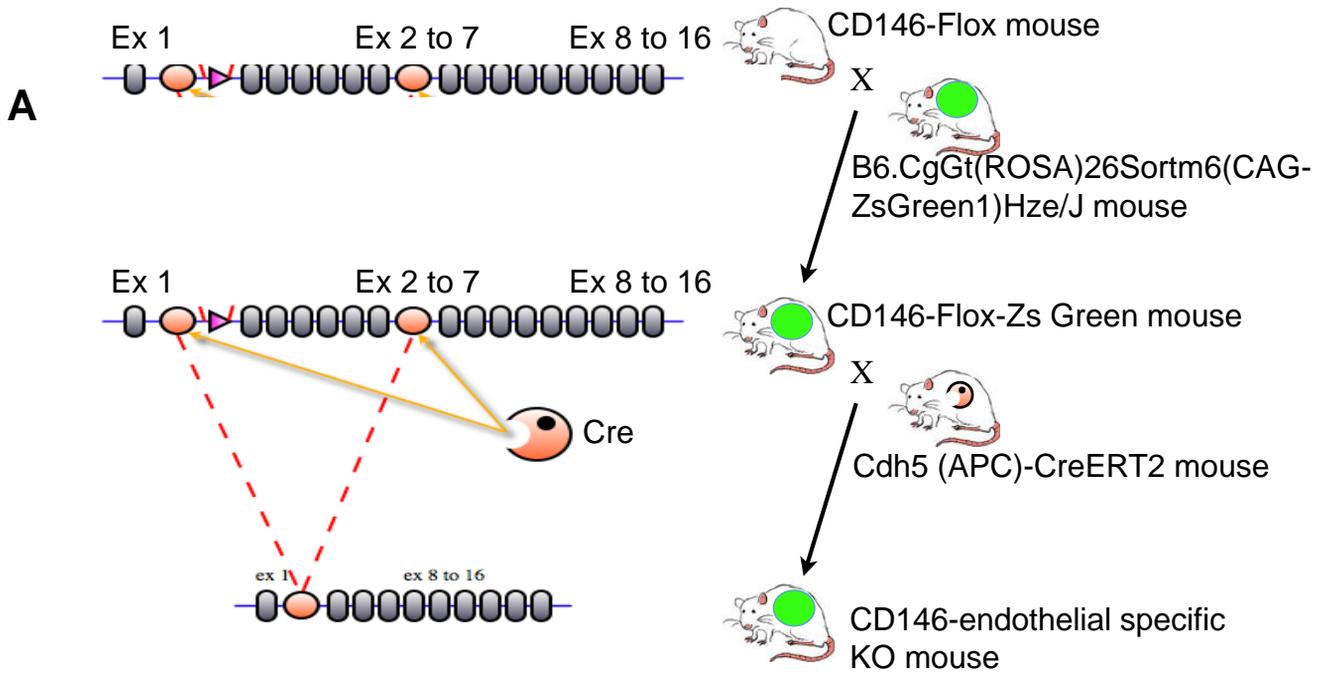


Figure 5

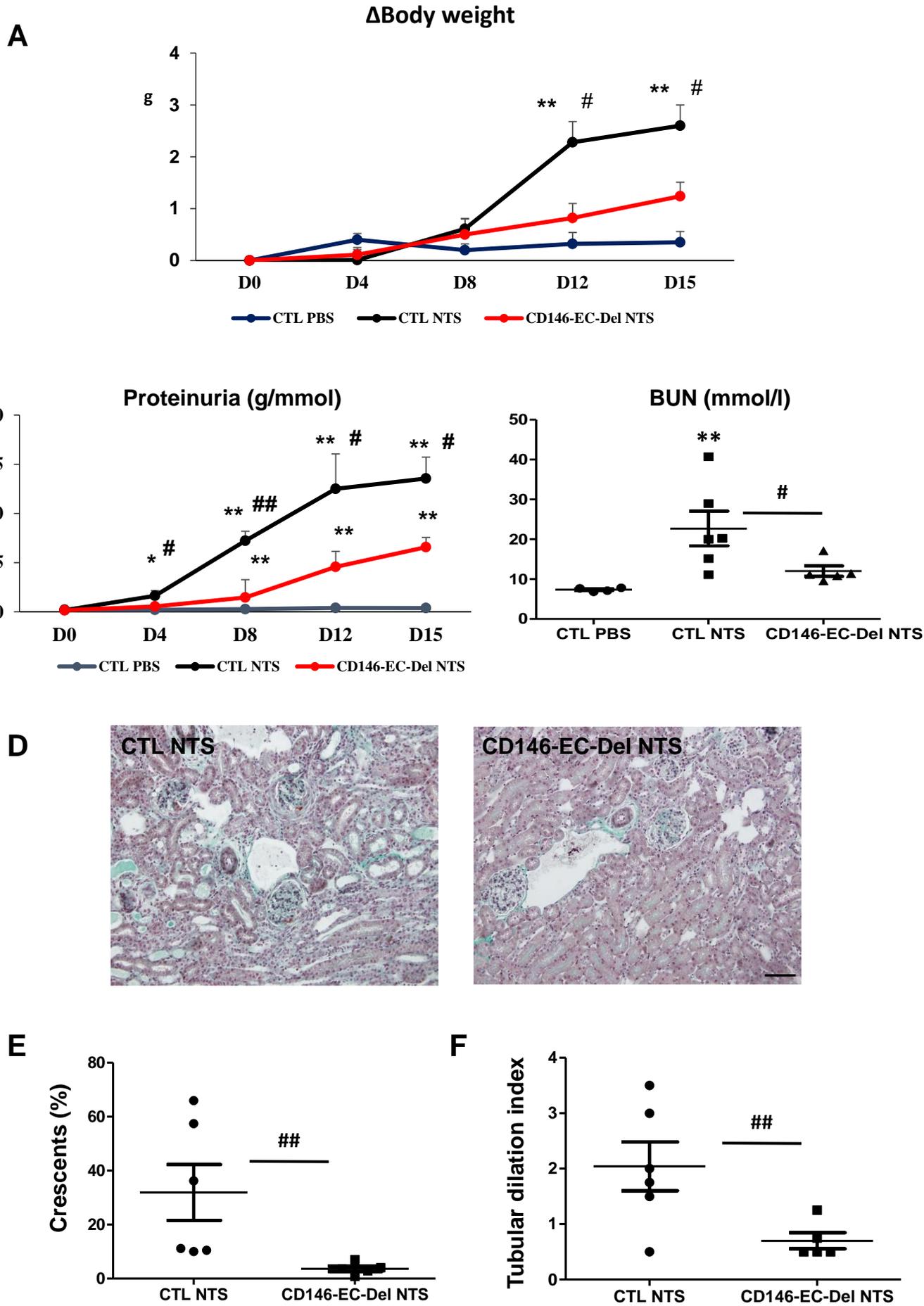
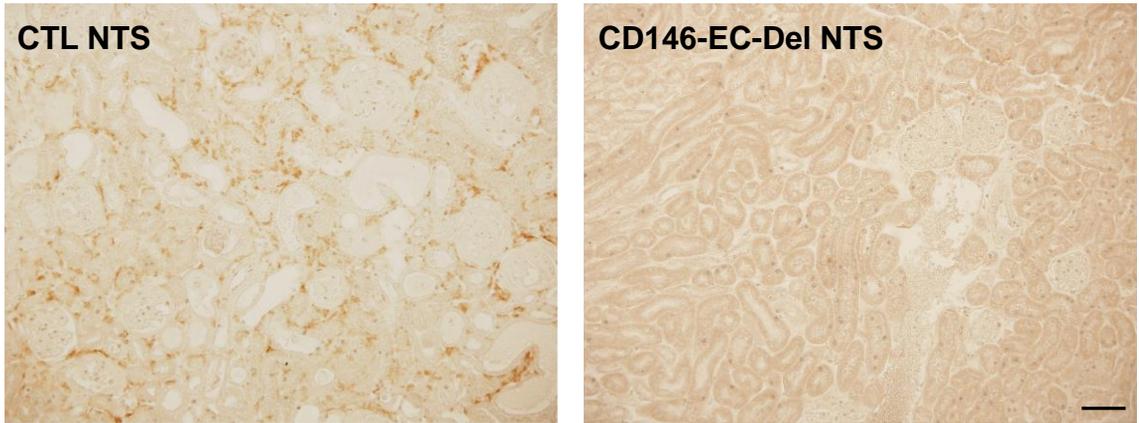
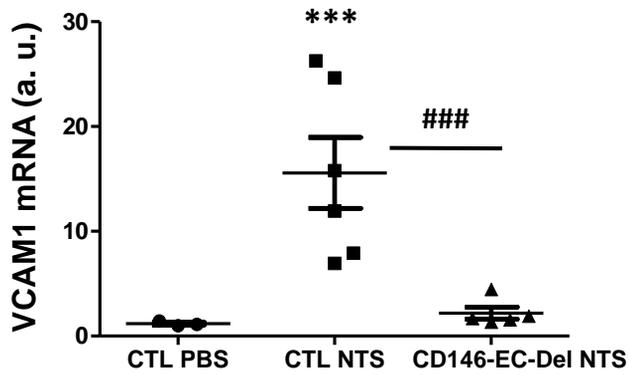
