Induction of leukocyte adhesion molecules and renal physiologically active molecules in *Porphyromonas gingivalis* lipopolysaccharide-induced diabetic nephropathy

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Research article

Keywords: P. gingivalis, LPS, diabetic nephropathy, VCAM-1, E-selectin, ACE2, FGF23

Posted Date: October 12th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-43555/v1

Version of Record: A version of this preprint was published at BMC Nephrology on January 6th, 2021. See the published version at https://doi.org/10.1186/s12882-020-02203-y.

Abstract

Background

We recently reported that the glomerular endothelium expresses toll-like receptor (TLR)2 and TLR4 in diabetic environments and established that the TLR2 ligand *Porphyromonas* (*P.*) *gingivalis* lipopolysaccharides (LPS) induces nephropathy in diabetic mice. It is thought that *P. gingivalis* LPS promotes the chronic inflammation with the overexpression of leukocyte adhesion molecules and renal-specific metabolic enzymes by the recognition of *P. gingivalis* LPS via TLR in the diabetic kidneys. The present study aims to examine the expression of leukocyte adhesion molecules and renal metabolic factors in mouse kidneys with periodontal pathogen *P. gingivalis* LPS-induced diabetic nephropathy that was recently established.

Methods

The immunohistochemical investigation was performed on mouse kidney with *P. gingivalis* LPS-induced diabetic nephropathy model with glomerulosclerosis in glomeruli.

Results

There were no vessels which expressed vascular cell adhesion molecule-1 (VCAM-1), E-selectin, or fibroblast growth factor (FGF) 23 in diabetic mice, or in healthy mice administered *P. gingivalis* LPS. However, in diabetic mouse kidneys with *P. gingivalis* LPS-induced nephropathy the expression of VCAM-1 and the accumulation of FGF23 were established in renal tubules and glomeruli, and the expression of E-selectin was established in renal parenchyma and glomeruli. The angiotensin-converting enzyme 2 (ACE2) was detected in the proximal tubules but not in other regions including not in distal tubules of diabetic mice without LPS, and not in healthy mice administered *P. gingivalis* LPS. In diabetic mouse kidneys with *P. gingivalis* LPS-induced nephropathy ACE2 was detected both in renal tubules as well as in glomeruli. The macrophage-1 (Mac-1) and podoplanin-positive cells increased in the renal parenchyma with diabetic condition and there was accumulation in *P. gingivalis* LPS-induced diabetic nephropathy. As the expression of VCAM-1 and E-selectin is upregulated in glomeruli, tubules, and intertubular capillaries, it is thought that the inflammatory infiltration of the monocyte-macrophage lineage promoted in kidneys with *P. gingivalis* LPS-induced the diabetic nephropathy.

Conclusions

P. gingivalis LPS may progressively accelerate the development of the renal inflammatory environment in LPS-accumulated glomeruli with the macrophage infiltration via the renal expression of VCAM-1 and E-

selectin, and with ACE2 overexpression and FGF23 accumulation. Periodontitis may be a critical factor in the progress of nephropathy in diabetic patients.

Background

Diabetic nephropathy is a serious complication in diabetes mellitus, caused by glomerulosclerosis, with renal failure arising from dysfunction of glomerular capillaries. Critical factors in diabetic nephropathy have been thought to be advanced glycation end products (AGE) and hydroxyl radicals which induce oxidative stress and the production of various cytokines through the recognition of AGE in a hyperglycemic environment (1–3). However, the factors which cause individual differences in the development of nephropathy in diabetic patients are not well elucidated. The renal metabolic recognition of AGE by the toll-like receptor (TLR) has been suggested as one candidate for the occurrence of diabetic nephropathy. The TLR is a sensor for bacterial components like lipopolysaccharide (LPS) and high levels of expression of TLR2 and TLR4 has been reported in blood cells of diabetic nephropathy patients (4–8). It has been established that the TLR ligand engagement induces the production of inflammatory cytokines as well as leukocyte adhesion molecules, which activate renal inflammation causing glomerulosclerosis (9–14). There are also reports that the periodontal pathogen *Porphyromonas* (*P.*) *gingivalis* becomes a risk factor in cerebrovascular diseases and atherosclerosis (15, 16). Large amounts of lipopolysaccharides (LPS) are produced in the outer membrane of *P. gingivalis* and act as a periodontal pathogen leading to periodontal tissue destruction as well as it is a risk factor in cardiovascular disorders (17, 18). There are reports that *P. gingivalis* LPS is also recognized by host defense systems via TLR in LPS-accumulated organs similar to renal glomeruli and that is a cause of inflammation with the induction of leukocyte adhesion molecule expression (19, 20).

We recently reported that the glomerular endothelium of streptozotocin (STZ)-induced diabetic mice expresses TLR2 and TLR4, and that all diabetic mice subjected to repeated *P. gingivalis* LPS administrations euthanized within the health survival period of all of diabetic mice not administered LPS and LPS-administered non-diabetic mice (21–23). In diabetic mice *P. gingivalis* LPS promoted the production of urinary protein and glomerulosclerosis with the accumulation of type 1 collagen and inflammatory cytokines in glomeruli. Further, the progress of diabetic nephropathy was suppressed in TLR blockage Eritoran-administered diabetic mice, where the albuminuria and glomerular hypertrophy were less pronounced than in diabetic mice not administered Eritoran (23). Severe periodontitis causes bacteremia and microorganisms of the oral cavity to enter the kidneys through the systemic circulation, and it appears that *P. gingivalis* LPS accumulated in glomeruli induce chronic renal inflammation as a result of the inflammatory leukocyte migration.

The expression of leukocyte adhesion molecules and renal metabolic enzymes has been reported in the kidneys of diabetic and other renal autoimmune diseases, such as erythematosus and IgA nephropathy. Vascular cell adhesion molecule-1 (VCAM-1) is expressed in the renal proximal tubules in renal immune diseases, with acute renal allograft rejection (tubulitis) as a prominent example (24–28); E-selectin is expressed as present on intertubular capillaries in glomerulonephritis (29, 30). Osteocyte-derived hormone

fibroblast growth factor (FGF) 23 acts as a key regulator of the renal phosphate metabolism which reduces renal phosphate uptake. In chronic kidney disease serum FGF23 levels are massively elevated (31–33). Angiotensin-converting enzyme 2 (ACE2), a monocarboxypeptidase that cleaves a typical renal pressor hormone angiotensin (Ang) into Ang 1–7 and degrades Ang I to Ang 1–9, is expressed in proximal tubular epithelial cells, and increases in diabetic kidney and hypertensive renal diseases (34, 35). Considering these findings, the overexpression of leukocyte adhesion molecules, renal-specific metabolic enzymes and cytokines may be induced in diabetic kidneys by the recognition of *P. gingivalis* via host defense systems which promote the chronic inflammation. The present study aims to examine the expression of leukocyte adhesion molecules and renal-specific metabolic factors in the mouse kidney with *P. gingivalis* LPS-induced diabetic nephropathy.

