

References

- Tolonen N, Forsblom C, Thorn L, Waden J, Rosengard-Barlund M, Saraheimo M, Feodoroff M, Makinen VP, Gordin D, Taskinen MR, Groop PH. Lipid abnormalities predict progression of renal disease in patients with type 1 diabetes. *Diabetologia*. 2009;52:2522–30.
- Perkins BA, Ficociello LH, Silva KH, Finkelstein DM, Warram JH, Krolewski AS. Regression of microalbuminuria in type 1 diabetes. *N Engl J Med*. 2003;348:2285–93.
- Ravid M, Brosh D, Ravid-Safran D, Levy Z, Rachmani R. Main risk factors for nephropathy in type 2 diabetes mellitus are plasma cholesterol levels, mean blood pressure, and hyperglycemia. *Arch Intern Med*. 1998;158:998–1004.
- Retnakaran R, Cull CA, Thorne KI, Adler AI, Holman RR. Risk factors for renal dysfunction in type 2 diabetes: UK prospective diabetes study 74. *Diabetes*. 2006;55:1832–9.
- Kuwabara T, Mori K, Mukoyama M, Kasahara M, Yokoi H, Saito Y, Ogawa Y, Imamaki H, Kawanishi T, Ishii A, Koga K, Mori KP, Kato Y, Sugawara A, Nakao K. Exacerbation of diabetic nephropathy by hyperlipidaemia is mediated by Toll-like receptor 4 in mice. *Diabetologia*. 2012;55:2256–66.
- Maeda S, Kobayashi MA, Araki S, Babazono T, Freedman BI, Bostrom MA, Cooke JN, Toyoda M, Umezono T, Tarnow L, Hansen T, Gaede P, Jorsal A, Ng DP, Ikeda M, Yanagimoto T, Tsunoda T, Unoki H, Kawai K, Imanishi M, Suzuki D, Shin HD, Park KS, Kashiwagi A, Iwamoto Y, Kaku K, Kawamori R, Parving HH, Bowden DW, Pedersen O, Nakamura Y. A single nucleotide polymorphism within the acetyl-coenzyme A carboxylase beta gene is associated with proteinuria in patients with type 2 diabetes. *PLoS Genet*. 2010;6:e1000842.
- Tang SC, Leung VT, Chan LY, Wong SS, Chu DW, Leung JC, Ho YW, Lai KN, Ma L, Elbein SC, Bowden DW, Hicks PJ, Comeau ME, Langefeld CD, Freedman BI. The acetyl-coenzyme A carboxylase beta (ACACB) gene is associated with nephropathy in Chinese patients with type 2 diabetes. *Nephrol Dial Transplant*. 2010;25:3931–4.
- Murea M, Freedman BI, Parks JS, Antinozzi PA, Elbein SC, Ma L. Lipotoxicity in diabetic nephropathy: the potential role of fatty acid oxidation. *Clin J Am Soc Nephrol*. 2010;5:2373–9.
- Fazio S, Linton MF. Mouse models of hyperlipidemia and atherosclerosis. *Front Biosci*. 2001;6:D515–25.
- Park L, Raman KG, Lee KJ, Lu Y, Ferran LJ Jr, Chow WS, Stern D, Schmidt AM. Suppression of accelerated diabetic atherosclerosis by the soluble receptor for advanced glycation endproducts. *Nat Med*. 1998;4:1025–31.
- Hou CJ, Tsai CH, Su CH, Wu YJ, Chen SJ, Chiu JJ, Shiao MS, Yeh HI. Diabetes reduces aortic endothelial gap junctions in ApoE-deficient mice: simvastatin exacerbates the reduction. *J Histochem Cytochem*. 2008;56:745–52.
- Fledderus JO, van Oostrom O, de Kleijn DP, den Ouden K, Penders AF, Gremmels H, de Bree P, Verhaar MC. Increased amount of bone marrow-derived smooth muscle-like cells and accelerated atherosclerosis in diabetic apoE-deficient mice. *Atherosclerosis*. 2013;226:341–7.
- Lassila M, Seah KK, Allen TJ, Thallas V, Thomas MC, Candido R, Burns WC, Forbes JM, Calkin AC, Cooper ME, Jandeleit-Dahm KA. Accelerated nephropathy in diabetic apolipoprotein e-knockout mouse: role of advanced glycation end products. *J Am Soc Nephrol*. 2004;15:2125–38.
- Watson AM, Gray SP, Jiaye L, Soro-Paavonen A, Wong B, Cooper ME, Bierhaus A, Pickering R, Tikellis C, Tsorotes D, Thomas MC, Jandeleit-Dahm KA. Alagebrium reduces glomerular fibrogenesis and inflammation beyond preventing RAGE activation in diabetic apolipoprotein E knockout mice. *Diabetes*. 2012;61:2105–13.
- Lopez-Parra V, Mallavia B, Lopez-Franco O, Ortiz-Munoz G, Oguiza A, Recio C, Blanco J, Nimmerjahn F, Egido J, Gomez-Guerrero C. Fcγ receptor deficiency attenuates diabetic nephropathy. *J Am Soc Nephrol*. 2012;23:1518–27.
- Berault DR, Sharma S, Shi Y, Khan MI, Werstuck GH. Glucosamine-supplementation promotes endoplasmic reticulum stress, hepatic steatosis and accelerated atherogenesis in apoE^{-/-} mice. *Atherosclerosis*. 2011;219:134–40.
- McAlpine CS, Bowes AJ, Khan MI, Shi Y, Werstuck GH. Endoplasmic reticulum stress and glycogen synthase kinase-3β activation in apolipoprotein E-deficient mouse models of accelerated atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2012;32:82–91.
- Goldberg IJ, Hu Y, Noh HL, Wei J, Huggins LA, Rackmill MG, Hamai H, Reid BN, Blazer WS, Huang LS. Decreased lipoprotein clearance is responsible for increased cholesterol in LDL receptor knockout mice with streptozotocin-induced diabetes. *Diabetes*. 2008;57:1674–82.
- Spencer MW, Muhlfeld AS, Segerer S, Hudkins KL, Kirk E, LeBoeuf RC, Alpers CE. Hyperglycemia and hyperlipidemia act synergistically to induce renal disease in LDL receptor-deficient BALB mice. *Am J Nephrol*. 2004;24:20–31.
- Sassy-Prigent C, Heudes D, Mandet C, Bélair MF, Michel O, Perdereau B, Bariéty J, Bruneval P. Early glomerular macrophage recruitment in streptozotocin-induced diabetic rats. *Diabetes*. 2000;49:466–75.
- Sauve M, Ban K, Momen MA, Zhou YQ, Henkelman RM, Husain M, Drucker DJ. Genetic deletion or pharmacological inhibition of dipeptidyl peptidase-4 improves cardiovascular outcomes after myocardial infarction in mice. *Diabetes*. 2010;59:1063–73.
- Hazra S, Rasheed A, Bhatwadekar A, Wang X, Shaw LC, Patel M, Caballero S, Magomedova L, Solis N, Yan Y, Wang W, Thinschmidt JS, Verma A, Li Q, Levi M, Cummins CL, Grant MB. Liver x receptor modulates diabetic retinopathy outcome in a mouse model of streptozotocin-induced diabetes. *Diabetes*. 2012;61:3270–9.
- Guilford BL, Ryals JM, Wright DE. Phenotypic changes in diabetic neuropathy induced by a high-fat diet in diabetic C57BL/6 mice. *Exp Diabetes Res*. 2011;2011:848307.
- Zeng XY, Wang YP, Cantley J, Iseli TJ, Molero JC, Hegarty BD, Kraegen EW, Ye Y, Ye JM. Oleic acid reduces hyperglycemia beyond treatment period with Akt/FoxO1-induced suppression of hepatic gluconeogenesis in type-2 diabetic mice. *PLoS One*. 2012;7:e42115.
- Moitra J, Mason MM, Olive M, Krylov D, Gavrilo O, Marcus-Samuels B, Feigenbaum L, Lee E, Aoyama T, Eckhaus M, Reitman ML, Vinson C. Life without white fat: a transgenic mouse. *Genes Dev*. 1998;12:3168–81.
- Kim JK, Gavrilo O, Chen Y, Reitman ML, Shulman GI. Mechanism of insulin resistance in A-ZIP/F-1 fatless mice. *J Biol Chem*. 2000;275:8456–60.
- Suganami T, Mukoyama M, Mori K, Yokoi H, Koshikawa M, Sawai K, Hidaka S, Ebihara K, Tanaka T, Sugawara A, Kawachi H, Vinson C, Ogawa Y, Nakao K. Prevention and reversal of renal injury by leptin in a new mouse model of diabetic nephropathy. *FASEB J*. 2005;19:127–9.
- Keren P, George J, Keren G, Harats D. Non-obese diabetic (NOD) mice exhibit an increased cellular immune response to glycated-LDL but are resistant to high fat diet induced atherosclerosis. *Atherosclerosis*. 2001;157:285–92.
- Fox TE, Bewley MC, Unrath KA, Pedersen MM, Anderson RE, Jung DY, Jefferson LS, Kim JK, Bronson SK, Flanagan JM,