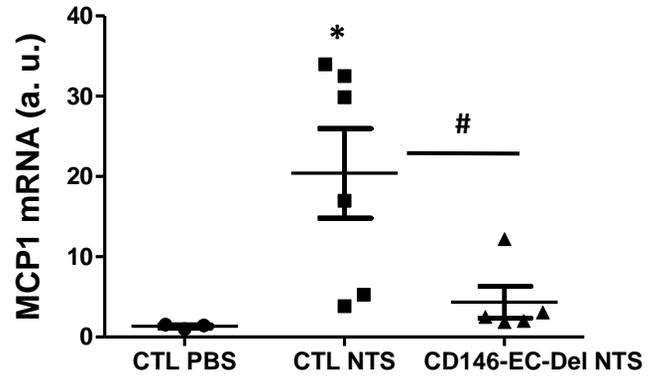
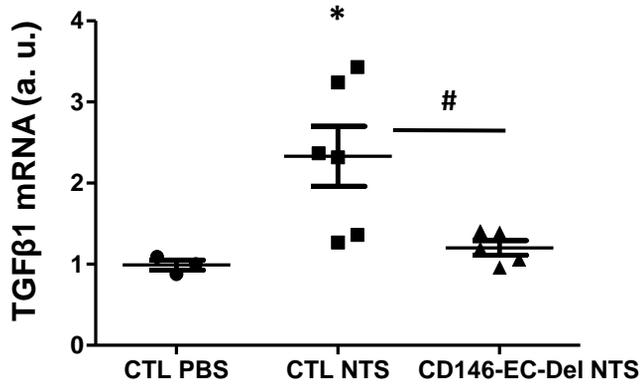
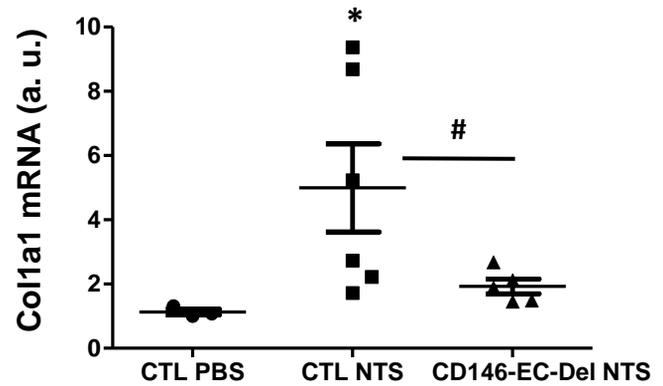


Figure 6

A**B****C****D****E****Figure 7**

Disease	<u>DN</u>	<u>MCD</u>	<u>IgA</u>	<u>FSGS</u>	<u>MGN</u>	<u>SLE</u>	<u>RPGN</u>
Average fold increase	1,91	1,28	1,29	1,64	1,31	1,37	1,45

Figure 8