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Leptin deficiency down-regulates IL-23 production in glomerular podocytes resulting in an attenuated immune response in nephrotoxic serum nephritis

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Abstract

Leptin, one of the typical adipokines, is reported to promote T_h17 cell responses and to enhance production of proinflammatory cytokines. To clarify the role of leptin in the regulation of the IL-23/IL-17 axis and the development of kidney disease, we used a murine model of nephrotoxic serum (NTS) nephritis (NTN). Sheep NTS was administered in wild-type C57BL/6J mice and food-restricted, leptin-deficient C57BL/6J-*ob*/*ob* (FR-*ob*/*ob*) mice after preimmunization with sheep IgG. The profile of mRNA expression relevant to T helper lymphocytes in the kidneys was analyzed by quantitative real-time PCR (qRT–PCR). Cultured murine glomerular podocytes and peritoneal exudate macrophages (PEMs) were used to investigate the direct effect of leptin on IL-23 or MCP-1 production by qRT–PCR. Kidney injury and macrophage infiltration were significantly attenuated in FR-*ob*/*ob* mice 7 days after NTS injection. The T_h17-dependent secondary immune response against deposited NTS in the glomeruli was totally impaired in FR-*ob*/*ob* mice because of deteriorated IL-17 and proinflammatory cytokine production including IL-23 and MCP-1 in the kidney. IL-23 was produced in glomerular podocytes in NTN mice and cultured murine glomerular podocytes produced IL-23 under leptin stimulation. MCP-1 production in PEMs was also promoted by leptin. Induction of MCP-1 expression was observed in PEMs regardless of Ob-Rb, and the leptin signal was transduced without STAT3 phosphorylation in PEMs. Leptin deficiency impairs the secondary immune response against NTS and down-regulates IL-23 production and T_h17 responses in the NTN kidney, which is accompanied by decreased MCP-1 production and macrophage infiltration in the NTN kidney.

Keywords: CCL2, IL-17, IL-22, Ob-R, T_h17

Introduction

Obesity is a risk factor for chronic kidney disease (CKD). The numbers of obese patients with CKD or end-stage renal disease have significantly increased worldwide (1, 2). Obesity is associated with the altered production of many adipokines and is linked to a number of pathologies (3). Leptin, one of the typical adipokines, is a 16-kDa nonglycosylated polypeptide, which is mainly produced in adipose tissue and plays an important role in the regulation of food intake, energy expenditure and adiposity (4). In addition, leptin has a variety of other functions, including the regulation of neuroendocrine function, angiogenesis, bone formation and the immune and inflammatory response (5–9). Leptin has been considered to shift the T helper balance toward a T_h1 phenotype (10, 11) and enhance susceptibility for experimentally induced autoimmune diseases including experimental autoimmune encephalomyelitis (12, 13), antigen-induced arthritis (14) and type 1 diabetes (15). Recent studies have demonstrated that leptin promotes not only T_h1 cell responses, but also T_h17 cell responses (16) and exacerbates autoimmune arthritis (14) and systemic lupus erythematosus lesions in MRL/Mp-Fas *lpr* mice (17) or NZB ×

NZW mice (18). The murine nephrotoxic serum (NTS) nephritis (NTN) model resembles human Goodpasture's disease (19) by virtue of its dependence on the targeting effect of anti-glomerular basement membrane (GBM) antibodies to direct immune complex deposition to the GBM and initiate an inflammatory response (20). It is also a leptin-dependent disease model (21), and IL-17-producing CD4$^+$ T cells (T_h17 cells), as well as γδ T cells have been shown to play important roles in the development of NTN that had been thought to be T_h1-dominant previously (22–24). IL-17 drives germinal center formation and class switch recombination (25); therefore, IL-17 is critical in the generation of a humoral immune response (25) and triggers various inflammatory diseases, including the NTN model (26). IL-23 is a potent inducer of IL-17 in murine T cells (27–29), and is also reported to be essential in the development of NTN (23), even though the producer of IL-23 in the nephritis model is still to be elucidated. Furthermore, the effect of leptin on macrophages infiltrating the nephritic kidney and playing a pivotal role as direct effector cells (20) is still unknown. In the current study, the effect of leptin on regulation of the IL-23/IL-17 axis and macrophage activation was investigated in the NTN mouse model.

Methods

Mice

Male C57BL/6J mice, male C57BL/6J-*ob/ob* mice, male BKS Cg-+*Lepr^{db}*/+*Lepr^{db}*/Jcl (*db/db*) mice and male BKS Cg-*m*+/*m*+/Jcl (*misty*) mice at 5–8 weeks of age were purchased from Charles River Japan (Yokohama, Japan) and maintained in our animal facility. *Ob/ob* mice were under food restriction (2.5 g of food per day) from 5 to 12 weeks of age. Eight hundred micrograms of pentobarbital was injected intraperitoneally for anesthesia before sacrifice. All animal experiments were performed following protocols approved by the Institutional Animal Care and Ethics Committee at Niigata University.

Induction of NTN

Sheep NTS was prepared as described previously (20). NTS was heat inactivated, and then absorbed with an excess of murine red blood cells. Twelve-week-old mice were preimmunized intraperitoneally with 200 μg of sheep IgG (AbD Serotec, Oxford, UK) in a 50:50 mix with complete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO, USA), followed by intravenous injection of sheep NTS (2.0 μl of serum per gram of mouse) 4 days later. Mice were sacrificed 7 days after NTS administration to collect blood, kidney, and spleen samples under anesthesia. Blood urea nitrogen level in sera was measured using a DetectX Urea Nitrogen Colorimetric Detection Kit (Arbor Assays, Ann Arbor, MI, USA). Serum creatinine and cystatin C concentration was measured using Creatinine Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI, USA) and Mouse/Rat Cystatin C Immunoassay (R & D Systems, Minneapolis, MN, USA), respectively. The serum leptin level was quantified using Mouse/Rat Leptin Quantikine ELISA Kit (R & D Systems).

Histological analysis

Kidneys were removed from mice, fixed in 10% buffered formalin, and embedded in paraffin. Four-micrometer paraffin sections were stained with periodic acid-Schiff (PAS) and assessed in 20 glomeruli per mouse by light microscopy in a blind manner. The clinical scores of glomerular injury were graded into five grades; grade 0 (no PAS-positive material), grade 1 (0 to 25% of glomerular cross-section PAS-positive), grade 2 (25 to 50%), grade 3 (50 to 75%) and grade 4 (75 to 100%) (20).

Immunohistochemical and immunofluorescence staining

For immunohistochemical analysis, 4-μm paraffin sections were subjected to heat-mediated antigen retrieval and stained for F4/80 (AbD Serotec), CD3 (HistoBioTec, Miami Beach, FL, USA), IL-17 (Abcam, Cambridge, UK), leptin receptor (Abcam) and IL-23 (Abnova, Taipei, Taiwan). These specific antibodies were incubated either HRP-conjugated goat anti-rabbit IgG or alkaline phosphatase (ALP)-conjugated goat anti-rat IgG (Sigma-Aldrich). Detection of immune complexes was performed using 3,3′-diaminobenzidine for HRP (Nichirei Biosciences, Tokyo, Japan) or Vector Red for ALP (Vector laboratories, Burlingame, CA, USA). The number of F4/80 positive cells was assessed in a minimum of 20 randomly selected high-power fields per animal. For immunofluorescence staining, 4-μm frozen sections were fixed in acetone and stained with FITC-conjugated anti-sheep IgG and tetramethylrhodamine-conjugated anti-mouse IgG (Sigma-Aldrich), respectively. For quantification of immunofluorescence, blinded sections were examined at 100-fold magnification and the mean intensity of 20 glomeruli for each sample was measured for evaluation using ImageJ software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). CD3 and IL-17A-double positive cells in 20 glomerular cross-sections and in 20 tubulointerstitial high-power fields (×400) per animal were counted by light microscopy in a blinded manner.