- Kester M. Circulating sphingolipid biomarkers in models of type 1 diabetes. *J Lipid Res.* 2011;52:509–17.
30. Colombo C, Haluzik M, Cutson JJ, Dietz KR, Marcus-Samuels B, Vinson C, Gavrilova O, Reitman ML. Opposite effects of background genotype on muscle and liver insulin sensitivity of lipotrophic mice. Role of triglyceride clearance. *J Biol Chem.* 2005;278:3992–9.
 31. Breyer MD, Bottinger E, Brosius FC 3rd, Coffman TM, Harris RC, Heilig CW, Sharma K. Mouse models of diabetic nephropathy. *J Am Soc Nephrol.* 2005;16:27–45.
 32. Brosius FC 3rd, Alpers CE, Bottinger EP, Breyer MD, Coffman TM, Gurley SB, Harris RC, Kakoki M, Kretzler M, Leiter EH, Levi M, McIndoe RA, Sharma K, Smithies O, Susztak K, Takahashi N, Takahashi T. Mouse models of diabetic nephropathy. *J Am Soc Nephrol.* 2009;20:2503–12.
 33. Qi Z, Fujita H, Jin J, Davis LS, Wang Y, Fogo AB, Breyer MD. Characterization of susceptibility of inbred mouse strains to diabetic nephropathy. *Diabetes.* 2005;54:2628–37.
 34. Agellon LB, Walsh A, Hayek T, Moulin P, Jiang XC, Shelanski SA, Breslow JL, Tall AR. Reduced high density lipoprotein cholesterol in human cholesteryl ester transfer protein transgenic mice. *J Biol Chem.* 1991;266:10796–801.
 35. Nakamura M, Oka K, Krushkal J, Kobayashi K, Yamamoto M, Li WH, Chan L. Alternative mRNA splicing and differential promoter utilization determine tissue-specific expression of the apolipoprotein B mRNA-editing protein (ApoBec1) gene in mice. Structure and evolution of ApoBec1 and related nucleoside/nucleotide deaminases. *J Biol Chem.* 1995;270:13042–56.
 36. Prentki M, Corkey BE. Are the beta-cell signaling molecules malonyl-CoA and cystolic long-chain acyl-CoA implicated in multiple tissue defects of obesity and NIDDM? *Diabetes.* 1996;45:273–83.
 37. Brun T, Roche E, Assimacopoulos-Jeannet F, Corkey BE, Kim KH, Prentki M. Evidence for an anaplerotic/malonyl-CoA pathway in pancreatic beta-cell nutrient signaling. *Diabetes.* 1996;45:190–8.
 38. Poyntout V, Robertson RP. Minireview. Secondary beta-cell failure in type 2 diabetes—a convergence of glucotoxicity and lipotoxicity. *Endocrinology.* 2002;143:339–42.
 39. Maedler K, Oberholzer J, Bucher P, Spinass GA, Donath MY. Monounsaturated fatty acids prevent the deleterious effects of palmitate and high glucose on human pancreatic beta-cell turnover and function. *Diabetes.* 2003;52:726–33.
 40. El-Asaad W, Buteau J, Peyot ML, Nolan C, Roduit R, Hardy S, Joly E, Dbaibo G, Rosenberg L, Prentki M. Saturated fatty acids synergize with elevated glucose to cause pancreatic beta-cell death. *Endocrinology.* 2003;144:4154–63.
 41. Martinez-Garcia C, Izquierdo A, Velagapudi V, Vivas Y, Velasco I, Campbell M, Burling K, Cava F, Ros M, Oresic M, Vidal-Puig A, Medina-Gomez G. Accelerated renal disease is associated with the development of metabolic syndrome in a glucolipotoxic mouse model. *Dis Model Mech.* 2012;5:636–48.
 42. Yamabe N, Noh JS, Park CH, Kang KS, Shibahara N, Tanaka T, Yokozawa T. Evaluation of loganin, iridoid glycoside from Corni Fructus, on hepatic and renal glucolipotoxicity and inflammation in type 2 diabetic db/db mice. *Eur J Pharmacol.* 2010;648:179–87.
 43. Urano F, Wang X, Bertolotti A, Zhang Y, Chung P, Harding HP, Ron D. Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science.* 2000;287:664–6.
 44. Hirosumi J, Tuncman G, Chang L, Gorgun CZ, Uysal KT, Maeda K, Karin M, Hotamisligil GS. A central role for JNK in obesity and insulin resistance. *Nature.* 2002;420:333–6.
 45. Ozcan U, Cao Q, Yilmaz E, Lee AH, Iwakoshi NN, Ozdelen E, Tuncman G, Gorgun C, Glimcher LH, Hotamisligil GS. Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science.* 2004;306:457–61.
 46. Bachar E, Ariav Y, Ketzinel-Gilad M, Cerasi E, Kaiser N, Leibowitz G. Glucose amplifies fatty acid-induced endoplasmic reticulum stress in pancreatic beta-cells via activation of mTORC1. *PLoS One.* 2009;4:e4954.
 47. Lee SJ, Choi SE, Hwang YC, Jung IR, Yi SA, Jung JG, Ku JM, Jeoung K, Han SJ, Kim HJ, Kim DJ, Lee KW, Kang Y. A compound (DW1182v) protecting high glucose/palmitate-induced glucolipotoxicity to INS-1 beta cells preserves islet integrity and improves hyperglycemia in obese db/db mouse. *Eur J Pharmacol.* 2012;696:187–93.
 48. Lu M, Seufert J, Habener JF. Pancreatic beta-cell-specific repression of insulin gene transcription by CCAAT/enhancer-binding protein beta. Inhibitory interactions with basic helix-loop-helix transcription factor E47. *J Biol Chem.* 1997;272:28349–59.
 49. Fontes G, Semache M, Hagman DK, Tremblay C, Shah R, Rhodes CJ, Rutter J, Poitout V. Involvement of Per-Arnt-Sim kinase and extracellular-regulated kinases-1/2 in palmitate inhibition of insulin gene expression in pancreatic beta-cells. *Diabetes.* 2009;58:2048–58.
 50. Zador IZ, Hsieh CC, Papaconstantinou J. Renal CCAAT/enhancer-binding proteins in experimental diabetes mellitus. *Nephron.* 1998;79:312–6.
 51. Zenz R, Eferl R, Kenner L, Florin L, Hummerich L, Mehic D, Scheuch H, Angel P, Tschachler E, Wagner EF. Psoriasis-like skin disease and arthritis caused by inducible epidermal deletion of Jun proteins. *Nature.* 2005;437:369–75.
 52. Yao D, Brownlee M. Hyperglycemia-induced reactive oxygen species increase expression of the receptor for advanced glycation end products (RAGE) and RAGE ligands. *Diabetes.* 2010;59:249–55.
 53. Endoh Y, Chung YM, Clark IA, Geczy CL, Hsu K. IL-10-dependent S100A8 gene induction in monocytes/macrophages by double-stranded RNA. *J Immunol.* 2009;182:2258–68.
 54. Kuruto-Niwa R, Nakamura M, Takeishi K, Nozawa R. Transcriptional regulation by C/EBP alpha and -beta in the expression of the gene for the MRP14 myeloid calcium binding protein. *Cell Struct Funct.* 1998;23:109–18.
 55. Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H, Flier JS. TLR4 links innate immunity and fatty acid-induced insulin resistance. *J Clin Invest.* 2006;116:3015–25.
 56. Suganami T, Mieda T, Itoh M, Shimoda Y, Kamei Y, Ogawa Y. Attenuation of obesity-induced adipose tissue inflammation in C3H/HeJ mice carrying a Toll-like receptor 4 mutation. *Biochem Biophys Res Commun.* 2007;354:45–9.
 57. Kim JK. Fat uses a TOLL-road to connect inflammation and diabetes. *Cell Metab.* 2006;4:417–9.
 58. Pal D, Dasgupta S, Kundu R, Maitra S, Das G, Mukhopadhyay S, Ray S, Majumdar SS, Bhattacharya S. Fetuin-A acts as an endogenous ligand of TLR4 to promote lipid-induced insulin resistance. *Nat Med.* 2012;18:1279–85.
 59. Ix JH, Biggs ML, Mukamal KJ, Kizer JR, Zieman SJ, Siscovick DS, Mozaffarian D, Jensen MK, Nelson L, Ruderman N, Djousse L. Association of fetuin-a with incident diabetes mellitus in community-living older adults: the cardiovascular health study. *Circulation.* 2012;125:2316–22.
 60. Ou HY, Wu HT, Hung HC, Yang YC, Wu JS, Chang CJ. Endoplasmic reticulum stress induces the expression of fetuin-A to develop insulin resistance. *Endocrinology.* 2012;153:2974–84.
 61. Odink K, Cerletti N, Bruggen J, Clerc RG, Tarcsay L, Zwadlo G, Gerhards G, Schlegel R, Sorg C. Two calcium-binding proteins in infiltrate macrophages of rheumatoid arthritis. *Nature.* 1987;330:80–2.
 62. Vogl T, Tenbrock K, Ludwig S, Leukert N, Ehrhardt C, van Zoelen MA, Nacken W, Foell D, van der Poll T, Sorg C, Roth J.

- Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. *Nat Med.* 2007;13:1042–9.
63. Croce K, Gao H, Wang Y, Mooroka T, Sakuma M, Shi C, Sukhova GK, Packard RR, Hogg N, Libby P, Simon DI. Myeloid-related protein-8/14 is critical for the biological response to vascular injury. *Circulation.* 2009;120:427–36.
 64. Loser K, Vogl T, Voskort M, Lueken A, Kupas V, Nacken W, Klenner L, Kuhn A, Foell D, Sorokin L, Luger TA, Roth J, Beissert S. The Toll-like receptor 4 ligands Mrp8 and Mrp14 are crucial in the development of autoreactive CD8+ T cells. *Nat Med.* 2010;16:713–7.
 65. Nguyen MT, Favelukis S, Nguyen AK, Reichart D, Scott PA, Jenn A, Liu-Bryan R, Glass CK, Neels JG, Olefsky JM. A subpopulation of macrophages infiltrates hypertrophic adipose tissue and is activated by free fatty acids via Toll-like receptors 2 and 4 and JNK-dependent pathways. *J Biol Chem.* 2007;282:35279–92.
 66. Solinas G, Vilcu C, Neels JG, Bandyopadhyay GK, Luo JL, Naugler W, Grivennikov S, Wynshaw-Boris A, Scadeng M, Olefsky JM, Karin M. JNK1 in hematopoietically derived cells contributes to diet-induced inflammation and insulin resistance without affecting obesity. *Cell Metab.* 2007;6:386–97.
 67. Brown HJ, Lock HR, Wolfs TG, Buurman WA, Sacks SH, Robson MG. Toll-like receptor 4 ligation on intrinsic renal cells contributes to the induction of antibody-mediated glomerulonephritis via CXCL1 and CXCL2. *J Am Soc Nephrol.* 2007;18:1732–9.
 68. Allam R, Lichtnekert J, Moll AG, Taubitz A, Vielhauer V, Anders HJ. Viral RNA and DNA trigger common antiviral responses in mesangial cells. *J Am Soc Nephrol.* 2009;20:1986–96.
 69. Hagele H, Allam R, Pawar RD, Reichel CA, Krombach F, Anders HJ. Double-stranded DNA activates glomerular endothelial cells and enhances albumin permeability via a toll-like receptor-independent cytosolic DNA recognition pathway. *Am J Pathol.* 2009;175:1896–904.
 70. Banas MC, Banas B, Hudkins KL, Wietecha TA, Iyoda M, Bock E, Hauser P, Pippin JW, Shankland SJ, Smith KD, Stoelcker B, Liu G, Grone HJ, Kramer BK, Alpers CE. TLR4 links podocytes with the innate immune system to mediate glomerular injury. *J Am Soc Nephrol.* 2008;19:704–13.
 71. Pawar RD, Castrezana-Lopez L, Allam R, Kulkarni OP, Segerer S, Radomska E, Meyer TN, Schwesinger CM, Akis N, Grone HJ, Anders HJ. Bacterial lipopeptide triggers massive albuminuria in murine lupus nephritis by activating Toll-like receptor 2 at the glomerular filtration barrier. *Immunology.* 2009;128:e206–21.
 72. Sica A, Mantovani A. Macrophage plasticity and polarization: in vivo veritas. *J Clin Invest.* 2012;1(122):787–95.
 73. Ricardo SD, van Goor H, Eddy AA. Macrophage diversity in renal injury and repair. *J Clin Invest.* 2008;118:3522–30.
 74. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol.* 2004;25:677–86.
 75. Lee S, Huen S, Nishio H, Nishio S, Lee HK, Choi BS, Ruhrberg C, Cantley LG. Distinct macrophage phenotypes contribute to kidney injury and repair. *J Am Soc Nephrol.* 2011;22:317–26.
 76. Fujii K, Manabe I, Nagai R. Renal collecting duct epithelial cells regulate inflammation in tubulointerstitial damage in mice. *J Clin Invest.* 2011;121:3425–41.
 77. Ito A, Suganami T, Yamauchi A, Degawa-Yamauchi M, Tanaka M, Kouyama R, Kobayashi Y, Nitta N, Yasuda K, Hirata Y, Kuziel WA, Takeya M, Kanegasaki S, Kamei Y, Ogawa Y. Role of CC chemokine receptor 2 in bone marrow cells in the recruitment of macrophages into obese adipose tissue. *J Biol Chem.* 2008;19(283):35715–23.
 78. Lumeng CN, Bodzin JL, Saltiel AR. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J Clin Invest.* 2007;117:175–84.
 79. Mahnke K, Bhardwaj R, Sorg C. Heterodimers of the calcium-binding proteins MRP8 and MRP14 are expressed on the surface of human monocytes upon adherence to fibronectin and collagen. Relation to TNF-alpha, IL-6, and superoxide production. *J Leukoc Biol.* 1995;57:63–71.

Predictive Significance of Kidney Myeloid-Related Protein 8 Expression in Patients with Obesity- or Type 2 Diabetes-Associated Kidney Diseases

Takashige Kuwabara¹, Kiyoshi Mori^{1,2*}, Masato Kasahara^{1,3}, Hideki Yokoi¹, Hirotaka Imamaki¹, Akira Ishii¹, Kenichi Koga¹, Akira Sugawara⁴, Shinji Yasuno³, Kenji Ueshima³, Takashi Morikawa⁵, Yoshio Konishi⁵, Masahito Imanishi⁵, Akira Nishiyama⁶, Kazuwa Nakao^{1,2}, Masashi Mukoyama¹

1 Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine, Kyoto, Japan, **2** Medical Innovation Center, Kyoto University Graduate School of Medicine, Kyoto, Japan, **3** Department of EBM Research, Institute for Advancement of Clinical and Translational Science, Kyoto University Hospital, Kyoto, Japan, **4** Department of Nephrology, Osaka Red Cross Hospital, Osaka, Japan, **5** Division of Nephrology and Hypertension, Osaka City General Hospital, Osaka, Japan, **6** Department of Pharmacology, Kagawa University Medical School, Kagawa, Japan

Abstract

Background and Objective: We have reported that toll-like receptor 4 (TLR4) and one of its endogenous ligands, myeloid-related protein 8 (MRP8 or S100A8), play an important role in the progression of diabetic nephropathy in mice. The aim of this study was to evaluate significance of kidney MRP8 expression in patients with obesity- or type 2 diabetes-associated kidney diseases.

Methods: In diabetic, obese or control subjects, MRP8 mRNA and protein expression levels in renal biopsy samples were determined by real-time RT-PCR and immunohistochemistry (n=28 and 65, respectively), and their associations with baseline and prognostic parameters were analyzed. Effects of MRP8 upon pro-inflammatory gene expressions were examined using macrophages.

Results: Kidney MRP8 gene and protein expression levels were elevated in obese or diabetic groups compared to control group. Among all subjects, by univariate linear regression analysis, glomerular MRP8-positive cell count and tubulointerstitial MRP8-positive area at baseline were both, respectively, correlated not only with various known risk factors for diabetic nephropathy (such as systolic blood pressure, proteinuria and serum creatinine) but also with extent of glomerulosclerosis and tubulointerstitial fibrosis. Independent factors predicting urinary protein levels a year later were examined by multivariate analysis, and they included glomerular MRP8-positive cell count ($\beta = 0.59$, $P < 0.001$), proteinuria ($\beta = 0.37$, $P = 0.002$) and systolic blood pressure ($\beta = 0.21$, $P = 0.04$) at baseline, after adjustment for known risk factors. MRP8 protein expression was observed in CD68-positive macrophages and atrophic tubules. In cultured mouse macrophages, MRP8 protein induced proinflammatory cytokine expressions and also triggered auto-induction of MRP8 in a TLR4-dependent manner.

Conclusions: Glomerular MRP8 expression appears to be associated with progression of proteinuria in obese or type 2 diabetic patients, possibly by inducing inflammatory changes in macrophages through TLR4 signaling.

Citation: Kuwabara T, Mori K, Kasahara M, Yokoi H, Imamaki H, et al. (2014) Predictive Significance of Kidney Myeloid-Related Protein 8 Expression in Patients with Obesity- or Type 2 Diabetes-Associated Kidney Diseases. PLoS ONE 9(2): e88942. doi:10.1371/journal.pone.0088942

Editor: Utpal Sen, University of Louisville, United States of America

Received: September 11, 2013; **Accepted:** January 14, 2014; **Published:** February 18, 2014

Copyright: © 2014 Kuwabara et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported in part by Grant-in-Aid for Diabetic Nephropathy and Nephrosclerosis Research from the Ministry of Health, Labour and Welfare of Japan (to KM), research grants from the Japanese Ministry of Education, Culture, Sports, Science and Technology (to TK, KM, and MM), from the Japan Foundation for Applied Enzymology (to TK) and from the Smoking Research Foundation (to MM). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: keyem@kuhp.kyoto-u.ac.jp

Introduction

Chronic inflammation plays an important role in the pathogenesis of diabetes or obesity and their cardiovascular complications [1]. Involvement of innate immune receptors and the endogenous ligands in the process of chronic inflammation has been implicated. Myeloid-related protein 8 (MRP8, also known as S100A8 or calgranulin A) was originally identified as a cytoplasmic calcium-binding protein in neutrophils and monocytes [2], and

has become widely recognized as a potent endogenous ligand for toll-like receptor 4 (TLR4) in various diseases including septic shock, vascular and autoimmune disorders [3,4,5]. We have recently proposed that MRP8/TLR4 signaling plays an important role in hyperlipidemia-induced progression of diabetic nephropathy [6]. Glomerular macrophages and collecting duct cells are major sources of MRP8 in mouse models of diabetic nephropathy [6] and renal fibrosis [7], respectively. Plasma levels of MRP8, which usually forms a heterodimeric complex with a binding

partner MRP14 in the bloodstream, are increased in obese subjects [8,9]. However, there have been no reports investigating renal expression of MRP8 in patients with obesity or type 2 diabetes and its association with renal prognosis.

The aim of this study was to determine mRNA and protein expression levels of MRP8 in the kidney of Japanese patients with diabetic nephropathy (DN), obesity-related glomerulopathy (ORG), minimal change nephrotic syndrome (MCNS) or minor glomerular abnormality (MGA), which were all diagnosed by renal biopsy, and to evaluate whether renal MRP8 expression can predict renal outcomes.

Materials and Methods

Ethics statement

The human study was conducted according to the principles expressed in the Declaration of Helsinki, and was approved by the Ethical Committees on Human Research of Kyoto University Graduate School of Medicine and Osaka City General Hospital, respectively. All participants gave written informed consent. The animal study protocol was approved by the Animal Research Committee of Kyoto University Graduate School of Medicine (Permit Number: Med Kyo 13318). All animal surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Study subjects

Proteinuric patients with obesity or type 2 diabetes who underwent renal biopsy were enrolled in this study. Patients with infectious disease, cancer, liver disease or collagen disease were excluded. Proteinuria was defined as urinary protein greater than 0.5 g/g creatinine or urinary albumin greater than 300 mg/g creatinine in at least two consecutive measurements. Obesity was defined as body mass index (BMI) greater than 25.0 (kg/m²). Type 2 diabetes was diagnosed in accordance with the criteria of the World Health Organization. Biochemical measurements on admission for renal biopsy were used as baseline characteristics for cross-sectional analysis. Estimated glomerular filtration rate (eGFR) was calculated using a simplified prediction equation proposed by the Japanese Society of Nephrology: $eGFR \text{ (ml/min/1.73 m}^2\text{)} = 194 \times [\text{age (years)}]^{-0.287} \times [\text{serum creatinine (mg per dl)}]^{-1.094} \times 0.739$ (for females), which is a validated local modification of MDRD [10]. The serum concentrations of creatinine were measured using an enzymatic method.

