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EGF Receptor-dependent TLR7 Signaling in Macrophages Promotes Glomerular Injury in Crescentic Glomerulonephritis

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Abstract

Glomerulonephritis (GN) is a group of inflammatory diseases, and an important cause of morbidity and mortality worldwide. The initiation of inflammatory process is quite different for each type of GN, however, each GN is characterized commonly and variably by acute inflammation with neutrophils and macrophages, and crescent formation, leading to glomerular death. Toll-like receptor 7 (TLR7) is a sensor for self-RNA and implicated in the pathogenesis of human and murine GN. Here we show that TLR7 exacerbates glomerular injury in nephrotoxic serum nephritis (NTN), a murine model of severe crescentic GN. $TLR7^{-/-}$ mice are resistant to NTN although $TLR7^{-/-}$ mice manifest comparable immune-complex deposition to wild type (WT) mice without significant defects in humoral immunity, suggesting that endogenous TLR7 ligands accelerates glomerular injury. TLR7 is expressed exclusively in macrophages in glomeruli in GN, but not in glomerular resident cells or neutrophils. Furthermore, we discovered that

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Author Contributions

D.N.L. and S.S. performed experiments. I.R.R., R.G.B., G.C.S. and M.A. performed study concept and design. N.T. and M.Y. performed study concept, design and experiments, provided acquisition, analysis, interpretation of data and statistical analysis, and performed writing, review and revision of the paper.

Ethics Approval / Consent to Participate

All animal experiments were performed under the animal protocol (IACUC#: 007158) approved by the Institutional Animal Care & Use Committee at CSMC.

EGF receptor (EGFR), a receptor-type tyrosine kinase, is essential for the TLR7 signaling in macrophages. Mechanistically, EGFR physically interacts with TLR7 upon TLR7 stimulation, and EGFR inhibitor completely blocks the phosphorylation of TLR7 tyrosine residue(s). EGFR inhibitor attenuated glomerular damage in WT mice, and no additional glomerular protective effects by EGFR inhibitor were observed in TLR7$^{-/-}$ mice. Finally, mice lacking EGFR in macrophages were resistant to NTN. This study clearly demonstrated that EGFR-dependent TLR7 signaling in macrophages is essential for glomerular injury in crescentic GN.

INTRODUCTION

Glomerulonephritis (GN) is a group of inflammatory disease in glomeruli, that includes lupus nephritis, IgA nephropathy, ANCA-associated crescentic GN, and anti-glomerular basement membrane (GBM) GN. GN is a major cause of chronic kidney disease, and is an important cause of morbidity and mortality worldwide: it affects 0.12 % of the general population, and 1.2 % of the older population(1); 66.3 % and 23.9 % of native kidney biopsies in the United States were diagnosed with glomerular disease and glomerulonephritis, respectively(2).

Although the initiation of inflammatory process is quite different for each subtype of GN, each GN is characterized by acute glomerular inflammation with neutrophils and macrophages (MΦs) and variably develops crescent formation, leading to global glomerulosclerosis, glomerular death. The glomerular crescent is composed of proliferating epithelial cells and inflammatory cells (3–5), and typically forms in sequential steps: endocapillary inflammation, fibrinoid necrosis and GBM rupture, and crescent formation (6). Of these steps, GBM rupture is the critical (point-of-no-return) step for glomerular death, and the crescentic lesion identifies various GN patients with an especially poor prognosis(7–10).

Toll-like receptor 7 (TLR7) (11, 12) is an evolutionarily conserved sensor involved in innate and inflammatory immune responses, and recognizes RNAs, broadly found in pathogens as Pathogen-Associated Molecular Patterns (PAMPs) and released by injured cells as Damaged-Associated Molecular Patterns (DAMPs). Polymorphisms in TLR7 are linked with the development of systemic lupus erythematosus (SLE) (13, 14), and mouse genetics support a role for TLR7 in lupus nephritis (15, 16). In addition, TLR7 signaling enhances IgG deposition in glomeruli(17) and modulates severity of lupus nephritis (18–21). However, the role of TLR7 and its mechanism in crescentic GN remain unknown.