Measurement of sheep-IgG-specific circulating IgG levels

Ninety-six-well ELISA plates were coated overnight at 4°C with 5 μg ml^{-1} of sheep IgG. After blocking for 1 h with 5% BSA in PBS, 1:500 diluted test sera were incubated for 1 h. After washing with PBS containing 0.05% Tween 20, the plates were incubated with HRP-conjugated anti-mouse IgG1, IgG2a, IgG2b and IgG3 antibodies, respectively (Bethyl Laboratories, Montgomery, TX, USA). For color development, 3,3′,5,5′-tetramethylbenzidine was used.

Quantitative real-time PCR analysis

Total RNA was extracted from kidneys or cultured cells using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Quantitative real-time PCR (qRT–PCR) analysis was performed using the Thermal Cycler Dice Real Time System II with One Step SYBR PrimeScript Plus RT–PCR Kit (Takara Bio, Shiga, Japan) following the manufacturer's protocol. Target amplicons and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were reverse transcribed and quantified using the same template RNA for relative quantification analysis. The primer sequences are shown in Table 1.

Renal and splenic single-cell preparation

Kidneys were finely minced and digested with 0.4 ng ml^{-1} collagenase D (Roche, Mannheim, Germany) and 0.01 ng ml^{-1} DNAse I in RPMI 1640 supplemented with 10%

Table 1. Sequences of primers used in the study

Gene	Sense (5′-3′)	Antisense (3′-5′)
F4/80	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
IL-17A	GCTCCAGAAGGCCCTCAGACT	CCAGCTTTCCCTCCGCATTGA
IL-22	ATACATCGTCAACCGCACCTTT	AGCCGGACATCTGTGTTGTTAT
IFN-γ	TCAAGTGGCATAGATGTGGAAGAA	TGGCTCTGCAGGATTTTCATG
IL-4	ACAGGAGGAGGGACGCCAT	GAAGCCCTACAGACGAGCTCA
IL-23p19	TGCTGGATTGCAGAGCAGTAA	GCATGCAGAGATTCCGAGAGA
IL-12p35	AAATGAAGCTCTGCATCCTGC	TCACCCTGTTGATGGTCACG
TNF-α	CATGAGCACAGAAAGCATGATCCG	AAGCAGGAATGAGAAGAGGCTGAG
IL-6	ACAACCACGGCCTTCCCTACTT	CACGATTTCCCAGAGAACATGTG
IL-1β	CTTCAGGCAGGCAGTATCACTCAT	TCTAATGGGAACGTCACACACCAG
MCP-1	CTTCTGGGCCTGCTGTTCA	CCAGCCTACTCATTGGGATCA
GAPDH	TGTGTCCGTCGTGGATCTGA	TTGCTGTTGAAGTCGCAGGAG

heat-inactivated FCS for 45 min at 37°C (22). Cell suspensions were sequentially filtered through 70-μm nylon mesh, resuspended in 33% Percoll solution, and centrifuged at 2000 rpm for 20 min at room temperature. After red blood cell lysis, kidney-infiltrating cells, as well as splenic cells, were activated by incubation at 37°C for 4 h with PMA (50 ng ml^{-1}; Sigma-Aldrich) and ionomycin (1 μg ml^{-1}; Sigma-Aldrich) in X-VIVO 10 medium (Lonza, Basel, Switzerland). Brefeldin A (10 μg ml^{-1}; Sigma-Aldrich) was added after 30 min of incubation (24). Activated kidney-infiltrating cells and splenic cells were stained with the indicated monoclonal antibodies, and were subjected to flow cytometric analysis. Anti-mouse TCR β chain (clone H57-597), TCR γδ chain (clone GL3), CD3ε chain (clone 145-2C11), IFN-γ (clone XMG1.2), IL-4 (clone 11B11) and IL-17A (clone TC11-18H10) were purchased from BD Biosciences (San Jose, CA, USA).

Cell culture

An immortalized mouse podocyte cell line was established by Mundel *et al*. (30). For propagation, podocytes between passage 14 and 20 were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin in the presence of 10 U ml^{-1} recombinant murine IFN-γ (Peprotech, London, UK) at 33°C with 5% CO_2 (permissive condition). For differentiation, the podocytes were plated on type I collagen at a density of 1×10^4 cells cm^{-2} and cultured with 1% FCS in the absence of IFN-γ at 37°C (nonpermissive condition). Two days later, the concentration of FCS was reduced to 0.5% and the podocytes were maintained under the nonpermissive condition for 10–14 days. These cells were exposed to recombinant murine leptin (Sigma-Aldrich) at 0, 10, 100 or 1000 ng ml^{-1} for 6 h. For detection of leptin receptor on cell surface, PE-conjugated rabbit anti-leptin receptor (Bioss, Woburn, MA, USA) was reacted. PE-conjugated normal rabbit IgG was used as a control.

Isolation of murine peritoneal exudate macrophages

Mice were injected intraperitoneally with 2 ml of 4% (w/v) Brewer's thioglycollate medium, and were sacrificed by bleeding under anesthesia 4 days later. Peritoneal exudate macrophages (PEMs) were collected in an intraperitoneal injection of 5 ml PBS, centrifuged at 1000 rpm for 5 min and resuspended in RPMI 1640 medium without FCS. PEMs were incubated at 37°C in a 5% CO_2 cell culture incubator for 2 h in culture plates. Nonadherent cells were removed by washing with PBS and adherent cells were used as PEMs.

Western blot

PEMs were incubated with recombinant murine leptin (1000 ng ml^{-1}; Sigma-Aldrich) for indicated times, or recombinant murine IFN-γ (100 ng ml^{-1}; Peprotech), IL-6 (100 ng ml^{-1}; R & D Systems) and IL-21 (100 ng ml^{-1}; R & D Systems) for 30 min in RPMI 1640 medium without FCS, and then were washed in ice-cold PBS and then lysed using lysis buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol) containing protease inhibitor and phosphatase inhibitor followed by brief sonication. After centrifugation at 12 000 × *g* for 15 min at 4°C, the supernatant was carefully removed and its protein concentration was measured. Cell extracts were applied to 10% SDS-PAGE gels and were transferred onto polyvinylidene difluoride membranes. The membrane was blocked with western blocking reagent (Dako, Glostrup, Denmark) for 30 min at room temperature. The blocked membranes were incubated with specific antibodies against STAT1, phospho-STAT1 (Tyr701), STAT3, phospho-STAT3 (Try705), phospho-STAT5a (Tyr694), p44/42 MAPK (ERK 1/2), phospho-p44/42 MAPK (Thr202/Tyr204), Akt, phospho-Akt (Ser473) (Cell Signaling, Danvers, MA, USA) or STAT5a (Abcam), overnight at 4°C. Signals were detected with western blot hyper-HRP substrate (Takara Bio).

Statistical analysis

Values are expressed as means ± SD. Statistical differences were calculated with Student's *t* test. $P < 0.05$ was considered significant.