Methods

Animals

The animal study reported here was conducted to investigate the expression of leukocyte adhesion molecules and renal molecules, physiologically active, in *P. gingivalis* LPS-induced diabetic nephropathy. The animal use protocol of the experiments was approved by the Animal Experiment Committee of Fukuoka Dental College (No. 19010). The study in the present report used 4 groups (non-treated control, LPS-administered non-diabetic control, diabetic control, LPS-administered diabetic experimental) with 6 mice in each group. All experimental specimens were harvested from euthanized mice and the experimental protocol followed ARRIVE guidelines. The 4-week-old male mice of the ICR closed line were purchased from a commercial vendor (Kyudo, Fukuoka, Japan). Animal upkeep and experiments were performed in the Fukuoka Dental College Animal Center following the conditions and procedures described elsewhere (23): normal feeding in a 100% controlled atmosphere which had passed an examination for bacteria in a room where the temperature and humidity were completely controlled. The health status and humane endpoints of the mice were assessed daily and mice which had lost the ability to ambulate and to access food or water were euthanized by induction anesthesia followed by cervical dislocation and intraperitoneal injections with sodium pentobarbital. The experimental drug administration to the mice were performed under anesthesia according to the method described elsewhere (23). All mice in the experimental groups were maintained till euthanization.

The STZ-injected ICR mice were used as a diabetic model and STZ and *P. gingivalis* LPS injected ICR mice were used as a diabetic nephropathy model (23). Following procedures described elsewhere (23). Briefly, mice were given a single intraperitoneal injection of STZ (Sigma-Aldrich, St. Louis, MO) under inhalation anesthesia and the blood glucose of mice were checked by a Glutest Sensor (Sanwa Kagaku Kenkyusyo CO., LTD., Nagoya, Japan) once a week after the injection. The STZ-injected ICR mice with blood glucose above 600 mg/dl were used as STZ-induced diabetic mice. After establishment as diabetic, *P. gingivalis* LPS (Invivogen, San Diego, California, USA) was consecutively administered in the buccal mucosa of the mice once a week to the STZ-induced diabetic mice for four months under inhalation anesthesia and simultaneously the urine was analyzed for sugar, protein, and bleeding by urine reagent

strips (Uriace, Terumo Corporation, Tokyo, Japan), and the blood was collected from the tail vein and analyzed for blood urea nitrogen (BUN) and creatinine (CRE) by Kyudo Co., LTD (Tosu, Japan). In summary, the present study was performed with 24 ICR mice divided into four groups (n = 6 per group) which is the smallest unit to achieve reliable statistical processing: groups of mice without any treatment (healthy control), with only LPS treatment (experimental infection model), with only STZ treatment (experimental diabetes model), and with LPS and STZ treatment (experimental diabetic nephropathy model). All mice were euthanized at the end of the designated period of the experiments, and tissue from the mice was collected.

Immunohistochemistry

The present study performed the investigation by immunohistochemistry following the method described elsewhere (23). Briefly, frozen mouse kidney tissue sections were fixed in 100% methanol and treated with primary antibodies: hamster monoclonal anti-mouse podoplanin clone 8.8.1 (BioLegend Inc., San Diego, CA) as a podocyte marker, rat monoclonal anti-mouse VCAM-1 (R&D Systems Inc., Minneapolis, MN), rat monoclonal anti-mouse E-selectin (R&D Systems), rabbit polyclonal anti-mouse FDF23 (Abcam plc., Cambridge, UK), rabbit polyclonal AGE2 (Abcam), and rat polyclonal anti-mouse CD11b clone M1/70 (macrophage-1, Mac-1a; BD Biosciences, San Jose, CA). After the treatment the sections were exposed with Alexa Fluor 488 or 568-conjugated goat anti-hamster, goat anti-rabbit, or goat anti-rat IgGs (Probes Invitrogen Com., Eugene, OR). The immunostained sections were examined by microscope digital camera systems with a CFI Plan Apo Lambda lens series and DS-Ri2/Qi2 (Nikon Corp., Tokyo, Japan). All experiments were replicated several times (5–10) with different sections.

Measurements of the immunostained areas of tissue sections

Immunostained areas were measured (10/section) in microscopic images at 200x magnification by ImageJ (National Institutes of Health, Bethesda, MD). The relative reaction amounts of primary antibodies were expressed by arbitrary units of the ratio: positive area of antibody reactions in the sections of diabetic mice and of LPS-administered diabetic mice/positive area of antibodies in the sections of LPS-administered non-diabetic mice.

Statistics

All experiments were repeated five times, and data are expressed as the mean + SD. The statistical significance of differences ($P < 0.01$) was determined by one-way ANOVA and the two-tailed unpaired Student's *t* test with STATVIEW 4.51 software (Abacus concepts, Calabasas, CA, USA). Mean values were calculated with standard deviation (STDEV). The corresponding author is fully aware of the group allocation at the different stages of the experiments. The data analysis and assessments were performed by all co-authors.

Results

Immunostaining of leukocyte adhesion molecules and leukocytes

Immunoreaction products of anti-E-selectin and VCAM-1 were not detected in non-diabetic mouse kidneys with *P. gingivalis* LPS administration or in diabetic mouse kidney without the LPS administration. In diabetic mouse kidneys with *P. gingivalis* LPS-induced nephropathy immunoreaction products of anti-E-selectin were identified in the whole renal parenchyma and in glomeruli, while immunoreaction products of anti-VCAM-1 were identified in renal tubules and also located in the glomeruli. The glomeruli were discriminated by immunoreaction with anti-podoplanin to glomerular epithelial cells. Mac-1-positive cells were identified in the whole renal parenchyma in non-diabetic mouse kidneys with *P. gingivalis* LPS administration and the number of Mac-1-positive cells increased in the diabetic mouse kidneys. Podoplanin-positive cells were identified in diabetic mouse kidneys.

Immunostaining of renal physiologically active molecules

Immunoreaction products of anti-FGF23 were not detected in non-diabetic mouse kidneys with *P. gingivalis* LPS administration or in diabetic mouse kidneys without the LPS administration. In diabetic mouse kidneys with *P. gingivalis* LPS-induced nephropathy immunoreaction products of anti-FGF23 were identified in the whole of the renal parenchyma and in glomeruli. Immunoreaction products of anti-ACE2 were only observed in proximal tubular cells with brush borders, but in no other region including distal tubular cells reacted with anti-ACE2 in non-diabetic mouse kidneys with *P. gingivalis* LPS administration as well as diabetic mouse kidneys without the LPS administration. In diabetic mouse kidneys with *P. gingivalis* LPS-induced nephropathy immunoreaction products of anti-ACE2 were identified both in tubules as well as in glomeruli.