We, here, show that TLR7 is essential for glomerular injury in nephrotoxic serum nephritis (NTN), a murine model of severe crescentic GN. NTN is initiated by nephrotoxic IgG deposition in glomeruli, and shares the pathological features of anti-GBM GN and human proliferative lupus nephritis. TLR7$^{-/-}$ mice were resistant to NTN without significant defects in IgG priming effects. TLR7 is exclusively expressed in macrophages (MΦs) in glomeruli. Furthermore, we showed that EGF receptor (EGFR) is essential for the TLR7 signaling in MΦs. EGFR physically interacts with TLR7 only after TLR7 stimulation, and EGFR inhibitor completely blocks the phosphorylation of TLR7 tyrosine residue(s). Pharmacologically, EGFR inhibitor attenuated glomerular damage in wild type (WT) mice,

but it did not show any additional protective effects on glomerular damages in TLR7$^{-/-}$ mice. Finally, mice lacking EGFR only in MΦs are resistant to NTN. These data support that endogenous danger signaling exacerbates glomerular injury in crescentic GN via TLR7-EGFR axis in MΦs.

MATERIALS AND METHODS

Ethical considerations and animal care

All animal experiments were performed under the animal protocol (IACUC#: 007158) approved by the Institutional Animal Care & Use Committee at CSMC. Female 8–12 weeks mice were used for all experiments. Wild type (WT; C57Bl/6) and TLR7$^{-/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). EGFR$^{floxed/floxed}$ mice were obtained from David Threadgill, PhD at Texas A&M University, and crossbred to CSFR1^{Cre} mice (The Jackson Laboratory) to generate EGFR$^{floxed/floxed}$- CSFR1^{Cre} mice. Mice were bred and kept in a specific pathogen-free condition with 12-h day/night cycles and allowed free access to food and water.

Induction of nephrotoxic serum nephritis

Nephrotoxic serum (NTS) was generated by immunizing rabbit with glomeruli from Sprague-Dawley rats (Lampire Biological Laboratory, Pipersville, PA) and was heat-inactivated at 56°C for 30 min. On Day −5, WT and TLR7$^{-/-}$ mice were sensitized by intraperitoneal injection of normal rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) emulsified with Incomplete Freund's Adjuvant (IFA; Sigma-Aldrich, St. Louis, MO). On Day 0, the mice were intravenously injected with NTS or PBS. On Day 7, urine samples were collected, and all mice were euthanized and kidneys and blood samples were collected. In some experiments, WT and TLR7$^{-/-}$ mice were treated with 2.4 mg/day of Gefitinib (Selleck Chemicals, Houston, TX), an EGFR inhibitor by oral gavage, everyday from 12 hrs before the NTS injection.

Histological assessment of glomerular injury

Two-micrometer sections from paraffin-embedded kidney were deparaffinized and rehydrated, following by periodic acid-methenamine silver staining, with which fibrinoid necrosis (a precursor lesion of crescent and defined with two or more of glomerular basement membrane rupture, fibrin deposition, and karyorrhexis (3, 22–24)) and crescent formation were assessed in all glomeruli on one section for each mouse in a blinded manner. Representative images were captured using a light microscope (Nikon Eclipse 50i, Nikon, Tokyo, Japan).

Assessment of proteinuria

To obtain protein to creatinine ratio (mg/mg) in urine, urinary protein was measured with protein assay dye (Bio-Rad, Hercules, CA) and urinary creatinine was assessed using Creatinine Assay Kit (BioAssay Systems, Hayward, CA).

GFR measurements

GFR was described elsewhere ((25, 26)), determined in conscious mice using a transcutaneous detector (NIC-Kidney; MediBeacon GmbH, Mannheim, Germany) by monitoring fluorescent intensity for 90 min after a single intravenous bolus of FITC-sinistrin (15 mg/100 g body weight; Fresenius Kabi Austria GmbH, Linz, Austria). To determine GFR, the t1/2 time of FITC-sinistrin was calculated using a three-compartment model according to the manufacturer's instructions.