Results

Leptin deficiency attenuates disease activity in NTN nephrotoxic serum nephritis

Because of the lack of inhibition of excess appetite, *ob/ob* mice are remarkably obese relative to wild-type (WT) mice. In the present study, *ob/ob* mice were under food restriction for the normal amount of daily intake by WT mice from 5 weeks of age, to exclude the effect of body weight and metabolic factors induced by obesity. After 8 weeks of food restriction, the weight of food-restricted *ob/ob* (FR-*ob/ob*) mice was almost the same as WT mice (mean body weight: WT, 25.8 ± 1.1 g;

FR-*ob/ob*, 27.4 ± 1.0 g). To investigate the role of leptin in NTN, we administered NTS in WT and FR-*ob/ob* mice. Seven days after injection of NTS, the blood urea nitrogen level of WT mice was significantly elevated compared with FR-*ob/ob* mice (WT, 233.0 ± 17.9 mg dl^{-1}; FR-*ob/ob*, 83.6 ± 18.3 mg dl^{-1}; $P < 0.01$; Fig. 1A). The serum creatinine level of WT mice was also elevated but not significantly different from that of FR-*ob/ob* mice (WT, 1.43 ± 0.65 mg dl^{-1}; FR-*ob/ob*, 0.99 ± 0.20 mg dl^{-1}; Fig. 1A); however, the serum cystatin C level, which is a more sensitive marker of kidney function and shows kidney damage earlier than creatinine (31), was significantly increased in WT NTN mice (WT, 1.65 ± 0.15 mg l^{-1}; FR-*ob/ob*, 0.69 ± 0.17 mg l^{-1}; $P < 0.01$; Fig. 1A). The blood urea nitrogen creatinine ratio (BCR) was elevated in WT mice, indicating critical illness and anticipated higher mortality in WT mice caused by acute kidney injury together with a systemic inflammatory response (32) (Fig. 1A). Finally seven of eight WT mice died within 18 days after NTS injection, while all FR-*ob/ob* mice survived (Fig. 1B). Kidney sections stained with PAS on day 7 from NTN WT mice demonstrated severe tissue damage with intracapillary cellular proliferation and glomerular thrombosis. WT mice also showed a dilated tubule containing a proteinaceous cast. In contrast, kidney injury of FR-*ob/ob* mice was markedly attenuated (Fig. 1C). The clinical scores for glomerular injury and

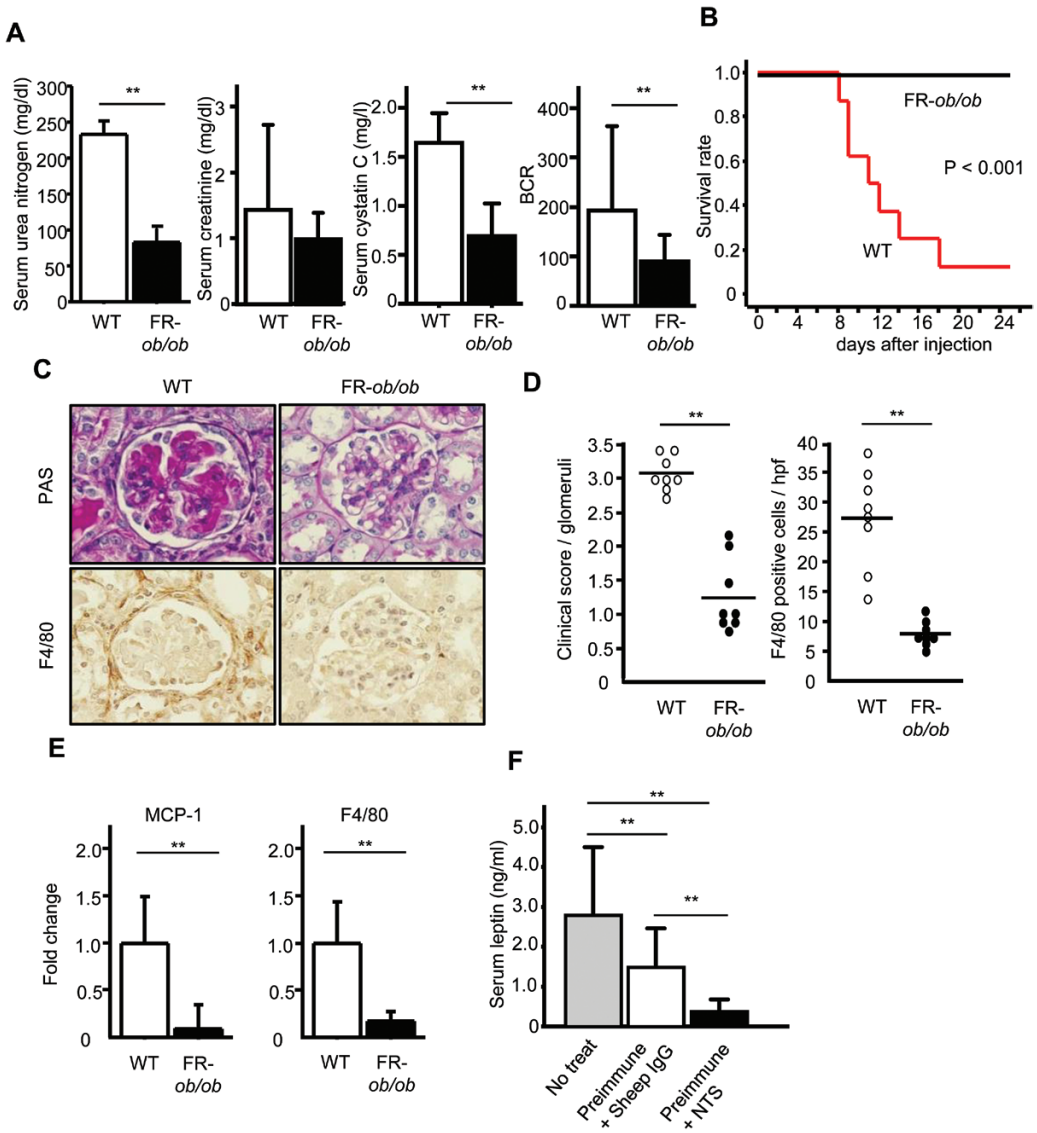

Fig. 1. Attenuated kidney injury and macrophage infiltration in FR-*ob/ob* mice. (A) Serum level of blood urea nitrogen, creatinine, cystatin C and blood urea nitrogen creatinine ratio (BCR) from WT and FR-*ob/ob* mice measured 7 days after NTS injection ($n = 8$). (B) Kaplan–Meier analysis of survival rate in WT and FR-*ob/ob* mice after NTS injection ($n = 8$). (C) Representative PAS-stained kidney sections of WT (upper left) and FR-*ob/ob* mice (upper right). Macrophage marker, F4/80, was detected in the kidney of WT (lower left) and FR-*ob/ob* mice (lower right). Kidneys were removed and examined on day 7. (D) Quantitative evaluation of glomerular injury (left) and infiltrated F4/80 positive cells per high-power field (hpf) (right) in the kidney sections of WT and FR-*ob/ob* mice 7 days after NTS injection ($n = 8$). (E) qRT–PCR analysis of MCP-1 (left) and F4/80 (right) mRNA expression in the kidneys of WT and FR-*ob/ob* mice on day 7 ($n = 8$). Each PCR product was quantified and standardized with the amount of PCR product for GAPDH and is shown as fold change. (F) Serum levels of leptin in WT mice preimmunized with sheep IgG were measured 7 days after NTS or sheep-IgG injection, or those without any treatment ($n = 8$). Values are mean ± SD. $^{**}P < 0.01$.

number of F4/80-positive macrophages infiltrating the glomeruli and interstitium were also remarkably decreased in FR-*ob/ob* mice compared with those in WT mice (Fig. 1C and D). Consistent with this observation, the renal mRNA expression of MCP-1 and F4/80 was significantly up-regulated in WT mice (Fig. 1E). Serum levels of leptin were measured in WT mice preimmunized with sheep IgG, treated with NTS or sheep IgG, or without any treatment. The serum leptin concentration was decreased by sheep-IgG-preimmunization, and was much diminished by NTS treatment on day 7 (Fig. 1F).