Quantitative analysis for immunostaining of renal physiologically active molecules, leukocyte adhesion molecules, and leukocytes

Expression of E-selectin, VCAM-1, and FGF23 were not detected in non-diabetic mouse kidneys with *P. gingivalis* LPS administration or in diabetic mouse kidneys without LPS administration, however reaction products were detected in diabetic mouse kidneys with *P. gingivalis* LPS-induced nephropathy. Reaction products of anti-ACE2 were detected in non-diabetic mouse kidneys with *P. gingivalis* LPS administration and in diabetic mouse kidneys without the LPS administration, while the reaction products increased in diabetic mouse kidneys with *P. gingivalis* LPS-induced nephropathy. Mac-1 or podoplanin-positive macrophages were detected in non-diabetic mouse kidneys with *P. gingivalis* LPS administration. The number of cells was larger in diabetic mouse kidneys without the LPS administration than in non-diabetic mouse kidneys with *P. gingivalis* LPS, and also larger in diabetic mouse kidneys with the LPS administration than in the diabetic mouse kidneys with no LPS administration.

Discussion

Expression of leukocyte adhesion molecules in diabetic mouse kidney with *P. gingivalis* LPS-induced nephropathy

An immunoglobulin superfamily member VCAM-1 binds to the integrins very late antigen-4/α4β7 on lymphocytes and monocytes, and provides leukocyte migration from the blood stream into tissue. The VCAM-1 expression is up-regulated in renal proximal tubules in several renal chronic diseases, and the renal proximal tubule is targeted in the renal infiltration of T cells and monocytes which are rarely found in normal kidneys (24–27). A member of selectin family E-selectin which expresses at the early inflammatory stage binds to sialylated glycoproteins on leukocytes and promotes the leukocyte weak adhesion, rolling on vessel walls. The E-selectin is present on intertubular capillaries in glomerulonephritis but never in renal tubules (28–30). In this study there were no vessels expressing VCAM-1 or E-selectin in diabetic mice, and in healthy mice administered *P. gingivalis* LPS only in amounts confirmed to have no effect on the health condition in mice in our previous study (Fig. 1,2,6). However, in diabetic mouse kidneys with *P. gingivalis* LPS-induced nephropathy the expression of VCAM-1 was identified in the renal tubules and glomeruli, and the expression of E-selectin was identified in the renal parenchyma and glomeruli (Fig. 1,2,6). These results suggest that *P. gingivalis* LPS give rise to the inflammatory conditions in renal intertubular and glomerular capillaries. Since renal proximal tubules express VCAM-1 in renal diseases, it is thought the tubulitis occurrs in *P. gingivalis* LPS-induced nephropathy (24–27). It has also been reported that the E-selectin is not expressed in tubules but expressed in renal intertubular capillaries in renal diseases, and that soluble E-selectin plays a role to promote glomerulonephritis (28–30). It appears that the immunoreaction of anti-E-selectin in diabetic kidneys with *P. gingivalis* LPS-induced nephropathy can be ascribed to a soluble E-selectin diffused in renal parenchyma or to E-selectin expression of intertubular capillaries.

Expression of renal physiologically active molecules in diabetic mouse kidneys with *P. gingivalis* LPS-induced nephropathy

The FGF23 directly targets proximal tubules to increase phosphate excretion by downregulating the cell surface expression of the sodium-dependent phosphate transporters in the proximal tubule. The FGF23 lowers serum phosphorus concentrations by the suppression of phosphorus reabsorption in proximal tubules and by active vitamin D reduction through 1α-hydroxylase suppression (31–33). In this study FGF23 was not detected in diabetic mice or in healthy mice administered *P. gingivalis* LPS (Fig. 3,6). However, in diabetic mouse kidneys with *P. gingivalis* LPS-induced nephropathy FGF23 was immunohistochemically detectable in renal tubules and in glomeruli (Fig. 3,6), suggesting that FGF23 accumulates and that excessive phosphate uptake is promoted in inflamed renal tubules of *P. gingivalis* LPS-induced diabetic nephropathy. It is thought that periodontitis may induce the increase in serum phosphorus concentration by the renal FGF23 accumulation, which contributes to the occurrence of cardiovascular failure in diabetic patients. The ACE2 is locally expressed in the brush border of proximal tubular epithelial cells and the expression amounts increase in diabetic kidneys, and in hypertensive renal diseases. In other organs ACE2 mRNA is expressed in cardiovascular and gastrointestinal tissues, and present at lower levels in many tissues. The ACE2 protein is detectable in blood vessels, upper airways,

and lungs, and the expression pattern in humans is similar to that in mice (34, 35). In this study ACE2 were only immunohistochemically detected in the proximal tubular cells and not in other regions including the distal tubules in diabetic mice without the LPS administration, and also not in healthy mice administered *P. gingivalis* LPS (Fig. 4,6). However, in diabetic mouse kidneys with *P. gingivalis* LPS-induced nephropathy ACE2 was immunohistochemically detected both in renal tubules as well as in glomeruli (Fig. 4,6), suggesting that renovascular hypertension based on the glomerular ACE2 overexpression may be promoted in inflamed glomeruli of *P. gingivalis* LPS-induced diabetic nephropathy.

Distribution of macrophages in diabetic mouse kidneys with P. gingivalis **LPS-induced nephropathy**

Macrophages and T17 express podoplanin, and activate platelet CLEC-2 under inflammatory conditions as well as they aggregate activated platelets. Macrophage antigen-1 (Mac-1) is a heterodimer integrin composed of the αM(CD11b) and β2(CD18) subunits, abundantly expressed on monocyte/macrophages. The Mac-1 is critical for the adhesion and migration into the extracellular matrix (36–39). In this study Mac-1 positive cells were detected in healthy mouse kidneys with *P. gingivalis* LPS administration (Fig. 5,6) at the same level as in healthy mice without LPS administration (not shown). The number of Mac-1 and podoplanin-positive cells increased in the renal parenchyma with diabetic conditions and accumulated in significant numbers in *P. gingivalis* LPS-induced diabetic nephropathy (Fig. 5,6). Since the expression of VCAM-1 and E-selectin is significantly upregulated in glomeruli, tubules and intertubular capillaries with *P. gingivalis* LPS-induced diabetic nephropathy (Fig. 1,2,6), it is thought that the inflammatory infiltration of monocyte-macrophage lineage in the kidney promoted with *P. gingivalis* LPS under the diabetic condition. Considering that the glomerular ACE2 overexpression and renal FGF23 accumulation promote the renovascular hypertension, it is thought that *P. gingivalis* LPS progressively accelerates the development of an inflammatory environment in renal proximal tubules, glomeruli, and intertubular capillaries by the LPS recognition via TLR in the diabetic kidneys. Periodontitis may be a critical factor in the progress of nephropathy in diabetic patients.

Conclusion

It is established that large amounts of LPS produced in *P. gingivalis* act as a risk factor in cardiovascular disorders. Severe periodontitis causes bacteremia and microorganisms of the oral cavity enter kidneys through the systemic circulation. It has been suggested that AGE induces TLR expression in several somatic cells and that AGE is recognized by TLR. We recently reported that the glomerular endothelium of diabetic mice expresses TLR2 and TLR4, and that the TLR2-legand *P. gingivalis* LPS causes glomerulosclerosis in diabetic mice with the accumulation of type 1 collagen and inflammatory cytokines in glomeruli (21–23). Considering the present study, it may be concluded that *P. gingivalis* LPS progressively accelerates the development of an inflammatory environment in LPS-accumulated glomeruli with the inflammatory infiltration of Mac-1/podoplanin positive macrophages via glomerular overexpression of VCAM-1 and E-selectin, and both simultaneously ACE2 overexpression and bone-derived FGF23 accumulation promote tubulitis and hypertensive renal diseases (Fig. 7).