For immunohistochemistry, 65 Japanese patients who underwent renal biopsy at Department of Medicine and Clinical Science, Kyoto University Hospital between 2000 and 2011 were analyzed. Biopsy-proven diagnoses of all patients during this period are listed in Table S1 in File S1. The subjects examined in this work included DN (n = 19), ORG (n = 10) and non-obese, non-diabetic control subjects who were diagnosed as MGA (n = 19) or MCNS (n = 17). Some cases in these categories were excluded because residual samples available contained less than 10 glomeruli. Definition of DN consisted of (1) more than 5 year duration after the onset of diabetes, (2) existence of micro- or macro-albuminuria, (3) compatible histopathological changes with DN such as glomerular basement membrane thickening, mesangial expansion, nodular sclerosis (Kimmelstiel-Wilson nodules) and/or arteriolar hyalinosis, and (4) exclusion of other causes for renal disorders [11]. ORG was defined morphologically as focal segmental glomerulosclerosis and/or glomerulomegaly in subjects having both obesity and proteinuria, whose definitions were described above [12,13].

For mRNA expression analysis, low quality samples, in which 18S ribosomal RNA (rRNA) levels were lower than the detection sensitivity limit by real-time RT-PCR, were excluded. Subjects enrolled consisted of 22 type 2 diabetic patients who underwent renal biopsy at Osaka City General Hospital between 2000 and 2010, and 6 non-diabetic control subjects, who had biopsy-proven MGA.

Tables 1 and 2 summarize the baseline clinical characteristics of the patients who were examined by immunohistochemical or gene expression analysis, respectively. For light microscopy, the tissue specimens were processed according to standard procedures. Sections were stained with haematoxylin-eosin, periodic acid-Schiff, periodic-acid methenamine silver or Masson trichrome (Fig. S1). The ratios of the number of glomeruli with global sclerosis among that of total glomeruli and the relative areas of tubulointerstitial fibrosis were evaluated independently by two pathologists unaware of diagnosis and clinical data.

Definition of renal outcomes

The following two prognostic indicators were examined by linear regression and logistic regression analyses, respectively: (1) the extent of proteinuria measured at one year post-biopsy, and (2) renal event defined as annual increase in serum creatinine by > 50% from baseline or initiation of chronic dialysis.

Immunohistochemistry

Immunohistochemistry of MRP8 and CD68 was carried out using kidney sections (thickness 4 μm) fixed with 4% buffered paraformaldehyde. After antigen retrieval by citrate buffer, kidney sections were incubated with 10% goat serum, followed by mouse anti-human MRP8 (1:100; BMA biomedical, Augst, Switzerland) [14] or mouse anti-human CD68 antibodies (1:50; DAKO, Ely, UK), respectively. Primary antibodies were visualized with horseradish peroxidase-conjugated secondary antibody and 3,3'-diaminobenzidine tetrahydrochloride (Dako USA, Carpinteria, CA). Nuclei were counterstained with hematoxylin. MRP8-positive cells were counted in more than 10 glomeruli, and MRP8-positive area in tubulointerstitium was measured quantitatively to obtain an average for each subject using MetaMorph 7.5 software (Molecular Devices, Downingtown, PA, USA). Colocalization of CD68- and MRP8-positive cells was evaluated with serial sections. There was neither MRP8 nor CD68 signal in negative controls stained without first antibody (Fig. S2). By preincubation of anti-MRP8 antibody with 20 molar excess of recombinant human MRP8 protein (Life Technologies, Carlsbad, CA, USA) at 4°C overnight, staining was markedly reduced, if not completely, further supporting the specificity of the antibody (Fig. S3).

Evaluation of mRNA expression

Frozen kidney sections were separated into glomeruli and non-glomerulus tissues by laser capture micro-dissection (LM200; Olympus, Tokyo, Japan) as previously described [15]. Total RNA was extracted with RNeasy mini kit (Qiagen, Tokyo, Japan). mRNA expression levels were determined by TaqMan real-time PCR (Applied Biosystems, Foster City, CA, USA) [16,17]. Expression levels of all genes were normalized by 18S rRNA (internal control) levels. See Table S2 in File S1 for primer and probe sequences. Eukaryotic 18S rRNA was detected with Pre-Developed TaqMan Assay Reagents (Applied Biosystems).

Table 1. Baseline clinical characteristics of patients at renal biopsy who were analyzed for MRP8 protein expression by immunohistochemistry.

	Non-obese, non-diabetic control				Between-group differences*
	MGA	MCNS	ORG	DN	
N	19	17	10	19	
Sex (male/female)	10/9	5/12	7/3	14/5	$\chi^2 = 8.1, P = 0.04$
Age (years)	35.5 ± 17.9	36.3 ± 17.4	49.3 ± 16.5	58.3 ± 9.0	P < 0.001
Diabetes duration (years)	-	-	-	11.3 ± 6.7	-
BMI (kg/m ²)	19.7 ± 2.1	23.7 ± 3.3	32.0 ± 5.6	24.7 ± 3.5	P < 0.001
HbA1c (NGSP, %)	5.6 ± 0.1	5.8 ± 0.2	6.0 ± 0.7	7.1 ± 1.5	P = 0.04
Systolic blood pressure (mmHg)	112.2 ± 11.4	113.0 ± 10.2	129.3 ± 10.4	149.4 ± 17.2	P < 0.001
Diastolic blood pressure (mmHg)	67.0 ± 6.8	70.2 ± 10.1	82.2 ± 12.7	82.8 ± 10.1	P < 0.001
Urinary protein (g/g creatinine)	0.30 ± 0.45	6.83 ± 3.37	1.29 ± 1.24	5.38 ± 4.02	P < 0.001
Creatinine (mg/dl)	0.66 ± 0.14	0.68 ± 0.15	0.84 ± 0.23	1.45 ± 0.66	P < 0.001
eGFR (ml/min/1.73 m ²)	100.7 ± 24.4	94.0 ± 20.3	75.0 ± 18.9	45.4 ± 19.6	P < 0.001
BUN (mg/dl)	13.3 ± 3.7	13.5 ± 6.8	15.6 ± 4.6	23.9 ± 10.3	P < 0.001
Total protein (g/dl)	7.0 ± 0.6	4.7 ± 0.8	7.0 ± 0.5	5.8 ± 1.0	P < 0.001
Albumin (g/dl)	4.3 ± 0.4	2.2 ± 0.8	4.1 ± 0.6	3.2 ± 0.8	P < 0.001
Total cholesterol (mg/dl)	184.7 ± 38.3	442.5 ± 123.2	206.1 ± 27.7	247.3 ± 50.8	P < 0.001
Triglyceride (mg/dl)	100.1 ± 84.4	230.8 ± 140.9	124.8 ± 63.7	175.8 ± 75.0	P = 0.001
HDL cholesterol (mg/dl)	55.1 ± 82.8	82.8 ± 16.8	53.7 ± 10.8	46.0 ± 10.8	P < 0.001
LDL cholesterol (mg/dl)	106.1 ± 30.2	298.2 ± 123.6	127.6 ± 26.0	161.8 ± 47.6	P < 0.001
CRP (mg/dl)	0.3 ± 0.9	0.3 ± 0.8	0.3 ± 0.2	0.1 ± 0.2	NS
Global glomerulosclerosis (%)	2.2 ± 4.2	2.5 ± 4.5	22.9 ± 15.9	33.2 ± 17.4	P < 0.001
Tubulointerstitial fibrosis (%)	0.6 ± 1.4	0.1 ± 0.5	15.5 ± 9.5	38.6 ± 18.6	P < 0.001

MGA: minor glomerular abnormality, MCNS: minimal change nephrotic syndrome, ORG: obesity-related glomerulopathy, DN: diabetic nephropathy, BMI: body mass index, BUN: blood urea nitrogen, CRP: C-reactive protein. Data are means ± SD. *Overall differences between MGA, MCNS, ORG and DN groups were compared by ANOVA.

doi:10.1371/journal.pone.0088942.t001

MRP8 treatment of macrophages

Bone marrow-derived macrophages were generated from wild-type or TLR4 knockout (KO) mice [18] on C57BL/6J genetic background (Oriental BioService, Kyoto, Japan) as described previously [6]. Briefly, following lysis of red blood cells, bone marrow cells were resuspended in medium containing 20% fetal calf serum and 50 ng/ml recombinant human macrophage colony-stimulating factor (Peprotech, Rocky Hill, NJ, USA), and cultured at 37°C in 5% CO₂ atmosphere. On day 7, macrophages were incubated with recombinant mouse MRP8 (Abnova, Taipei, Taiwan) or vehicle for 4 hours. Polymyxin B (25 µg/ml, Nacalai Tesque, Kyoto, Japan) was added to each well to minimize contamination of endotoxin as described previously [3,19]. No endotoxin was detected at any concentration of MRP8 tested here after incubation with 25 µg/ml of polymyxin B by ToxinSensor Chromogenic LAL Endotoxin Assay Kit (GenScript, Piscataway, NJ, USA). Total RNA from cells was extracted with RNeasy Mini Kit, and mRNA expression levels of interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNFα) and MRP8 were determined by TaqMan real-time RT-PCR. Expression levels of all genes were normalized by rodent GAPDH levels (internal control, Pre-Developed TaqMan Assay Reagents). Primer and Probe sequences for real-time PCR are listed in Table S2 in File S1.

Statistical analysis

Data are expressed as means ± SD, or means ± 95% confidence interval (CI) when appropriate. For the comparison among four groups, one-way or two-way ANOVA with Bonferroni[†] post-hoc analysis was used, and categorical variables were compared using χ^2 test. Student's unpaired t-test was applied for comparison between two groups as appropriate. Spearman's correlation coefficients were estimated to determine associations between two variables. To examine the effects of baseline covariates determining the extent of glomerular or tubulointerstitial MRP8 expression or urinary protein levels one year after biopsy, univariate and multivariate linear regression analyses were performed. Logistic regression analysis was used to analyze explanatory variables predicting the occurrence of renal event. All data were analyzed using StatView 5.0 software (SAS Institute Inc., Cary, NC, USA). P values < 0.05 were considered statistically significant.