*Deposition of mouse IgG in the glomeruli and serum titer of antigen-specific IgG were significantly reduced in FR-*ob/ob *mice*

Induction of heterologous sheep NTS into presensitized mice triggers the production of IgG anti-NTS antibodies and the glomerular deposition of pathogenic immune complexes, which leads to accelerated glomerular injury (20). To assess the humoral immune responses, deposition of heterologous sheep IgG and autologous mouse IgG in the glomeruli was quantitated by immunofluorescence on frozen kidney sections. There was no significant difference in the deposited glomerular heterologous sheep IgG between WT and FR-*ob/ob* mice 7 days after NTS administration; however, deposition of autologous mouse IgG in the glomeruli was remarkably reduced in FR-*ob/ob* mice (Fig. 2A and B). Furthermore, serum titers of sheep-IgG-specific IgG1, IgG2a, IgG2b and IgG3 were measured by ELISA and antigen-specific IgG1, IgG2b and IgG3 titers were significantly reduced in FR-*ob/ob* mice (Fig. 2C).

Helper T lymphocyte-related cytokine expression in renal tissue

To clarify the mechanism of the reduced immune response against sheep IgG in FR-*ob/ob* NTN mice, we investigated the expression of T_h17-related cytokines, which are reported to be crucial for the secondary immune response in NTN (22), as well as other helper T lymphocyte-related cytokines and proinflammatory cytokines in the kidney. As shown in Fig. 3, mRNA expression of IL-17A, which is mainly produced by T_h17 cells, was specifically up-regulated in WT NTN mice, but not IL-22, another cytokine produced from T_h17 cells. The up-regulation of IL-17A production was not observed in WT mice treated with sheep-IgG-preimmunization and following

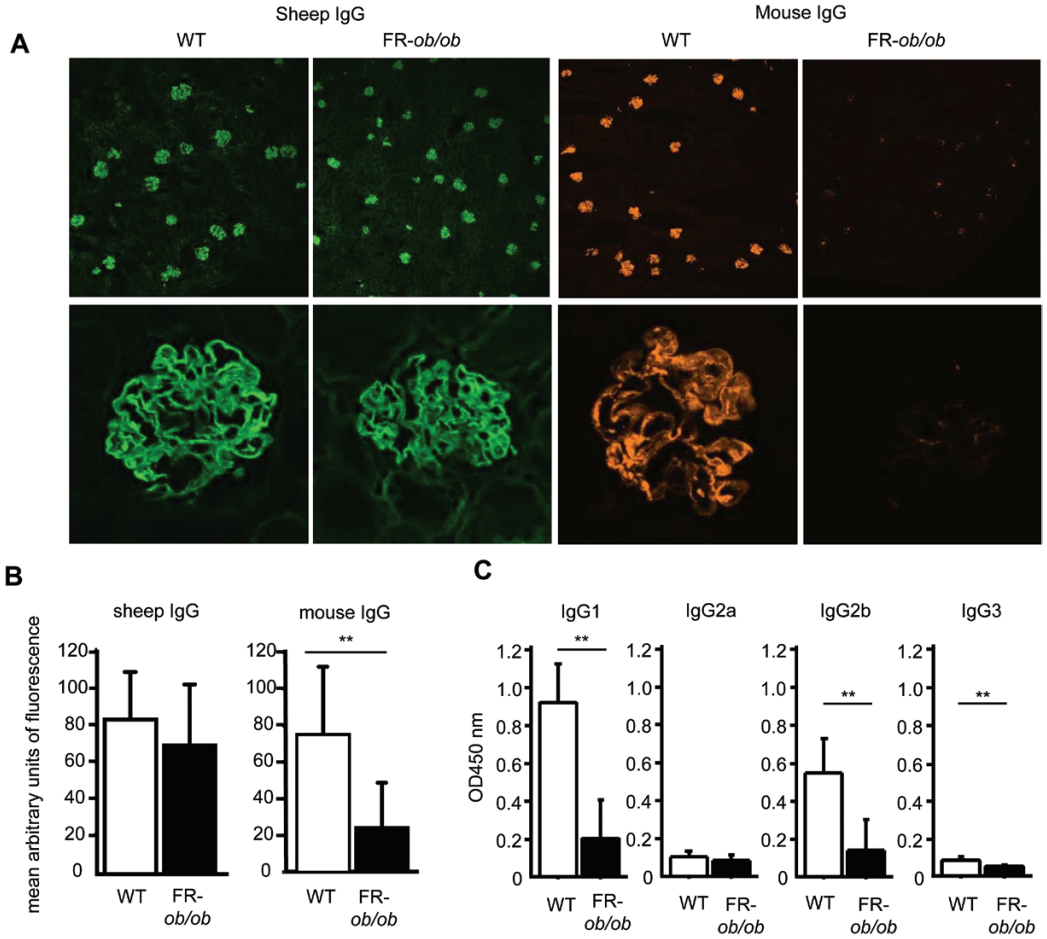

Fig. 2. Impaired secondary immune responses in FR-*ob/ob* mice. (A) Representative immunofluorescence microscopy of kidney sections for sheep IgG and mouse IgG deposited along GBM in the kidney of WT and FR-*ob/ob* mice 7 days after NTS injection. Upper row; ×40, Lower row; ×400. (B) Quantitative evaluation of immunofluorescence for sheep IgG and mouse IgG deposited in glomeruli on day 7 ($n = 8$). (C) Serum levels of IgG1-, IgG2a-, IgG2b- and IgG3-specific antibodies against sheep IgG measured by ELISA ($n = 8$). Values are mean ± SD. $^{**}P < 0.01$.