Abbreviations

AGE
advanced glycation end products
TLR
toll-like receptor
LPS
lipopolysaccharide
P. gingivalis
Porphyromonas gingivalis
STZ
streptozotocin
VCAM-1
Vascular cell adhesion molecule-1
FGF
fibroblast growth factor 23
ACE2
Angiotensin-converting enzyme 2
Ang
angiotensin
Mac-1
macrophage-1

Declarations

*Ethics approval

All methods were performed in accordance with the relevant guidelines and regulations. The animal experimental procedures were prepared following the ARRIVE guidelines. The protocol of the experiments for animal use was approved by the Animal Experiment Committee of Fukuoka Dental College (No. 19010).

*Consent to participate

Not applicable.

*Consent to publish

Not applicable.

*Availability of data and materials

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

***Competing interests**

The authors state that there are no potential conflicts of interest. The organizations funding the research had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

***Funding**

This work was supported in part by Grant-in-Aid for Scientific Research (B) 18H03015 (principal investigator: Sawa, Y) and Grant-in-Aid for Early-Career Scientists 18K17305 (principal investigator: Kajiwara, K) from the Japan Society for the Promotion of Science. None of the funders participated in or influenced the design of the study or collection, analysis and interpretation of data or writing of the manuscript.

***Author's contributions:** YS conceived this study; YS designed the study and wrote the manuscript; KK, YS, TF, and ST undertook statistical analyses; KK, YS, TF, and ST acquired data, edited and approved the manuscript.

***Acknowledgements:** Not Applicable.

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Figures

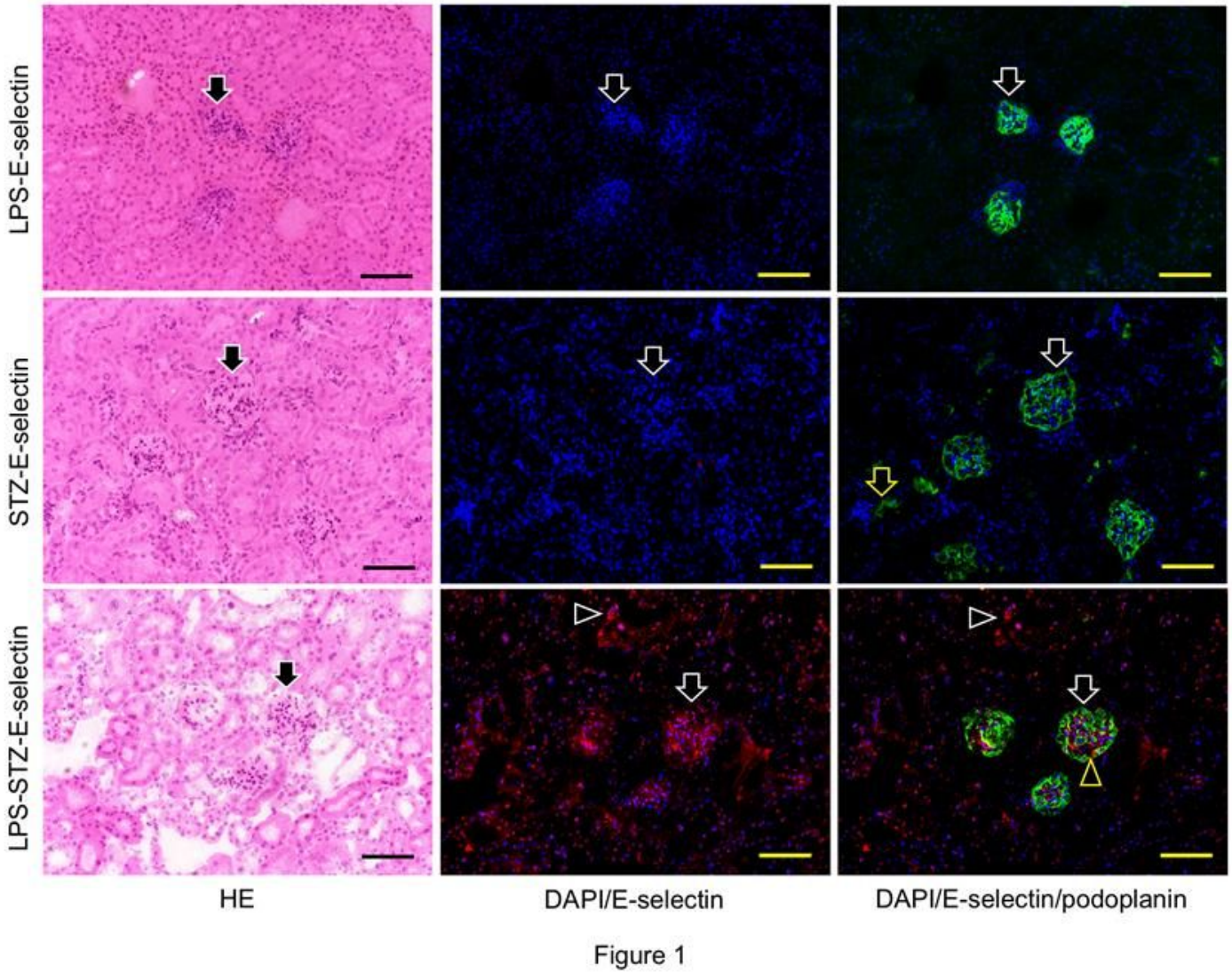

Figure 1

Immunostaining of E-selectin in STZ-induced type 1 diabetic mouse kidney with P. gingivalis LPS-induced nephropathy. HE staining (left column); immunostaining for E-selectin (red)(center column); and merged immunostaining for E-selectin and podoplanin (green)(right column), with DAPI staining of nuclei (blue). The glomerular epithelial cells were immunostained by anti-podoplanin to discriminate glomeruli (arrows). Reaction products of anti-E-selectin were not identified in non-diabetic mouse kidneys with P. gingivalis LPS administration (LPS, top row) or in diabetic mouse kidneys without LPS administration (STZ, middle row), however, the reaction products were identified in the whole renal parenchyma (arrowheads) and in glomeruli (yellow arrowheads) in diabetic mouse kidneys with P. gingivalis LPS-induced nephropathy (LPS-STZ, bottom row). Podoplanin-positive macrophages were identified in diabetic mouse kidneys (yellow arrows). Bars: 100 μm.