Results

We compared MRP8 protein expression levels in the kidneys among DN, ORG and non-obese, non-diabetic control (MGA and MCNS) groups. Immunohistochemical analysis revealed that both glomerular MRP8-positive cell count (Fig. 1A) and tubulointerstitial MRP8-positive area (Fig. 1B) in DN were significantly larger than those in other groups including MGA, MCNS and ORG (P <

Table 2. Baseline clinical characteristics of patients at renal biopsy who were analyzed for MRP8 mRNA expression by real-time RT-PCR.

	MGA	DN	Between-group differences*
N	6	22	
Sex (male/female)	0/6	15/7	$\chi^2 = 8.8$, $P = 0.003$
Age (years)	38.3 ± 10.4	57.0 ± 11.1	$P = 0.001$
Diabetes duration (years)	-	14.1 ± 6.8	-
RAS blockade (yes/no)	0/0	18/4	-
BMI (kg/m ²)	19.4 ± 2.0	25.0 ± 3.8	$P = 0.002$
HbA1c (NGSP, %)	-	7.9 ± 2.0	-
Systolic blood pressure (mmHg)	111.2 ± 13.5	157.9 ± 30.1	$P = 0.001$
Diastolic blood pressure (mmHg)	67.3 ± 9.0	86.8 ± 11.7	$P = 0.001$
Urinary protein (g/g creatinine)	0.10 ± 0.11	4.87 ± 4.09	$P = 0.009$
Creatinine (mg/dl)	0.6 ± 0.2	1.1 ± 0.4	$P = 0.004$
eGFR (ml/min/1.73 m ²)	92.8 ± 26.6	57.8 ± 27.2	$P = 0.009$
Total protein (g/dl)	7.0 ± 0.4	6.2 ± 0.9	NS
Albumin (g/dl)	4.2 ± 0.4	3.2 ± 0.7	$P = 0.002$
Total cholesterol (mg/dl)	213.5 ± 57.2	240.1 ± 71.2	NS
Triglyceride (mg/dl)	172.0 ± 161.4	241.0 ± 137.7	NS
HDL cholesterol (mg/dl)	61.3 ± 12.5	52.5 ± 12.0	NS
LDL cholesterol (mg/dl)	117.8 ± 33.6	137.3 ± 59.1	NS

RAS: renin-angiotensin system. Data are means ± SD. *Differences between MGA and DN groups were compared by unpaired t-test.
doi:10.1371/journal.pone.0088942.t002

0.01). ORG subjects also showed a tendency of elevated MRP8 expression compared to MGA and MCNS (Fig. 1A, 1B). Furthermore, glomerular MRP8 mRNA expression levels in DN subjects were significantly higher compared to non-DM control subjects ($P < 0.01$, Fig. 1C). In non-glomerulus tissues, MRP8 mRNA expression levels were much lower than those in glomeruli, both in non-DM and DM groups. Abundant MRP8 protein expression in the tubulointerstitium of DN cases was not clearly reflected into increased mRNA expression, which may be partly caused by deposition of blood-derived proteins in the tubulointerstitium as discussed in the next section. As shown in representative photos (Fig. 1D–G, see Fig. S4 in detail), renal biopsy samples from MGA and MCNS subjects showed few MRP8-positive cells in glomeruli (Fig. 1D, 1E and Fig. S4). In ORG subjects, some MRP8-positive cells appeared in glomeruli and tubulointerstitium (Fig. 1F and Fig. S4). In DN subjects, marked increase of MRP8-expressing cells in glomeruli and significant expansion of MRP8-positive areas in the tubulointerstitium were observed in a focal manner (Fig. 1G and Fig. S4). Of note, MRP8-positive cells were absent in nodular sclerosing lesions of diabetic glomeruli (Fig. S4: DN case 2, 3) as described previously for sclerotic lesions in ANCA-associated glomerulonephritis [20]. Paired immunohistochemistry for CD68 and MRP8 in serial sections suggested that MRP8 signals were, at least in part, observed in macrophages expressing CD68 (Fig. 2), as we reported in a mouse model of diabetic nephropathy [6]. Besides, focally injured atrophic tubular epithelial cells also strongly expressed MRP8, which were surrounded by MRP8(+), CD68(+)-positive macrophages (Fig. 2, Fig S4: DN case 3–5). In the cases with nephrotic range proteinuria, MRP8 staining was also observed along brush borders of proximal tubules both in MCNS and DN cases (Fig. S4). Since sample number of mRNA expression was too small for multivariate

analysis, the following analyses were performed using data from patients studied by immunohistochemistry.

The associations between kidney MRP8 signals and baseline clinical parameters at the time of renal biopsy were analyzed cross-sectionally (Table 3). By univariate analysis, glomerular and/or tubulointerstitial MRP8 protein expression was significantly correlated to age, systolic and diastolic blood pressures, urinary protein, serum levels of creatinine, BUN and HDL cholesterol, eGFR, and extent of global glomerulosclerosis and tubulointerstitial fibrosis. These parameters were further examined by multivariate analysis after excluding diastolic blood pressure, eGFR and BUN because of collinearity. Percentage of tubulointerstitial fibrosis was independently correlated with glomerular MRP8 signals ($\beta = 0.62$, adjusted $P = 0.02$) and tubulointerstitial MRP8 signals ($\beta = 0.85$, adjusted $P < 0.001$), respectively. Additionally, tubulointerstitial MRP8 signals were also independently correlated with baseline proteinuria ($\beta = 0.20$, adjusted $P = 0.01$).

Scattered plot analyses between MRP8 signals in glomeruli or tubulointerstitium and clinical parameters indicated that MCNS group had a distinct distribution pattern from other groups, especially as to urinary protein and serum LDL cholesterol levels (Fig. S5A–D, S6A–D). Exclusion of MCNS group improved correlation between MRP8 signals and urinary protein or serum LDL-cholesterol levels (Fig. S5E–F, S6E–F). Therefore, we carried out sub-analysis excluding MCNS patients, and found that urinary protein was an independent factor correlated with glomerular MRP8 signals by multivariate analysis (Table 4; $\beta = 0.36$, adjusted $P = 0.03$).

Next, we performed linear regression or logistic regression analyses to identify explanatory factors predicting renal outcomes which were extent of proteinuria a year later and renal event within a year. Since there was a good association between glomerular and tubulointerstitial MRP8 signals (Fig. S7; $R = 0.67$,

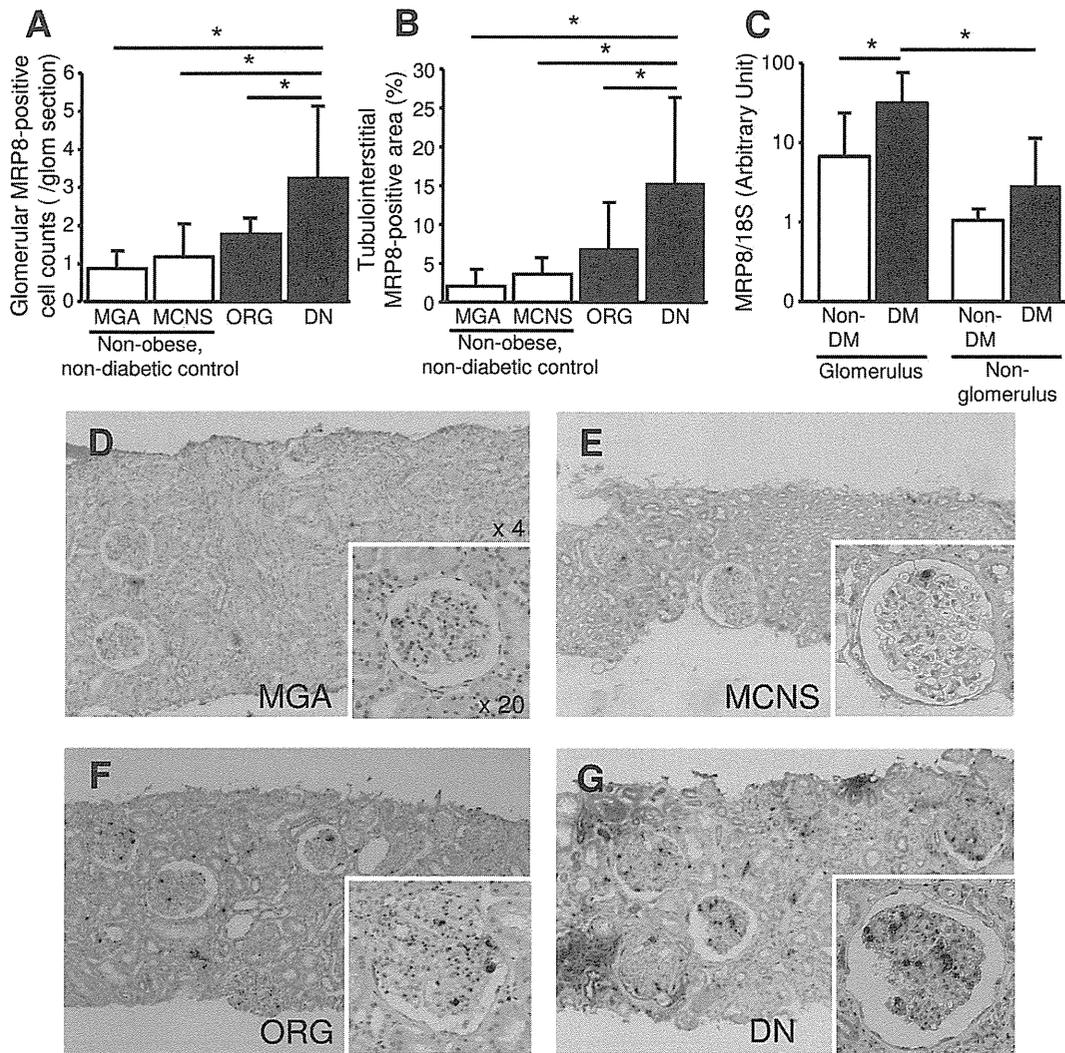


Figure 1. Immunohistochemical and mRNA analyses for MRP8 in kidney biopsy samples. Quantification of glomerular MRP8-positive cell count (A) and tubulointerstitial MRP8-positive area (B). mRNA expression of MRP8 in glomerular and non-glomerular fractions (C). Open bars: non-obese, non-diabetic controls which are MGA or MCNS, closed bars: ORG or DN (A–C). Representative pictures of MGA, MCNS, ORG and DN groups (D–G). MGA: minor glomerular abnormality, MCNS: minimal change nephrotic syndrome, ORG: obesity-related glomerulopathy, DN: diabetic nephropathy. * $P < 0.01$.