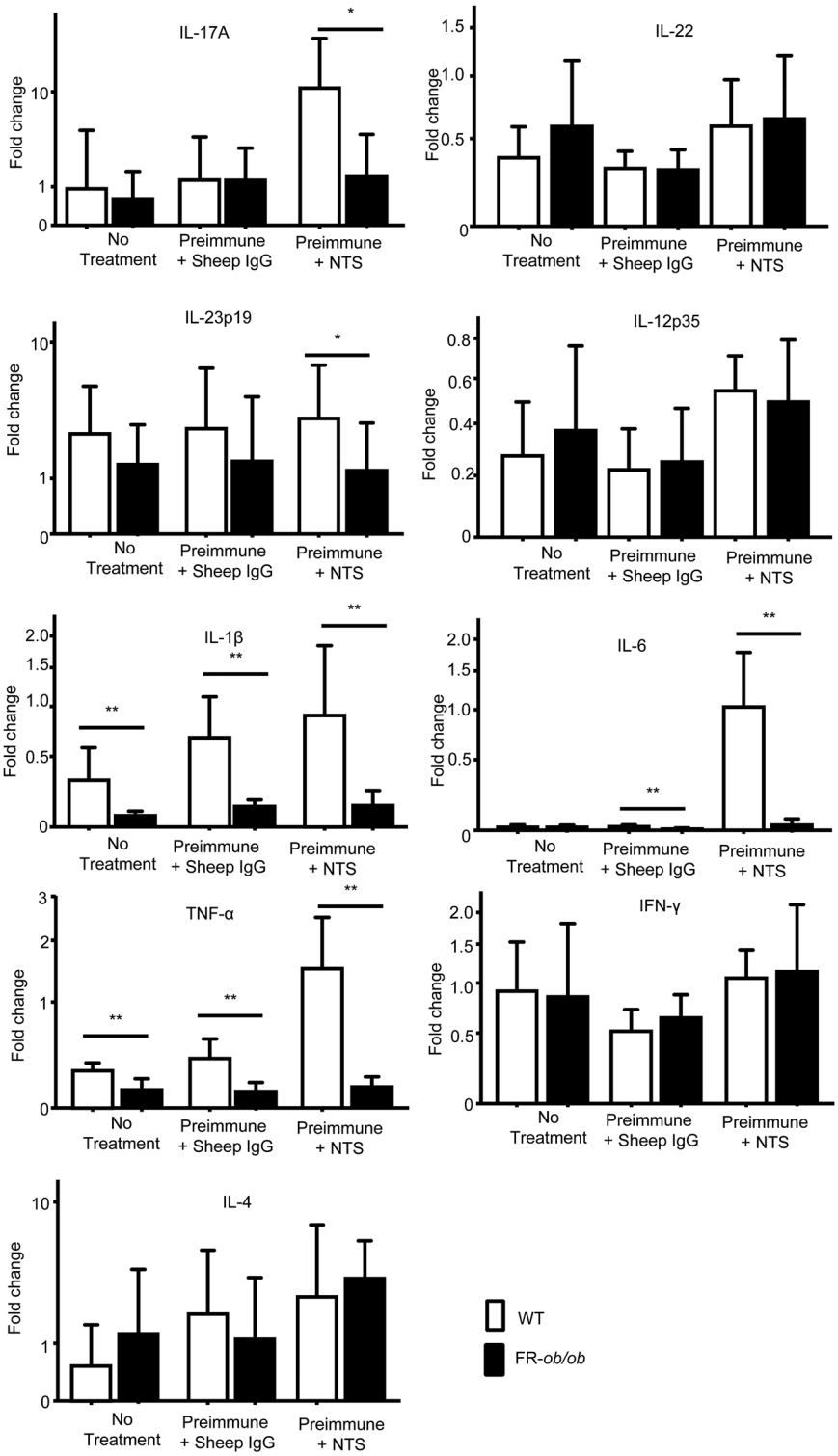

Fig. 3. qRT–PCR analysis of helper T lymphocyte-related and proinflammatory cytokine expressions in the kidney. Total RNA was extracted from the kidney of WT or FR-*ob/ob* mice without any treatment, or preimmunized with sheep IgG and following NTS or sheep IgG injection 7 days later. mRNA expression of IL-17A, IL-22, IL-23p19, IL-12p35, IL-1β, IL-6, TNF-α, IFN-γ and IL-4 and were quantitatively evaluated by qRT–PCR and standardized with the amount of PCR product for GAPDH and is shown as fold change (n = 8). Values are mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$.

sheep IgG injection as a control immune booster, which indicated that the leptin-prompted T_h17 response in the kidney would require a local response caused by immunocomplex deposition in the glomeruli. In contrast, expression levels of the T_h1 cytokine IFN-γ and the T_h2 cytokine IL-4 did not differ between WT and FR-*ob/ob* mice. Expression of renal

IL-23p19, which is essential for T_h17 expansion and survival, was detected at basal level in both WT and FR-*ob/ob* mice, but was also specifically up-regulated in WT NTN mice in accordance with IL-17A expression. Expression of the IL-12p35, an inducer of T_h1 cytokines, was similar between WT and FR-*ob/ob* NTN mice. IL-17A is known to stimulate fibroblasts, endothelial cells, macrophages and epithelial cells to secrete IL-1β, IL-6 and TNF-α (33). The expression of IL-1β and TNF-α mRNA was elevated at basal level in WT mice compared with FR-*ob/ob* mice, and synergic enhancement of IL-1β and TNF-α expression, as well as IL-6, was detected in WT NTN mice.

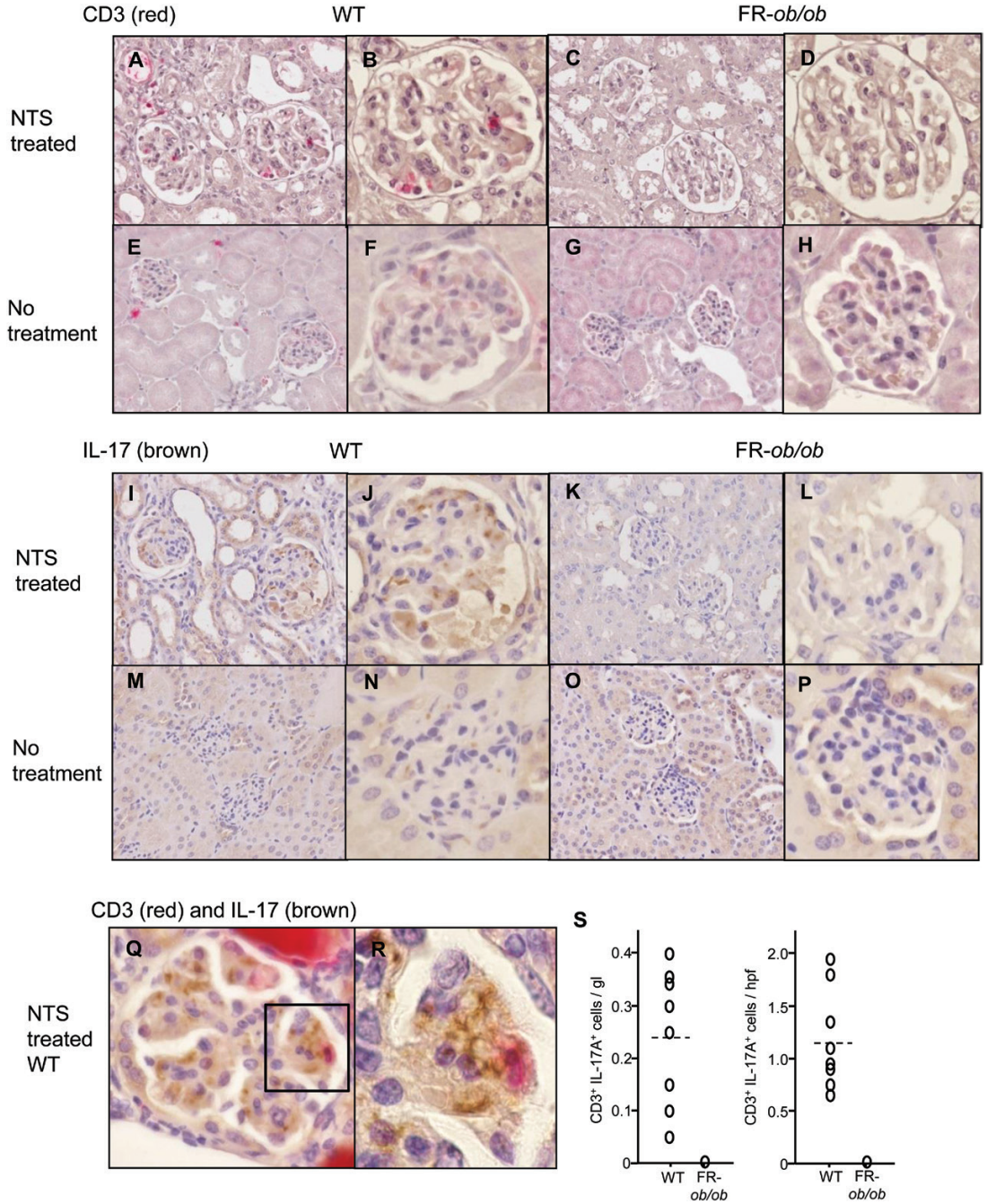

Fig. 4. Immunohistochemical analysis of IL-17-producing T lymphocytes in the kidney. (A–H) Representative kidney sections stained with CD3-specific antibody. (A, B) WT mice, (C, D) FR-*ob/ob* mice 7 days after NTS injection. (E, F) WT mice, (G, H) FR-*ob/ob* mice without any treatment. (I–P) Representative kidney sections stained with IL17-specific antibody. (I, J) WT mice, (K, L) FR-*ob/ob* mice 7 days after NTS injection. (M, N) WT mice, (O, P) FR-*ob/ob* mice without any treatment. (Q, R) Double staining of CD3 and IL-17A in the NTN WT mice kidney 7 days after NTS injection (red; CD3, brown; IL-17A). (A, C, E, G, I, K, M, O) ×200, (B, D, F, H, J, L, N, P) ×400, (Q) ×800, (R) ×2000. (S) Quantification of glomerular (left) and tubulointerstitial (right) CD3 and IL-17A-double positive cells in WT or FR-*ob/ob* mice treated with NTS (n = 8). Symbols represent individual data points, and the horizontal dashed lines indicate mean values. gl, glomerulus; hpf, high-power field.