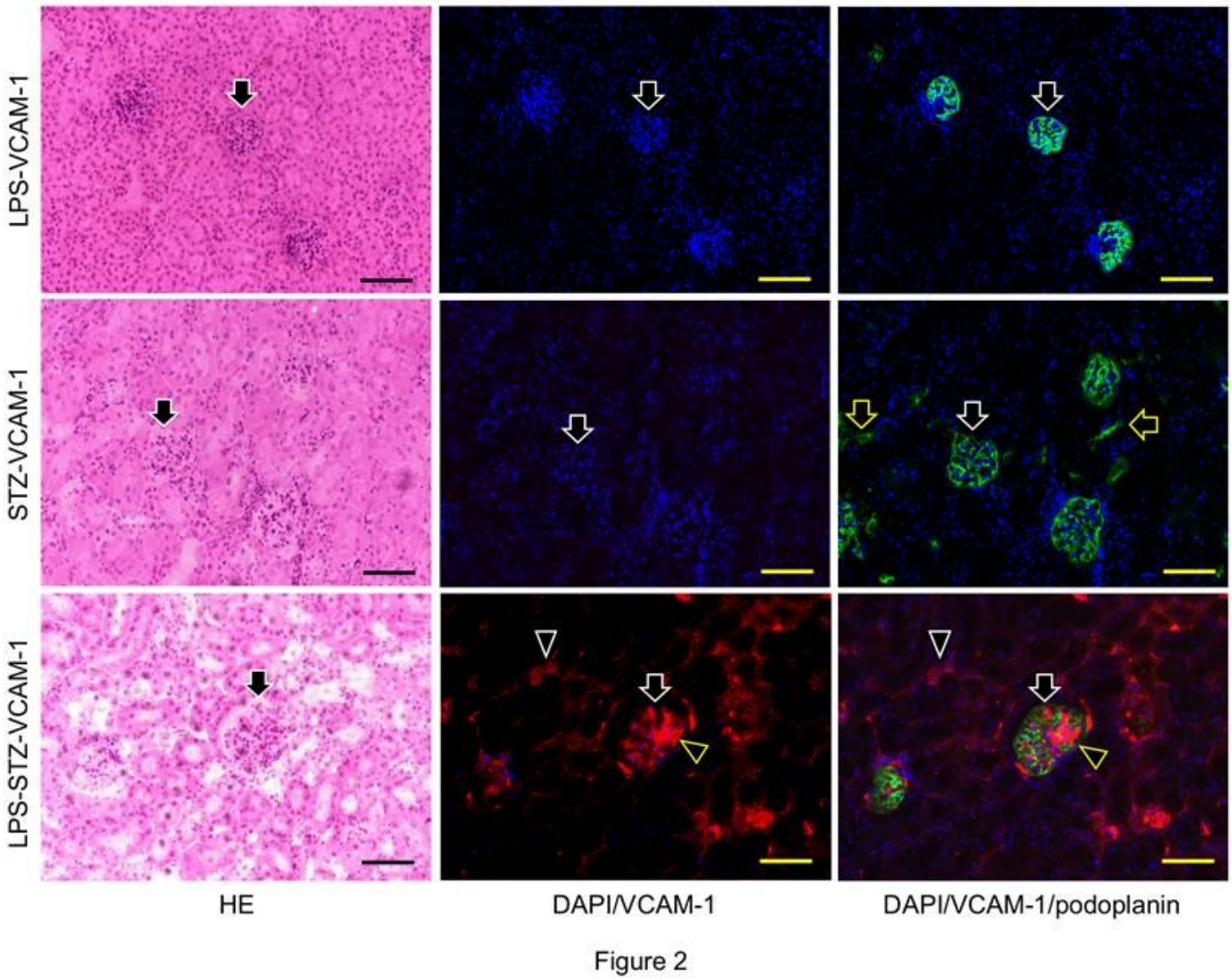

Figure 2

Figure 2

Immunostaining of VCAM-1 in STZ-induced type 1 diabetic mouse kidney with P. gingivalis LPS-induced nephropathy. HE staining (left column); immunostaining for VCAM-1 (red)(center column); and merged immunostaining for VCAM-1 and podoplanin (green)(right column), with DAPI staining of nuclei (blue). The glomerular epithelial cells were immunostained by anti-podoplanin to be able to discriminate glomeruli (arrows). Reaction products of anti-VCAM-1 were not identified in non-diabetic mouse kidneys with P. gingivalis LPS administration (LPS, top row) or in diabetic mouse kidneys without the LPS administration (STZ, middle row), however, reaction products were identified in renal tubules (arrowheads) and in glomeruli (yellow arrowheads) in diabetic mouse kidneys with P. gingivalis LPS-induced nephropathy (LPS-STZ, bottom row). Podoplanin-positive macrophages were identified in diabetic mouse kidneys (yellow arrow). Bars: 100 μm.