doi:10.1371/journal.pone.0088942.g001

$P < 0.001$), these parameters were alternatively enrolled in further analyses. We evaluated association between baseline parameters and urinary protein at 1 year after renal biopsy by multiple regression analysis. As shown in Table 5, glomerular MRP8 signal ($\beta = 0.59$, adjusted $P < 0.001$) was a predictive factor for the extent of proteinuria a year later, as well as baseline systolic blood pressure ($\beta = 0.21$, adjusted $P = 0.04$) and baseline proteinuria ($\beta = 0.37$, adjusted $P = 0.002$) were. These parameters were independent from other known diabetic nephropathy risk factors including renal dysfunction (serum creatinine) and extent of global sclerosis and tubulointerstitial fibrosis [11,21–24]. On the other hand, tubulointerstitial MRP8 signal ($\beta = 0.34$, adjusted $P = 0.09$) was not an independent predictive factor for urinary protein levels a year later. Renal events occurred in 7 patients (6 in DN and 1 in ORG cases) within a year after renal biopsy. By univariate analysis, not only extent of glomerulosclerosis and tubulointerstitial fibrosis, and glomerular and tubulointerstitial MRP8 signals, but also blood pressures, renal dysfunction and urinary protein levels

at baseline were significant predictive factors for the occurrence of renal events. However, by multivariate analysis, these covariates were cancelled out by each other (Table S3 in File S1), likely due to high correlations among these parameters.

Finally, we examined the potency of MRP8 as an endogenous ligand for TLR4 using cultured macrophages. In bone marrow-derived macrophages from wild-type mice, MRP8 protein induced upregulation of proinflammatory cytokine genes such as IL-1 β and TNF α and also triggered auto-induction of MRP8, in a dose-dependent manner between 10–1000 ng/ml. These effects of MRP8 were suppressed approximately by two-thirds in macrophages obtained from TLR4 KO mice ($P < 0.01$) (Fig. 3).

Discussion

The present study has demonstrated that MRP8 is abundantly expressed in glomeruli and tubulointerstitium of patients with DN as compared to ORG and non-obese, non-diabetic control (MGA and MCNS). Furthermore, in ORG subjects, MRP8 expression

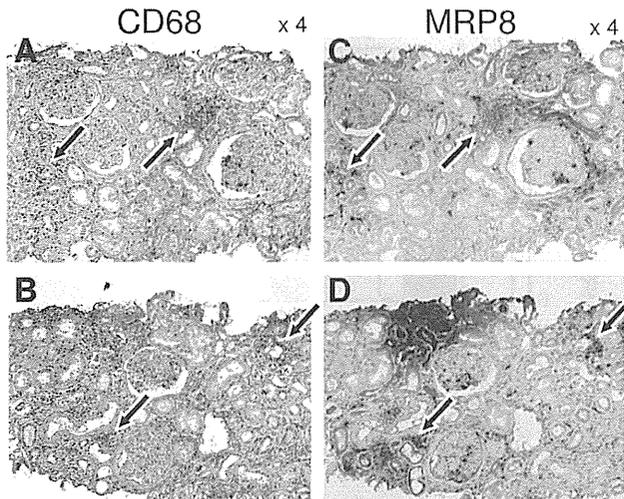


Figure 2. Localization of CD68 and MRP8 protein expression in serial sections of diabetic nephropathy cases. Expression of CD68 (A, B) and MRP8 expression (C, D) in paired renal specimens (A and C, or B and D). Arrows indicate colocalization of CD68 and MRP8 signals. doi:10.1371/journal.pone.0088942.g002

levels tended to be higher than MGA or MCNS subjects. In baseline cross-sectional investigation including all subjects, by univariate linear regression analysis, glomerular MRP8-positive cell count and tubulointerstitial MRP8-positive area were both,

respectively, correlated not only with various known risk factors for diabetic nephropathy (such as systolic blood pressure, proteinuria and serum creatinine) but also with extent of glomerulosclerosis and tubulointerstitial fibrosis. By multivariate analysis, tubulointerstitial MRP8-positive area was significantly correlated with proteinuria and tubulointerstitial fibrosis. Glomerular MRP8-positive cell count was significantly correlated with tubulointerstitial fibrosis in the primary analysis, and with proteinuria in a sub-analysis excluding MCNS group. Immunohistochemistry indicated that MRP8 was expressed, at least partly, by CD68(+)-expressing macrophages and atrophic tubules. These findings raise a possibility that kidney MRP8 signals in glomeruli or tubulointerstitium may serve as novel markers of diabetic nephropathy.

In prognostic study, multivariate analysis revealed that urinary protein levels at a year after renal biopsy were independently associated with glomerular MRP8-positive cell count, urinary protein and systolic blood pressure at baseline. Of note, glomerular MRP8 expression showed the strongest correlation with urinary protein a year later ($\beta=0.87$), even stronger than baseline urinary protein ($\beta=0.78$), by univariate analysis. It is partly because glomerular MRP8 expression is not largely elevated in 'benign' forms of proteinuria such as ones observed in MCNS patients, whose levels of proteinuria are extremely high at diagnosis by renal biopsy but are usually resolved within a year after initiation of immunosuppressive therapy. These findings suggest that glomerular MRP8 expression may possess a unique predictive nature as a disease marker, which cannot be substituted by baseline proteinuria or routine pathological analysis evaluating

Table 3. Relationship between baseline clinical parameters and MRP8 signals.

	Glomerular MRP8-positive cell count				Tubulointerstitial MRP8-positive area			
	Univariate		Multivariate*		Univariate		Multivariate [#]	
	β	P	β	P	β	P	β	P
Sex (male)	0.21	0.09			0.16	0.20		
Age (y)	0.37	0.002	0.02	0.87	0.39	0.001	0.03	0.74
Diabetes duration (y)	0.28	0.14			0.33	0.08		
BMI (kg/m ²)	0.14	0.27			0.09	0.48		
HbA1c (NGSP, %)	0.11	0.52			0.04	0.82		
Systolic BP (mmHg)	0.62	<0.001	0.18	0.29	0.64	<0.001	-0.12	0.37
Diastolic BP (mmHg)	0.43	<0.001			0.42	<0.001		
Urinary protein (g/gCr)	0.37	0.003	0.18	0.07	0.43	<0.001	0.20	0.01
Creatinine (mg/dl)	0.60	<0.001	0.01	0.10	0.75	<0.001	0.20	0.08
eGFR (ml/min/1.73 m ²)	-0.49	<0.001			-0.70	<0.001		
BUN (mg/dl)	0.56	<0.001			0.71	<0.001		
Total protein (g/dl)	-0.16	0.21			-0.13	0.30		
Albumin (g/dl)	-0.22	0.09			-0.21	0.09		
T-chol (mg/dl)	-0.06	0.66			-0.04	0.76		
Triglyceride (mg/dl)	0.02	0.84			0.11	0.38		
HDL-chol (mg/dl)	-0.24	0.06			-0.28	0.03	-0.03	0.73
LDL-chol (mg/dl)	0.00	0.98			0.00	0.99		
CRP (mg/dl)	0.03	0.79			0.12	0.34		
Global GS (%)	0.52	<0.001	-0.17	0.40	0.61	<0.001	-0.27	0.07
TI fibrosis (%)	0.68	<0.001	0.62	0.02	0.80	<0.001	0.85	<0.001

Coefficient of determination (R^2) calculated with explanatory parameters enrolled in multiple regression analysis was 0.52* and 0.74[#], respectively. y, years; BP, blood pressure; gCr, g creatinine; T-chol, total cholesterol; HDL-chol, HDL cholesterol; LDL-chol, LDL cholesterol; GS, glomerulosclerosis; TI, tubulointerstitial. doi:10.1371/journal.pone.0088942.t003

Table 4. Sub-analysis of relationship between baseline clinical parameters and MRP8 signals, after exclusion of MCNS group.

	Glomerular MRP8(+) cell count				Tubulointerstitial MRP8(+) area			
	Univariate		Multivariate*		Univariate		Multivariate [#]	
	β	P	β	P	β	P	β	P
Systolic BP (mmHg)	0.64	<0.001	0.16	0.38	0.63	<0.001	-0.16	0.23
Urinary protein (g/gCr)	0.70	<0.001	0.36	0.03	0.75	<0.001	0.47	<0.001
Creatinine (mg/dl)	0.62	<0.001	0.05	0.80	0.75	<0.001	0.24	0.09
Total protein (g/dl)	-0.51	<0.001	0.03	0.84	-0.49	<0.001	0.12	0.25
Triglyceride (mg/dl)	0.31	0.003			0.37	0.01		
HDL-chol (mg/dl)	-0.16	0.27			-0.20	0.18		
LDL-chol (mg/dl)	0.60	<0.001	0.13	0.35	0.52	0.001	-0.10	0.35
Global GS (%)	0.49	<0.001	-0.20	0.29	0.59	<0.001	-0.17	0.23
TI fibrosis (%)	0.68	<0.001	0.38	0.18	0.80	<0.001	0.70	0.002

R² was 0.60* and 0.77[#], respectively. BP, blood pressure; gCr, g creatinine; HDL-chol, HDL cholesterol; LDL-chol, LDL cholesterol; GS, glomerulosclerosis; TI, tubulointerstitial.

doi:10.1371/journal.pone.0088942.t004

Table 5. Multiple regression analysis for identification of factors predicting urinary protein levels 1 year after renal biopsy.