IL-17A-producing T lymphocyte infiltration in the diseased kidney

To identify the localization of IL-17A-producing cells in the kidney, firstly we conducted immunohistochemical analyses. Figure 4 demonstrates that CD3 positive T lymphocytes were infiltrating both in glomeruli and interstitium of the kidney in WT NTN mice (Fig. 4A–H). IL-17A was also detected in the glomeruli and tubular cells of the kidney in WT mice; however, it was difficult to clarify whether these IL-17A positive cells really produced IL-17A or this cytokine was just deposited on these cells (Fig. 4I–P). Double staining of CD3 and IL-17A revealed that only a small part of the IL-17A positive population of cells was T lymphocytes (Fig. 4Q and R), although $CD3^+$ $IL\text{-}17A^+$ cells were increased both in glomeruli and interstitium of WT NTN mice, and almost none of these cells was detected in FR-*ob/ob* mice treated with NTS in the histological analyses (Fig. 4S). To clarify the involvement of infiltrating IL-17A-producing T lymphocytes, renal mononuclear cells, as well as splenic cells, in the sheep IgG-preimmunized WT mice or FR-*ob/ob* mice, treated with NTS or control sheep IgG, were purified and stimulated with PMA and ionomycin. IL-17A-producing αβ T lymphocytes, as well as γδ T lymphocytes to a lesser extent, were significantly increased in the kidney of WT NTN mice (Fig. 5A and B). These IL-17A-producing αβ T lymphocytes were also increased in the spleen of WT NTN mice and in the kidney of WT mice treated with control sheep IgG (Fig. 5A and B). On the other hand, infiltration of IL-17A-producing T lymphocytes, especially of αβ T lymphocytes, was markedly reduced in the kidney of FR-*ob/ob* NTN mice (Fig. 5A and B). Considering these results, leptin deficiency would have caused a diminished T_h17 response at the basal level, and would have hampered both systemic and local activation of the T_h17-dependent immune system in the FR-*ob/ob* NTN mice. The expression of other helper T lymphocyte-related cytokines such as IFN-γ or IL-4 in the spleen was assessed, and neither T_h1 nor T_h2 response had been influenced by leptin deficiency (Fig. 5C).

Leptin induces IL-23 expression in glomerular podocytes

Considering that the elevated IL-23 mRNA expression and specific induction of IL-17A-producing T lymphocytes in the

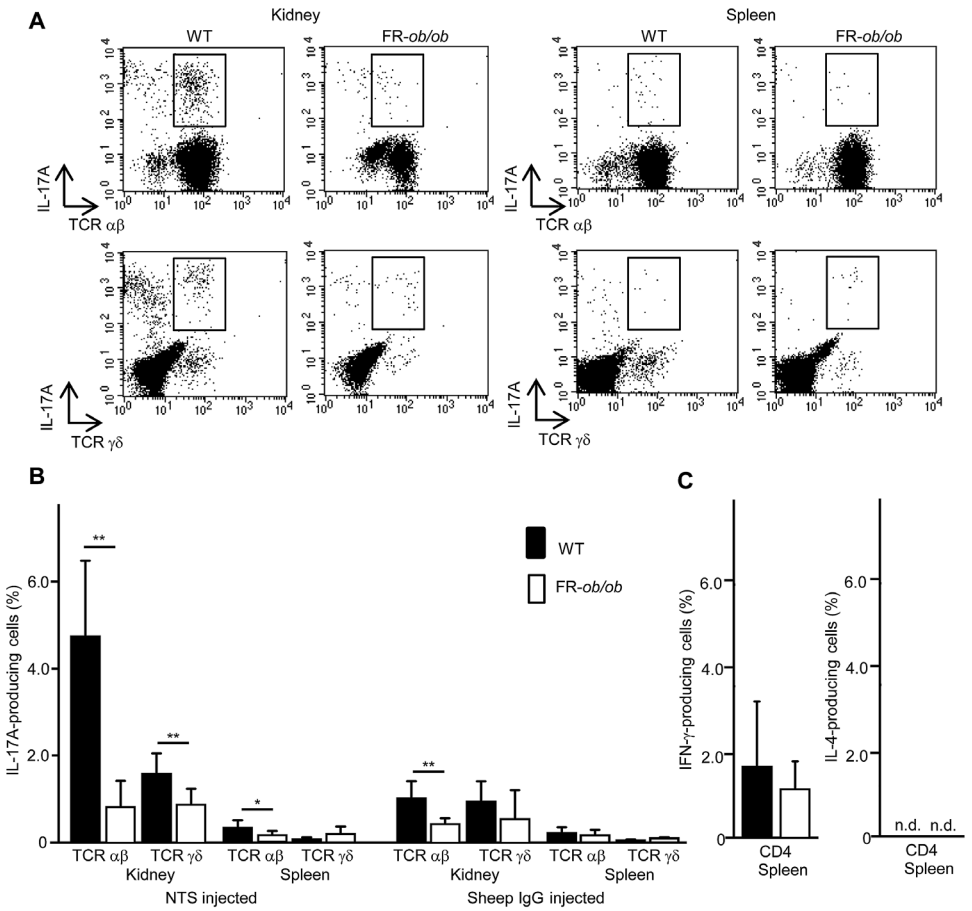

Fig. 5. Flow cytometric analysis of IL-17-producing T lymphocytes in the kidney. (A) Representative flow cytometry analysis of IL-17A-producing mononuclear cells bearing TCR αβ or TCR γδ extracted from kidney (left) or spleen (right) of WT or FR-*ob/ob* mice 7 days after NTS injection. (B) Quantitative evaluation of IL-17-producing cells per CD3-positive T lymphocytes bearing TCR αβ or γδ in the kidney or spleen of WT or FR-*ob/ob* mice on day 7 treated with NTS or control sheep IgG injection ($n = 4$). IL-17A and TCRβ or TCRγδ double positive cells were identified as IL-17-producing T lymphocytes. (C) Quantitative evaluation of IFN-γ- or IL-4-producing cells per CD4-positive T lymphocytes in the spleen of WT or FR-*ob/ob* mice on day 7 treated with NTS or control sheep IgG injection ($n = 4$). IFN-γ or IL-4 and CD4 double positive cells were identified as IFN-γ- or IL-4-producing T lymphocytes.

diseased kidney were attenuated in FR-*ob/ob* NTN mice (Figs 3 and 5), we hypothesized that leptin would induce IL-23 expression in some component cells in the kidney. Immunohistochemistry revealed that IL-23 was detected in glomerular podocytes which surrounded the epithelial side of the GBM in WT NTN mice (Fig. 6A and B). Leptin receptor was also detected in glomerular podocytes, as well as Bowman's capsules in normal WT mice (Fig. 6E and F). To investigate the direct effect of leptin on glomerular podocytes, cultured murine podocytes expressing leptin receptor (Fig. 6I) were stimulated with recombinant leptin and mRNA expression of IL-23p19 and IL-12p35 was quantified. Leptin induced IL-23p19, but not IL-12p35 in cultured podocytes in a dose-dependent manner (Fig. 6J).