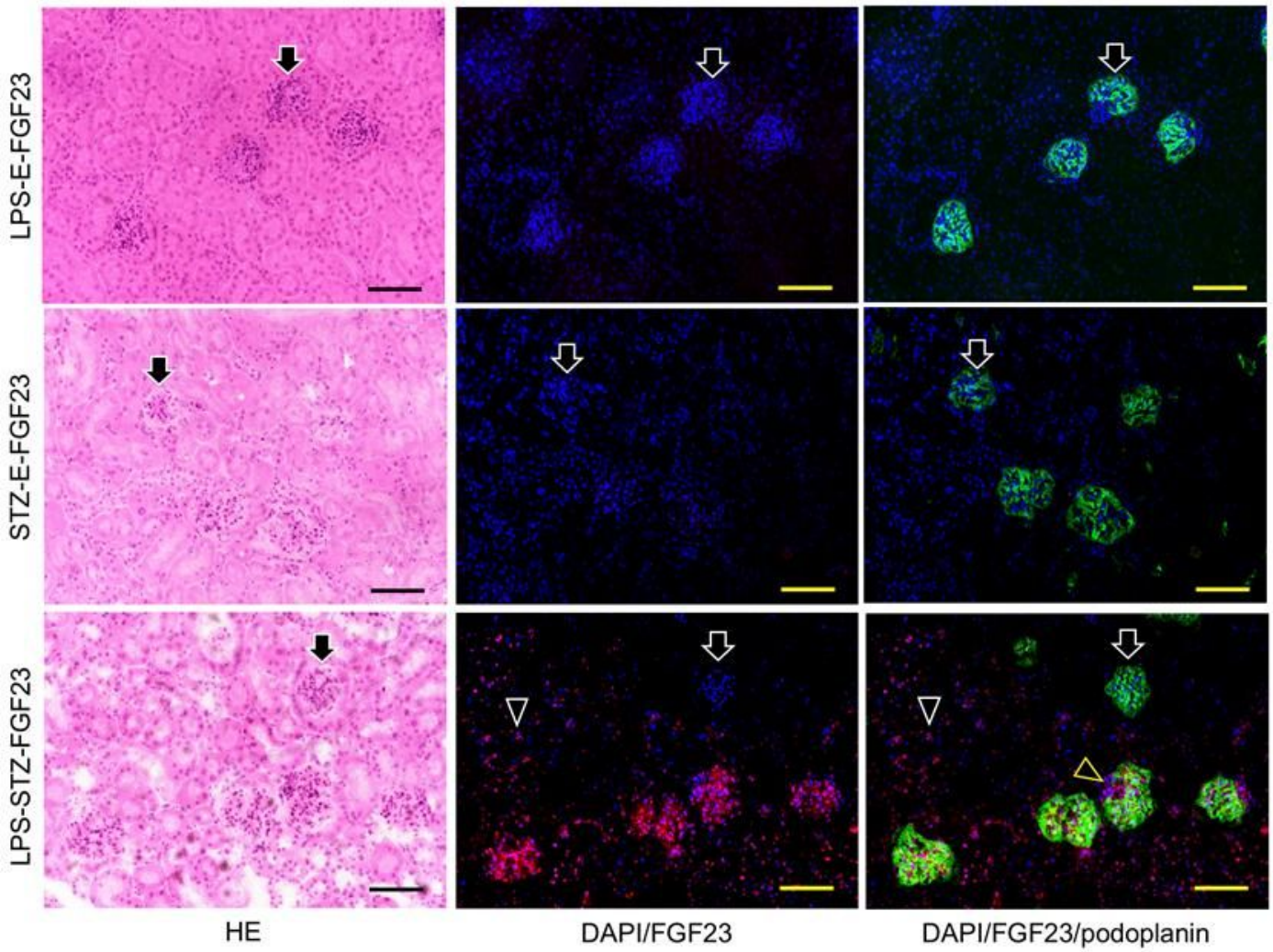

Figure 3

Immunostaining of FGF23 in STZ-induced type 1 diabetic mouse kidneys with P. gingivalis LPS-induced nephropathy. HE staining (left column); immunostaining for FGF23 (red)(center column); and merged immunostaining for FGF23 and podoplanin (green)(right column), with DAPI staining of nuclei (blue). The glomerular epithelial cells are immunostained by anti-podoplanin to be able to discriminate glomeruli (arrows). Reaction products of anti-FGF23 were not identified in non-diabetic mouse kidneys with P. gingivalis LPS administration (LPS, top row) or in diabetic mouse kidneys without the LPS administration (STZ, middle row), however, the reaction products were identified in the whole renal parenchyma (arrowheads) and in glomeruli (yellow arrowheads) in diabetic mouse kidneys with P. gingivalis LPS-induced nephropathy (LPS-STZ, bottom row). Bars: 100 μm.

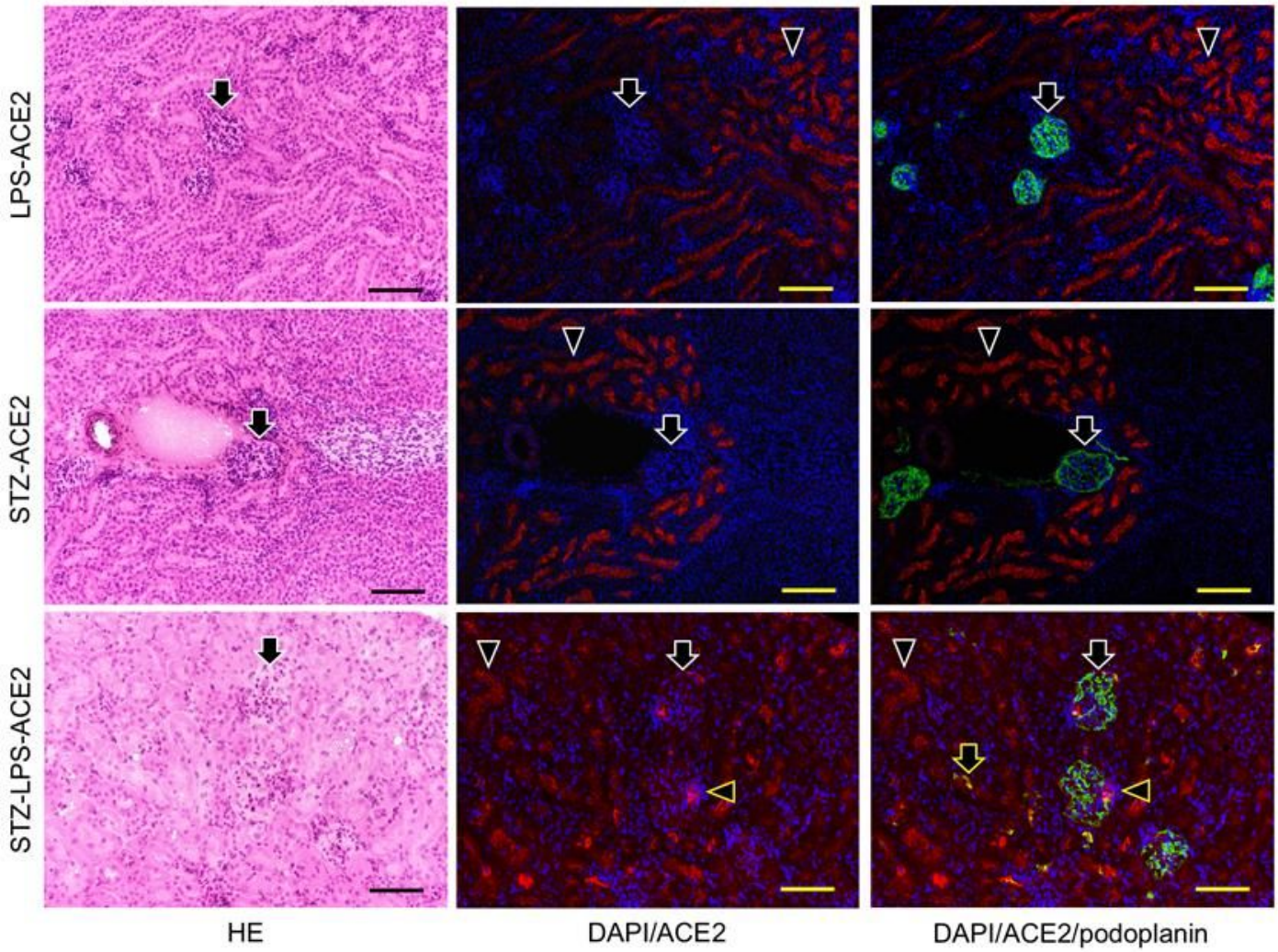

Figure 4

Figure 4

Immunostaining of ACE2 in STZ-induced type 1 diabetic mouse kidney with P. gingivalis LPS-induced nephropathy. HE staining (left column); immunostaining for ACE2 (red)(center column); and merged immunostaining for ACE2 and podoplanin (green)(right column), with DAPI staining of nuclei (blue). The glomerular epithelial cells are immunostained by anti-podoplanin to be able to discriminate glomeruli (arrows). Reaction products of anti-ACE2 (arrowheads) are only observed in the proximal tubular cells with brush borders, but in no other region including not in distal tubular cells reacted with anti-ACE2 in non-diabetic mouse kidneys with P. gingivalis LPS administration (LPS, top row) or in diabetic mouse kidneys without the LPS administration (STZ, middle row), however, reaction products were identified in tubules as well as in glomeruli (yellow arrowheads) of diabetic mouse kidneys with P. gingivalis LPS-induced nephropathy (LPS-STZ, bottom row). Podoplanin-positive macrophages were identified in diabetic mouse kidneys (yellow arrow).Bars: 100 μm.