	Urinary protein 1-year after renal biopsy					
	Univariate		Multivariate			
	β	P	Model 1*		Model 2 [#]	
	β	P	β	P	β	P
Sex (male)	-0.14	0.50				
Age (y)	-0.01	0.95				
Diabetes duration (y)	0.24	0.22				
BMI (kg/m ²)	-0.33	0.10				
HbA1c (NGSP, %)	0.08	0.70				
Systolic BP (mmHg)	0.43	0.03	0.21	0.04	0.30	0.10
Diastolic BP (mmHg)	0.07	0.73				
Urinary protein (g/gCr)	0.78	<0.001	0.37	0.002	0.55	0.002
Creatinine (mg/dl)	0.44	0.02				
eGFR (ml/min/1.73 m ²)	-0.56	0.003	-0.31	0.76	0.18	0.45
BUN (mg/dl)	0.46	0.02				
Total protein (g/dl)	-0.54	0.004				
Albumin (g/dl)	-0.69	<0.001				
T-chol (mg/dl)	0.66	<0.001				
Triglyceride (mg/dl)	0.18	0.38				
HDL-chol (mg/dl)	-0.02	0.91				
LDL-chol (mg/dl)	0.62	<0.001				
CRP (mg/dl)	-0.11	0.57				
Global glomerulosclerosis (%)	-0.05	0.79	-0.17	0.11	-0.31	0.06
Tubulointerstitial fibrosis (%)	0.43	0.02	-0.02	0.91	0.10	0.67
Glomerular MRP8(+) cell count	0.87	<0.001	0.59	<0.001		
Tubulointerstitial MRP8(+) area (%)	0.67	0.001			0.34	0.09

R² was 0.91* and 0.75[#], respectively. y, years; BP, blood pressure; gCr, g creatinine; T-chol, total cholesterol; HDL-chol, HDL cholesterol; LDL-chol, LDL cholesterol.

doi:10.1371/journal.pone.0088942.t005

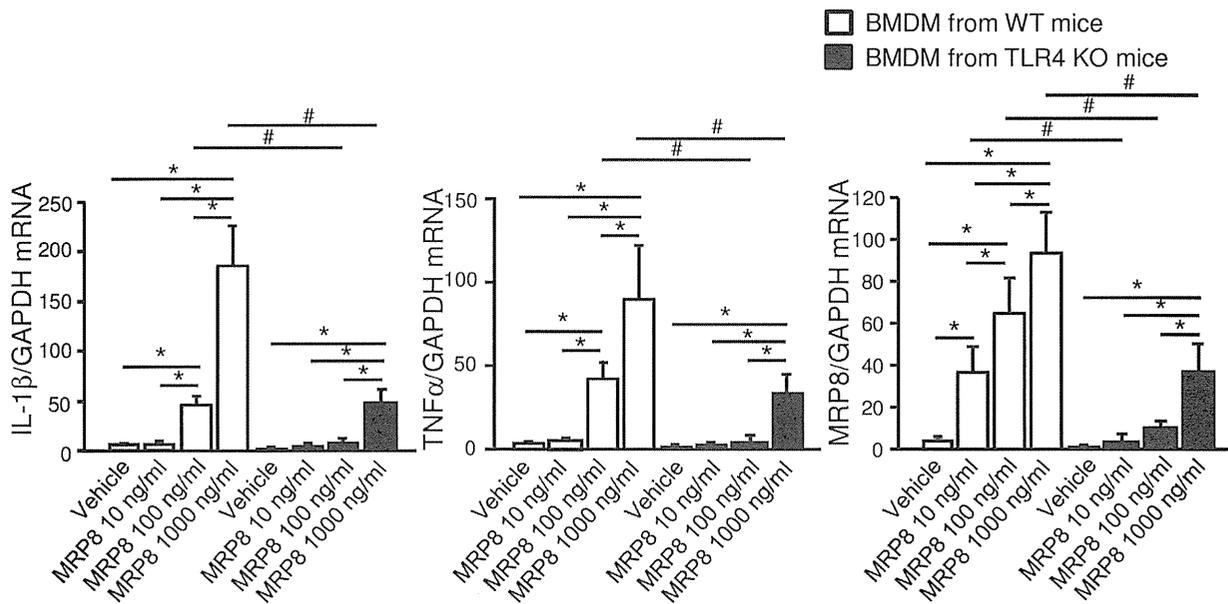


Figure 3. Effects of MRP8 upon bone marrow-derived macrophages. Bone marrow-derived macrophages (BMDM) were stimulated with recombinant mouse MRP8 for 4 hours. Error bars indicate 95% CI and statistical analyses were performed with log-transformed values. Two-way ANOVA revealed significant effects of genotypes, MRP8 concentrations and their interactions for expression of all 3 genes ($P < 0.001$ for all comparisons). $n = 4$. WT, wild-type; KO, knockout; IL-1 β , interleukin 1 beta; TNF α , tumor necrosis factor alpha. * $P < 0.01$ among different concentrations, # $P < 0.01$ among genotypes. doi:10.1371/journal.pone.0088942.g003

global glomerulosclerosis and tubulointerstitial fibrosis. Moreover, we speculate that glomerular MRP8 expression is not a simple marker or bystander but an active player in glomerular injury as discussed below.

In another attempt of longitudinal study, logistic regression analysis failed to find out any independent predictors for the occurrence of renal event within a year. We speculate that this is partly because tubulointerstitial MRP8 expression and tubulointerstitial fibrosis were two potent predictors for renal event in univariate analysis but their significances were canceled by each other in multivariate analysis. These two parameters showed strong correlation ($R = 0.68$, $P < 0.001$) (Fig. S8), suggesting that these two parameters might be equivalent to predict renal event. Indeed, interstitial MRP8 expression showed quite similar pattern to interstitial fibrosis evaluated by Masson trichrome staining. Quantity of interstitial MRP8 largely depends on the positive signals in atrophic tubules rather than those in macrophages, whose feature differs from that of glomerular MRP8 in punctate distribution. Furthermore, small sample size, short observation period and few subjects who developed renal event might have reduced the detection power. Since MRP8 expression in tubular epithelial cells plays a causative role in the progression of tubulointerstitial inflammation in a mouse model of renal fibrosis [7], further analysis will be needed to clarify the role of tubulointerstitial MRP8 in DN.

In accordance with our previous study [6], MRP8 mRNA was upregulated predominantly in the glomerular fraction of human DN subjects as compared to control subjects with MGA. On the other hand, MRP8 protein expression was observed not only in the glomerulus but also in the tubulointerstitium. In this regard, it should be noted that there were two distinct patterns of MRP8 staining in the tubulointerstitium of DN. One was intense and focal staining in severely atrophic tubules. The other was mild staining distributed along the brush border of proximal tubules, which was also found in ORG and MCNS. The latter signals likely

represent MRP8 protein derived from the blood and reabsorbed by proximal tubules, which should not be accompanied by increased MRP8 mRNA expression. Concerning proteins other than MRP8, we and others have recently reported similar phenomena of immunoreactive protein detection in the proximal tubules caused by reabsorption but not by renal synthesis [25,26]. On the other hand, since a little MRP8 staining remained in antibody absorption test, especially in glomerular exudative lesions and severely-scarred, fibrotic lesions around atrophic tubules, presence of non-specific signals cannot be completely negated (Fig. S3).

Glomerular MRP8 signals mainly showed punctuate pattern in DN subjects (Fig. 1, Fig. S4). Since both of CD68 and MRP8 were detected by mouse monoclonal antibodies, localization of these molecules were evaluated by serial sections, not by double-immunostaining. Staining patterns of MRP8 were compatible with those in other inflammatory renal disorders including IgA nephritis [27], membrano-proliferative glomerulonephritis [14], and ANCA-related glomerulonephritis [20], in which macrophages were suggested as a major source of MRP8, as we reported in a rodent model [6]. In addition, neutrophils could be considered as another source of MRP8 affecting vascular complications [28]. Currently, we are investigating the molecular mechanism why MRP8 is predominantly upregulated in myeloid-lineage cells infiltrating glomeruli. In vitro study revealed that MRP8 induced inflammatory cytokine expression and also potentiated expression of MRP8 itself in macrophages in a TLR4-dependent manner. Additionally, MRP8-positive cells were absent in nodular sclerosing lesion, suggesting that glomerular MRP8 might reflect ongoing glomerular damage [20]. Importantly, a largest-scale human study reported that MRP8 gene expression in blood mononuclear cells of type 1 diabetic patients is significantly elevated in subjects with diabetic complications including nephropathy [29].

Since inhibition of renin-angiotensin system (RAS) is an important determinant of renal outcomes, we examined the effects of RAS blockade on kidney MRP8 expression. We found no significant

difference in kidney MRP8 mRNA expression between DN patients treated with or without RAS blockade (Fig. S9), probably because cases treated with RAS blockade tended to have more severe hypertension and proteinuria than cases without RAS blockade.

In obese humans and mice, increased plasma MRP8/14 complex may reflect a degree of obesity and originate from adipocytes as well as leukocytes [8,9]. Our ORG cases had mild staining of MRP8 in proximal tubules, suggesting increased plasma levels of MRP8. In contrast, there was no significant correlation between tubulointerstitial MRP8 expression and body mass index (Fig. S6G). Therefore, local MRP8 expression in the kidney may serve better as a marker for renal injury rather than for obesity [8,9].

Our study has several limitations. Sample number of each group studied was small. Non-identical subjects were enrolled in the mRNA and immunohistochemical analyses. Since we only analyzed patients who underwent renal biopsy, the composition of patients investigated here may not reflect those in general type 2 diabetic patients or in general chronic kidney disease subjects. Although age was not retained as an independent factor associated with MRP8 signals in our data (Table 3), it is known that aging associates chronic inflammation [30]. The effects of age cannot be completely neglected. Although most MRP8 signals were lost in antibody absorption test, there were some positive signals remaining, which might be caused by a non-specific binding of the first antibody. As discussed above, investigation of renal MRP8 expression by renal biopsy helps us understand the pathophysiology and prognosis of chronic kidney diseases, especially associated with obesity and diabetes, but has a disadvantage for routine and repeated use in out-patient clinics.