Leptin induces MCP-1 expression in macrophages in Ob-Rb/STAT3-independent signaling pathways

Finally, we investigated the effect of leptin on macrophages, which infiltrate in the glomeruli, produce proinflammatory cytokines and chemokines, and generate proteinases such as matrix metalloproteinases leading to glomerular injury in NTN. Leptin induced MCP-1 production in PEMs dose-dependently, and MCP-1 mRNA expression was also induced in PEMs from *db/db* mice, which genetically lack the long isoform of leptin receptor Ob-Rb (Fig. 7A and B). We hypothesized that the leptin signal would be transmitted through the short isoform of the leptin receptor, which is independent of STAT3 phosphorylation. PEMs from WT mice were incubated with leptin and the phosphorylation of signaling molecules was evaluated by western blot. As shown in Fig. 7C, leptin induced phosphorylation of ERK1/2 and Akt in PEMs, but not that of STATs, which could be activated by IFN-γ, IL-6 or IL-21. Leptin signaling was transduced without STAT3 phosphorylation, suggesting that macrophages were stimulated by leptin in the Ob-Rb/STAT3 pathway independently.

Discussion

The results of the present study demonstrate that leptin stimulates glomerular podocytes to produce IL-23. MCP-1 production in macrophages was induced independently via the Ob-Rb/STAT3 pathway. Leptin deficiency decreased the secondary immune response and led to a deteriorated T_h17

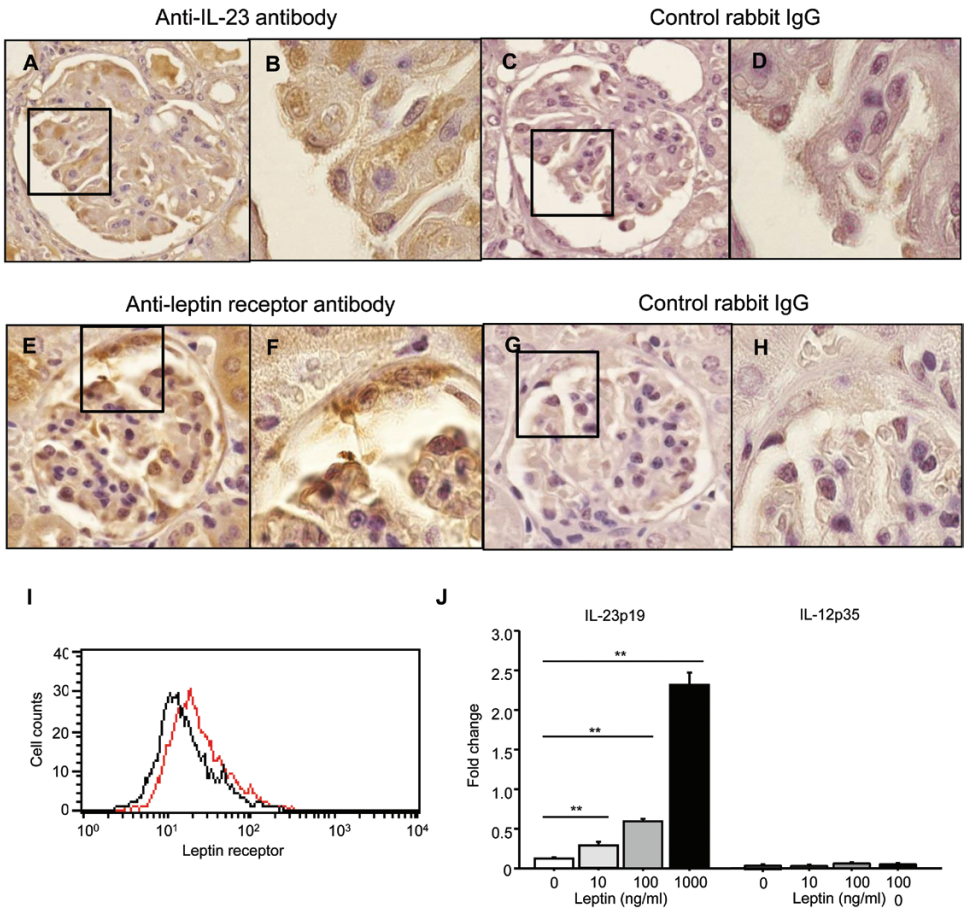

Fig. 6. IL-23 production and leptin receptor expression of glomerular podocytes. (A–D) Representative kidney sections of WT NTN mice 7 days after NTS injection stained with IL23-specific antibody (A, B) or with control rabbit IgG as a staining control (C, D). (E-H) Representative kidney sections of normal WT mice stained with leptin receptor-specific antibody (E, F) or with control rabbit IgG as a staining control (G, H). (A, C, E, G) ×600, (B, D, F, H) ×2000. (I) Cell surface expression of leptin receptor (red) and its control (black) on cultured mouse podocytes. (J) Quantitative analysis of IL-23p19 and IL-12p35. mRNA expression in cultured murine glomerular podocytes induced by leptin stimulation were quantitatively evaluated by qRT–PCR and standardized with the amount of PCR product for GAPDH and is shown as fold change. ($n = 3$). Values are mean ± SD. $^{**}P < 0.01$.