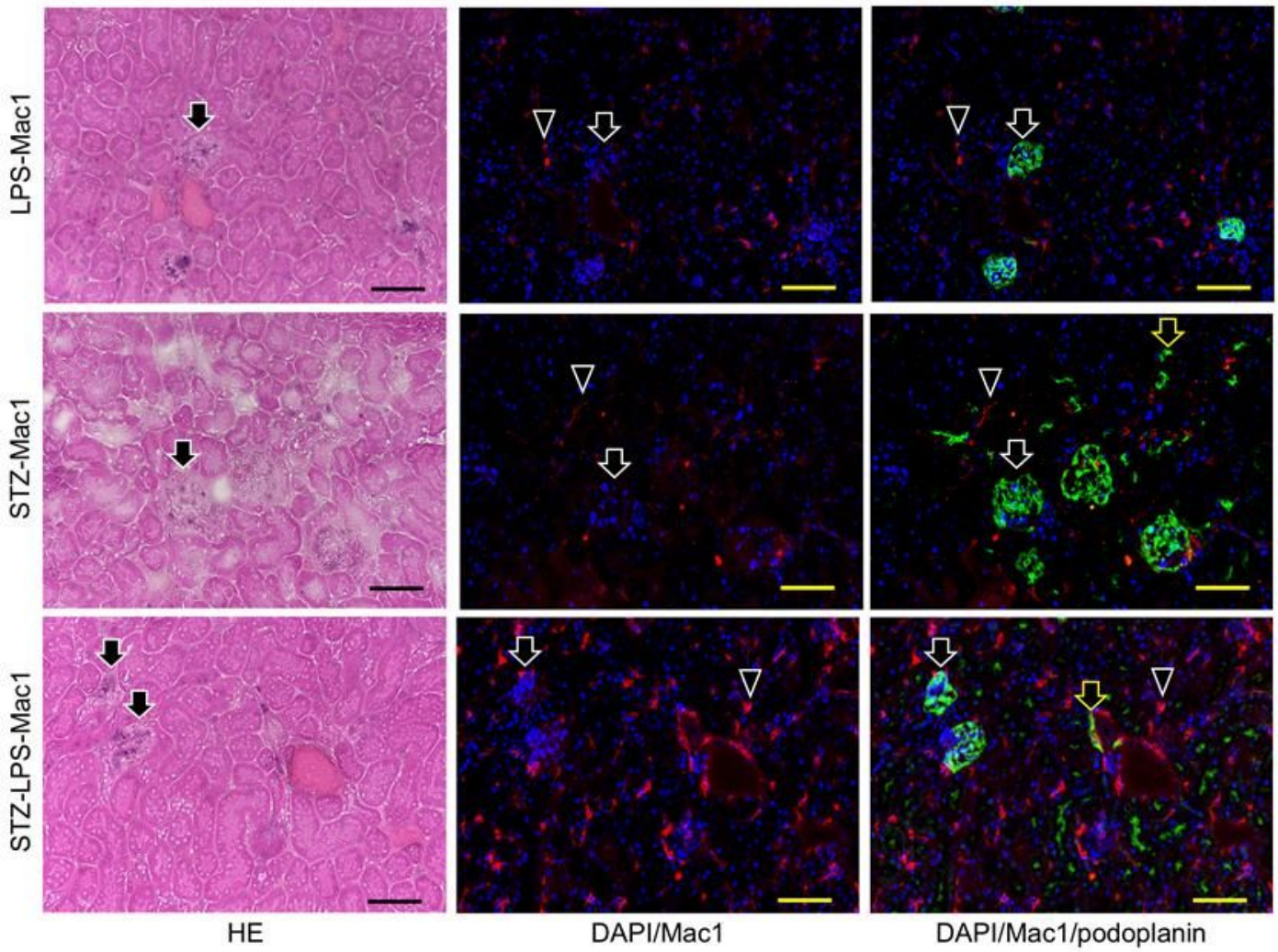

Figure 5

Figure 5

Immunostaining of Mac-1 in STZ-induced type 1 diabetic mouse kidney with P. gingivalis LPS-induced nephropathy. HE staining (left column), immunostaining for Mac-1 (red)(center column), and merged immunostaining for Mac-1 and podoplanin (green)(right column), with DAPI staining of nuclei (blue). The glomerular epithelial cells are immunostained by anti-podoplanin to be able to discriminate glomeruli (arrows). Mac-1-positive cells (arrowheads) were identified in the whole renal parenchyma (arrowheads) of non-diabetic mouse kidneys with P. gingivalis LPS administration (LPS, top row) and the number of Mac-1-positive cells was larger in diabetic mouse kidneys (STZ, middle row; LPS-STZ, bottom row). Podoplanin-positive macrophages were identified in diabetic mouse kidneys (yellow arrow). Bars: 100 μm.