In summary, the present study suggests that expression of MRP8 in the kidney reflects the current pathological status and also predicts renal outcomes in patients with obesity or type 2 diabetes. Further investigations studying urinary MRP8 levels among obese or diabetic patients in large scale may be warranted.

Supporting Information

Figure S1 Representative photos showing renal biopsy sections of a DN patient stained with (A) periodic acid-Schiff, (B) periodic-acid methenamine silver or (C) Masson trichrome. The ratio of the number of glomeruli with global sclerosis (arrows) among that of total glomeruli and the relative area of tubulointerstitial fibrosis were 33% and 65%, respectively, in this patient.
(TIFF)

Figure S2 Immunohistochemistry for MRP8 and CD68 proteins in DN patients. Photos in the right column show negative control experiments without 1st antibody.
(TIF)

Figure S3 Antibody absorption test for MRP8 staining. PBS: phosphate buffered saline, rhMRP8: recombinant human MRP8.
(TIF)

References

1. Donath MY, Shoelson SE (2011) Type 2 diabetes as an inflammatory disease. *Nat Rev Immunol* 11: 98–107.
2. Odink K, Cerletti N, Bruggen J, Clerc RG, Tarcsay L, et al. (1987) Two calcium-binding proteins in infiltrate macrophages of rheumatoid arthritis. *Nature* 330: 80–82.
3. Vogl T, Tenbrock K, Ludwig S, Leukert N, Ehrhardt C, et al. (2007) Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. *Nat Med* 13: 1042–1049.
4. Croce K, Gao H, Wang Y, Mooroka T, Sakuma M, et al. (2009) Myeloid-related protein-8/14 is critical for the biological response to vascular injury. *Circulation* 120: 427–436.
5. Loser K, Vogl T, Voskort M, Lueken A, Kupas V, et al. (2010) The Toll-like receptor 4 ligands Mrp8 and Mrp14 are crucial in the development of autoreactive CD8⁺ T cells. *Nat Med* 16: 713–717.
6. Kuwabara T, Mori K, Mukoyama M, Kasahara M, Yokoi H, et al. (2012) Exacerbation of diabetic nephropathy by hyperlipidaemia is mediated by Toll-like receptor 4 in mice. *Diabetologia* 55: 2256–2266.

Figure S4 Representative photos of MRP8 expression in MGA, MCNS, ORG and DN groups.
(TIF)

Figure S5 Correlation between glomerular MRP8-positive cell count and clinical parameters. The log-transformed values of MRP8 signals were used. The correlations were analyzed using all of 4 groups (A–D, G) or 3 groups excluding MCNS group (E, F). Open circles: minor glomerular abnormality (MGA), closed circles: minimal change nephrotic syndrome (MCNS), open triangles: obesity-related glomerulopathy (ORG), closed triangles: diabetic nephropathy (DN).
(TIF)

Figure S6 Correlation between tubulointerstitial MRP8-positive area and clinical parameters. The log-transformed values of MRP8 signals were used. These correlations were analyzed using all of the 4 groups (A–D, G) or 3 groups excluding MCNS group (E, F).
(TIF)

Figure S7 Correlation between glomerular and tubulointerstitial MRP8 expression. The log-transformed values of MRP8 signals were used.
(TIF)

Figure S8 Correlation between tubulointerstitial MRP8-positive area and tubulointerstitial fibrosis. The log-transformed values of MRP8 signals were used.
(TIF)

Figure S9 Renal mRNA expression of MRP8 in DN patients with or without renin-angiotensin blockade. N.S.: not significant. n = 15 (Yes), 6 (No). Among 22 DN cases, information about medication was not available in one patient.
(TIF)

File S1 Supporting Tables. Table S1, Pathological diagnoses of all cases who underwent renal biopsy at Department of Medicine and Clinical Science, Kyoto University Hospital between 2000 and 2011. Table S2, Primer and probe sequences for TaqMan real-time RT-PCR. Table S3, Logistic regression analysis for the occurrence of renal event within a year.
(DOC)

Acknowledgments

We gratefully acknowledge S. Tanaka for statistical advice, Y. Ogawa, N. Igarashi and C. Kimura for technical assistance, and A. Yamamoto and S. Ogino for secretary assistance.

Author Contributions

Conceived and designed the experiments: TK KM MK HY MI AN KN MM. Performed the experiments: TK HI AI KK TM YK MI AN. Analyzed the data: TK KM MK HY AS SY KU KN MM. Contributed reagents/materials/analysis tools: TK KM. Wrote the paper: TK KM MM.

7. Fujii K, Manabe I, Nagai R (2011) Renal collecting duct epithelial cells regulate inflammation in tubulointerstitial damage in mice. *J Clin Invest* 121: 3425–3441.
8. Sekimoto R, Kishida K, Nakatsuji H, Nakagawa T, Funahashi T, et al. (2012) High circulating levels of S100A8/A9 complex (calprotectin) in male Japanese with abdominal adiposity and dysregulated expression of S100A8 and S100A9 in adipose tissues of obese mice. *Biochem Biophys Res Commun* 419: 782–789.
9. Mortensen OH, Nielsen AR, Erikstrup C, Plomgaard P, Fischer CP, et al. (2009) Calprotectin—a novel marker of obesity. *PLoS One* 4: e7419.
10. Matsuo S, Imai E, Horio M, Yasuda Y, Tomita K, et al. (2009) Revised equations for estimated GFR from serum creatinine in Japan. *Am J Kidney Dis* 53: 982–992.
11. Gross JL, de Azevedo MJ, Silveiro SP, Canani LH, Caramori ML, et al. (2005) Diabetic nephropathy: diagnosis, prevention, and treatment. *Diabetes Care* 28: 164–176.
12. Kambham N, Markowitz GS, Valeri AM, Lin J, D'Agati VD (2001) Obesity-related glomerulopathy: an emerging epidemic. *Kidney Int* 59: 1498–1509.
13. Praga M, Morales E (2006) Obesity, proteinuria and progression of renal failure. *Curr Opin Nephrol Hypertens* 15: 481–486.
14. Kawasaki Y, Hosoya M, Takahashi A, Isome M, Tanji M, et al. (2005) Myeloid-related protein 8 expression on macrophages is a useful prognostic marker for renal dysfunction in children with MPGN type 1. *Am J Kidney Dis* 45: 510–518.
15. Nishiyama A, Konishi Y, Ohashi N, Morikawa T, Urushihara M, et al. (2011) Urinary angiotensinogen reflects the activity of intrarenal renin-angiotensin system in patients with IgA nephropathy. *Nephrol Dial Transplant* 26: 170–177.
16. Ogawa Y, Mukoyama M, Yokoi H, Kasahara M, Mori K, et al. (2012) Natriuretic peptide receptor guanylyl cyclase-A protects podocytes from aldosterone-induced glomerular injury. *J Am Soc Nephrol* 23: 1198–1209.
17. Yokoi H, Mukoyama M, Mori K, Kasahara M, Suganami T, et al. (2008) Overexpression of connective tissue growth factor in podocytes worsens diabetic nephropathy in mice. *Kidney Int* 73: 446–455.
18. Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, et al. (1999) Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J Immunol* 162: 3749–3752.
19. Suganami T, Tanimoto-Koyama K, Nishida J, Itoh M, Yuan X, et al. (2007) Role of the Toll-like receptor 4/NF-kappaB pathway in saturated fatty acid-induced inflammatory changes in the interaction between adipocytes and macrophages. *Arterioscler Thromb Vasc Biol* 27: 84–91.
20. Pepper RJ, Hamour S, Chavele KM, Todd SK, Rasmussen N, et al. (2013) Leukocyte and serum S100A8/S100A9 expression reflects disease activity in ANCA-associated vasculitis and glomerulonephritis. *Kidney Int* 83: 1150–1158.
21. Ravid M, Brosh D, Ravid-Safran D, Levy Z, Rachmani R (1998) Main risk factors for nephropathy in type 2 diabetes mellitus are plasma cholesterol levels, mean blood pressure, and hyperglycemia. *Arch Intern Med* 158: 998–1004.
22. Adler AI, Stratton IM, Neil HA, Yudkin JS, Matthews DR, et al. (2000) Association of systolic blood pressure with macrovascular and microvascular complications of type 2 diabetes (UKPDS 36): prospective observational study. *Bmj* 321: 412–419.
23. Ruggenenti P, Remuzzi G (1998) Nephropathy of type-2 diabetes mellitus. *J Am Soc Nephrol* 9: 2157–2169.
24. Taft JL, Nolan CJ, Yeung SP, Hewitson TD, Martin FI (1994) Clinical and histological correlations of decline in renal function in diabetic patients with proteinuria. *Diabetes* 43: 1046–1051.
25. Matsusaka T, Niimura F, Shimizu A, Pastan I, Saito A, et al. (2012) Liver angiotensinogen is the primary source of renal angiotensin II. *J Am Soc Nephrol* 23: 1181–1189.
26. Kuwabara T, Mori K, Mukoyama M, Kasahara M, Yokoi H, et al. (2009) Urinary neutrophil gelatinase-associated lipocalin levels reflect damage to glomeruli, proximal tubules, and distal nephrons. *Kidney Int* 75: 285–294.
27. Kawasaki Y, Suyama K, Go H, Imamura T, Ushijima Y, et al. (2009) Accumulation of macrophages expressing myeloid-related protein 8 associated with the progression of sclerotic changes in children with IgA nephropathy. *Tohoku J Exp Med* 218: 49–55.
28. Nagareddy PR, Murphy AJ, Stürzaker RA, Hu Y, Yu S, et al. (2013) Hyperglycemia promotes myelopoiesis and impairs the resolution of atherosclerosis. *Cell Metab* 17: 695–708.
29. Jin Y, Sharma A, Carey C, Hopkins D, Wang X, et al. (2013) The Expression of Inflammatory Genes Is Upregulated in Peripheral Blood of Patients with Type 1 Diabetes. *Diabetes Care*.
30. Longo VD, Finch CE (2003) Evolutionary medicine: from dwarf model systems to healthy centenarians? *Science* 299: 1342–1346.