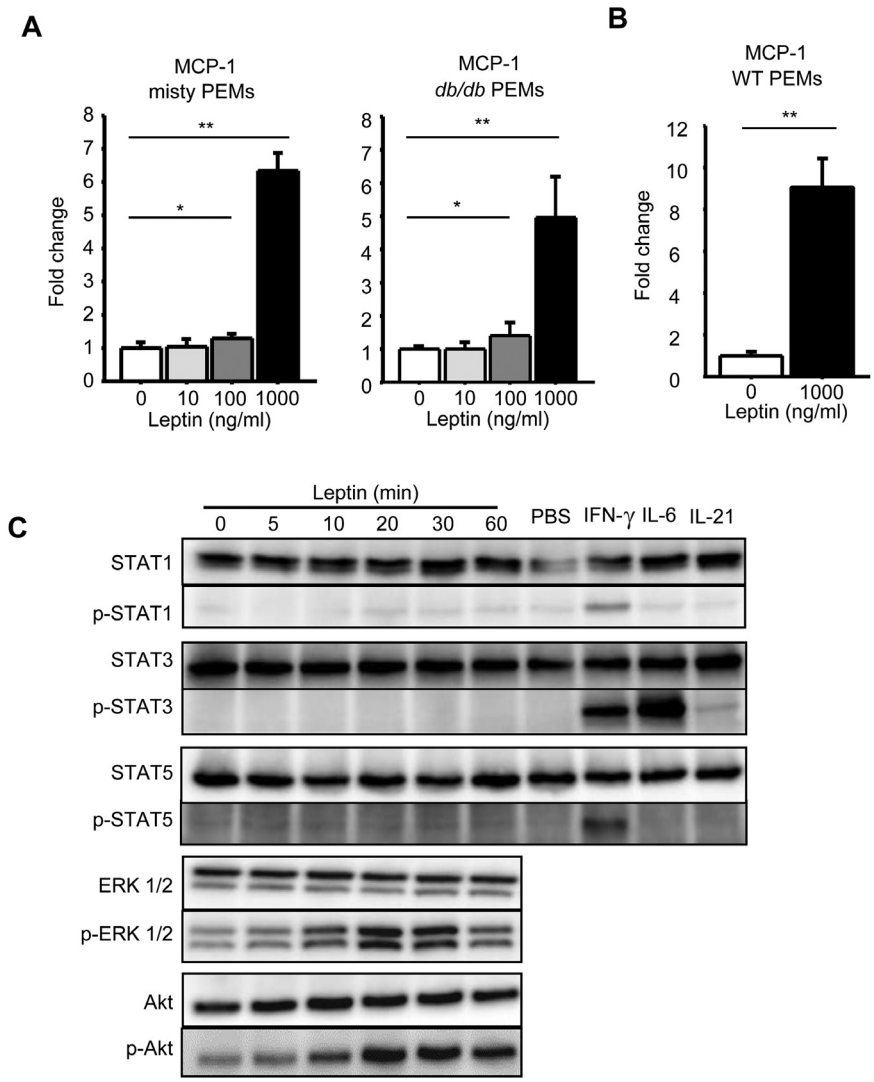

Fig. 7. Leptin induces MCP-1 production in PEMs in Ob-Rb/STAT3-independent signaling pathways. (A) Total RNA was extracted from PEMs of WT misty mice (left) or *db/db* mice (right) after leptin stimulation. MCP-1 mRNA expression was quantitatively evaluated by qRT–PCR and standardized with the amount of PCR product for GAPDH and is shown as fold change (n = 5). (B) PEMs of WT mice were cultured with the indicated doses of leptin and MCP-1 mRNA expression was quantitatively evaluated by qRT–PCR and standardized with the amount of PCR product for GAPDH and is shown as fold change (n = 5). (C) PEMs were incubated with leptin (1000 ng ml^{-1}) for the indicated times, or with IFN-γ (100 ng ml^{-1}), IL-6 (100 ng ml^{-1}), IL-21 (100 ng ml^{-1}) or PBS as their vehicle instead of leptin for 30 min. Total cellular protein was extracted to evaluate signal transduction induced by leptin. Total and phosphorylated STAT1, STAT3, STAT5, ERK 1/2 and Akt were detected by each specific antibody.

response, combined with reduced MCP-1 production and macrophage infiltration in the kidney, which resulted in attenuated disease activity in an NTN mouse model. Leptin would systemically promote a T_h17-dependent immune response, and local IL23 production in the kidney would synergically enhance a T_h17 response.

Ob-R is a high-affinity receptor for leptin and consists of at least six isoforms derived from different splicing. These receptors share an identical extracellular amino-terminal end, which binds leptin, but differ at their carboxy-terminal ends. The long form of the receptor, Ob-Rb, is principally found in the hypothalamus, and the short form of the receptor, Ob-Ra, is expressed in peripheral tissues and is much more abundant (34, 35). A study of *db/db* mice, which possess a point mutation in the genomic Ob-Rb sequence resulting in a lack of the STAT3-binding cytoplasmic domain (36), revealed that Ob-Rb signal deficiency led to a severe obese phenotype (37). However, the exact role of Ob-Ra and its mechanism of signal transduction are still undetermined. Both the long isoform Ob-Rb receptor and, in much larger amounts, the short isoform Ob-Ra receptor, are present in the kidney. The Ob-Ra receptor is reported to be present in endothelial cells including glomerulus, glomerular mesangial cells and podocytes (34, 38). So far leptin has been reported to promote proliferation and TGF-β1 production in glomerular endothelial cells, and to facilitate TGF-β receptor II expression and collagen type IV synthesis in mesangial cells, which finally leads to glomerulosclerosis (39). However, the effect of leptin on

glomerular podocytes with respect to immune responses is not fully understood. In the present study, we demonstrate that leptin induces IL-23 production in cultured murine podocytes and that leptin deficiency reduces IL-23 production in NTN mice kidneys. Judged by the impaired serum anti-sheep IgG production (Fig. 2), reduced production of proinflammatory cytokines such as IL-1β and TNF-α at basal level (Fig. 3) and reduced IL-17A positive αβ T lymphocytes in the kidney and spleen (Fig. 5B) in FR-*ob/ob* mice, leptin would promote proinflammatory and T_h17-dependent immune responses systemically. In addition, considering that glomerular podocytes would be one of the main producers of IL-23 in the NTN kidney (Fig. 6), we surmise that leptin would facilitate IL-23 production in the kidney with immunecomplex deposition, resulting in damaged kidney-specific enhancement of the T_h17 response, followed by accelerated kidney injury.

In addition to its direct effect on the component cells in the kidney, leptin also facilitates proinflammatory responses in various kinds of immune cells (40). One of the proinflammatory roles of leptin is to stimulate proliferation, phagocytosis and production of several inflammatory mediators such as inducible nitric oxide synthase, cyclooxygenase 2, cytokines and CC chemokine ligands in monocytes/macrophages (8, 9, 41). Macrophage accumulation is a prominent feature in most types of human glomerulonephritis (42) and in animal models of experimental glomerulonephritis in which macrophages induce glomerular (42, 43) or tubulointerstitial injury (44). Chemokines and their specific receptors are increased in the NTN model and play a major role in leukocyte infiltration in the diseased kidney (45). In particular, MCP-1 has been reported to play a major role in the pathogenesis of the progression of kidney disease in both human kidney disease and animal models, including NTN (46). MCP-1 is produced in tubular epithelial cells, mesangial cells, endothelial cells and infiltrating mononuclear cells (47). Leptin is reported to enhance MCP-1 expression in cultured human macrophages (48). In the present study, we clarified that leptin phosphorylated ERK 1/2 and Akt, but not STAT3, in PEMs derived from WT mice, and that leptin could induce MCP-1 expression in PEMs that lack Ob-Rb. Both short and long isoforms of Ob-R have the same sequence of the first intracellular 29 amino acids, and this sequence contains a constant box 1 motif and JAK tyrosine kinase. The Ras/Raf/MAPK pathway can be stimulated by either Ob-Ra or Ob-Rb, resulting phosphorylation of ERK. On the other hand, Ob-Rb specifically possesses a box 3 motif, which binds STAT transcription factor (49, 50). Bjørbæk *et al*. reported that using cells transfected with either the long or the short isoform of mouse Ob-R, both Ob-Rs mediated leptin-dependent tyrosine phosphorylation of JAK2 and ERK1/2, whereas STAT3 phosphorylation was observed in the cells with long isoform of Ob-R alone (51). These results indicated that Ob-Rb and phosphorylation of STAT3 were not involved with signal transduction in macrophages.

The leptin concentration was rather decreased in WT mice treated with sheep IgG-preimmunization, and was much diminished in NTN mice on day 7 (Fig. 1F). Considering the fact that leptin is an acute phase reactant and elevates at an early stage of inflammatory responses (52), we surmise that leptin was consumed by inflammatory cells and decreased 11 days after sheep IgG-preimmunization. Furthermore, during the development of severe NTN serum leptin would have been lost in the urine together with other serum proteins, resulting in a decreased serum concentration in the mouse treated with sheep-IgG-preimmunization and NTS.

Leptin is principally metabolized by the kidney through a megalin-mediated tubular uptake, followed by endocytosis and intracellular degradation (53). In this way, circulating leptin levels are increased in advanced CKD (39). In concert with hyperleptinemia in CKD, leptin could directly promote renal pathophysiological changes. The present study highlights the novel aspect of leptin's interactions in the kidney, adipose tissue and immune system of a nephritis model. This knowledge is of importance in identifying one of the molecular mechanisms in which obesity exacerbates the progression of kidney disease.

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