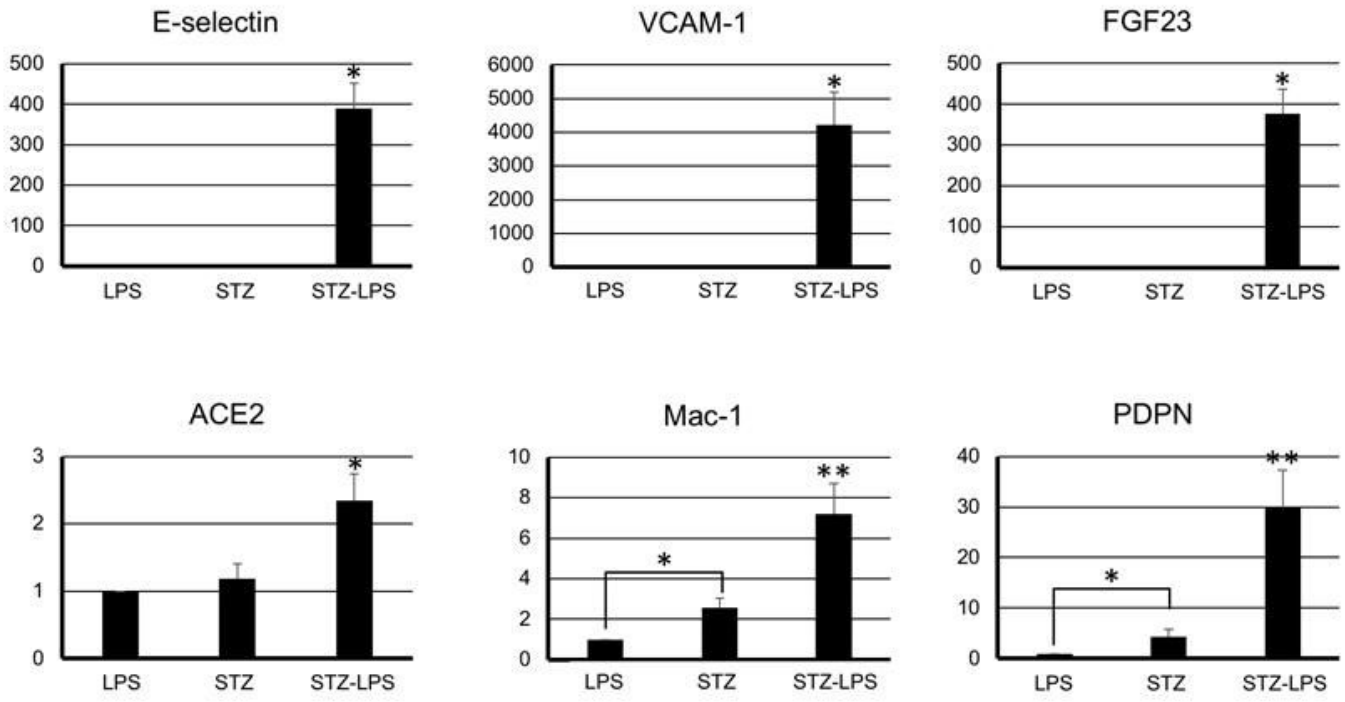

Figure 6

Figure 6

Quantitative analysis of the immunostaining of renal physiologically active molecules, leukocyte adhesion molecules, and leukocytes. Immunostained areas were measured in microscopic images by ImageJ. The relative reaction amounts of primary antibodies are expressed by arbitrary units of the ratio: positive areas of antibody reactions in the sections of diabetic mice and of LPS-administered diabetic mice/positive area of antibodies in the sections of LPS-administered non-diabetic mice. Reaction products of anti-E-selectin, anti-VCAM-1, and anti-FGF23 were not detected in the non-diabetic mouse kidneys with P. gingivalis LPS administration or in the diabetic mouse kidneys without LPS administration, however reaction products were detected in diabetic mouse kidneys with P. gingivalis LPS-induced nephropathy. Reaction products of anti-ACE2 were detected in non-diabetic mouse kidneys with P. gingivalis LPS administration and diabetic mouse kidneys without LPS administration, and the amounts of reaction products increased in diabetic mouse kidneys with P. gingivalis LPS-induced nephropathy. Mac-1 and/or podoplanin-positive macrophages were detected in non-diabetic mouse kidneys with P. gingivalis LPS administration. The number of Mac-1 and/or podoplanin-positive cells is larger in diabetic mouse kidneys without LPS administration than in non-diabetic mouse kidneys with P.

gingivalis LPS, and is larger in diabetic mouse kidney with the LPS administration than in diabetic mouse kidney without the administration. *Significantly different by one-way ANOVA (**) and the two-tailed unpaired Student's t test (*).

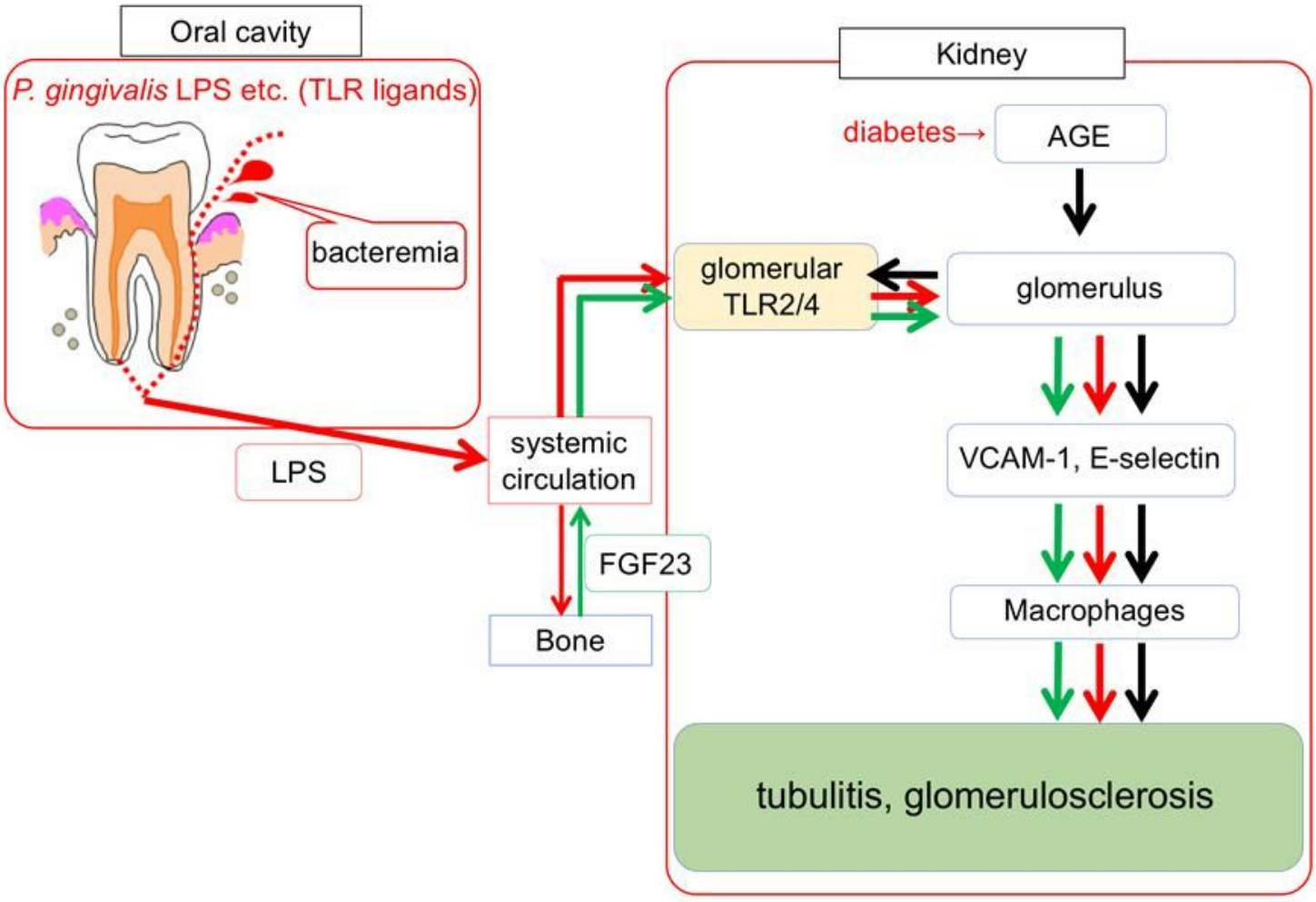

Figure 7

Figure 7

Prediction of complex etiology of P. gingivalis LPS-induced diabetic nephropathy Severe periodontitis causes bacteremia and microorganisms of the oral cavity enter kidneys through the systemic circulation. AGE induces renal TLR expression, recognized by TLR2/4, and promotes the expression of cytokines and leukocyte adhesion molecule VCAM-1, and E-selectin. Inflammatory infiltration of Mac-1/podoplanin positive macrophages causes with overexpression of leukocyte adhesion molecules in P. gingivalis LPS-accumulated renal glomeruli, and simultaneously ACE2 overexpression and bone-derived FGF23 accumulation promote tubulitis and diabetic nephropathy with hypertensive renal diseases.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- AuthorChecklistE10only.pdf