Immunohistochemistry and esterase staining

After antigen retrieval by heating in 0.01 M citrate buffer (pH 6.0) or 0.76 g/L pronase in TBS for 30 min, intrinsic peroxidases were inactivated by incubation in 3% hydrogen peroxidase for 10 min. To reduce non-specific background staining, sections were treated with 1% bovine serum albumin (BSA) for 30 min and Streptavidin/Biotin blocking kit (Vector Laboratories, Burlingame, CA) for 15 min each and then incubated for 24 h at 4°C with biotin anti-mouse/human Mac-2 (BioLegend, San Diego, CA) or biotin anti-TLR7 (G-BIOSCIENCES, St. Louis, MO), followed by the incubation with a horseradish peroxidase streptavidin (Vector Laboratories, Burlingame, CA) for 60 min at room temperature. Horseradish peroxidase was visualized by reaction with ImmPACT® DAB Peroxidase (HRP) Substrate (Vector Laboratories, Burlingame, CA). For periodic acid-Schiff staining, sections were incubated 0.5% periodic acid (Sigma-Aldrich, St. Louis, MO) for 10 min, following by placing in Schiff's Reagent (Sigma-Aldrich, St. Louis, MO) for 30 min at room temperature. For nuclei staining, Hematoxylin solution (Ricca Chemical Company, Arlington, TX) was used. To detect neutrophils, esterase reaction was performed on paraffin-embedded kidney sections as described (27), followed by nuclei staining with Hematoxylin solution (Ricca Chemical Company, Arlington, TX).

Serum mouse anti-rabbit IgG and recall assay to rabbit IgG in splenocytes

Serum titer of mouse anti-rabbit IgG was determined by ELISA kit of mouse IgG (Bethyl Laboratories, Montgomery, TX). For recall assay, 14 days after immunization with rabbit IgG and IFA, splenocytes were isolated from WT and $TLR7^{-/-}$ mice. Cells were seeded at 5.0×10^6 cells per well in 12-well plate and incubated with 500 μg/mL rabbit IgG for 24 h. Supernatants were collected and the concentration of mouse IL-6 or IFN-γ was measured using ELISA kits (eBioscience, San Diego, CA, and R&D systems, Minneapolis, MN, respectively).

Immunofluorescence study for mouse IgG and rabbit IgG

For immunofluorescence, 4 μm-thickness frozen kidney sections were fixed in acetone for 3 min, followed by blocking in 1% BSA for 30 min prior to overnight incubation at 4°C with Goat anti-Mouse IgG (H+L) Superclonal Secondary Antibody Alexa Fluor 488 (ThermoFisher Scientific, Waltham, MA) and Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody Alexa Fluor 568 (ThermoFisher Scientific, Waltham, MA). Immunofluorescence images were obtained with a fluorescence microscope BZ-X710 (Keyence, Osaka, Japan). Semi-quantifications (0 to 4+) were performed in 10 high power fields (magnification × 400) per kidney in a blinded manner.

Electron microscopy

Samples fixed in 3% glutaraldehyde were postfixed in OsO_4 and embedded in epoxy resin. Ultrathin sections were collected on carbon-coated formvar grids and stained with uranyl acetate and lead citrate. Transmission electron microscope images were obtained using JEOL 100 CX (JEOL, Peabody, MA) or Hitachi 7700 (Hitachi, Santa Clara, CA).

Isolation of glomeruli, neutrophils, and macrophages

Glomerular isolation was performed as previously described(28), using enzymatic digestion and perfusion with magnetic beads. Neutrophils were isolated from bone marrow leukocytes with a neutrophil isolation kit (Miltenyi Biotec). The purity of isolated neutrophils was checked by flow cytometry, and only neutrophils with >95% (CD11b+Ly-6G+) purity were used for experiments. Peritoneal macrophages were prepared as previously reported(29) with some modifications. The mice were intraperitoneally injected with 2.5 ml of 3% thioglycollate, and peritoneal leukocytes were harvested from the peritoneal cavity by washing with cell culture medium using a syringe 84–96 hs after the injection. The cell suspension was centrifuged at 300 rcf for 5 min, then erythrocytes in the cell pellets were lysed with 1xRBC lysis buffer at RT for 5 min. After one-time washing with culture medium, macrophages/monocytes were isolated by a peritoneal macrophage isolation kit, or EasySep Isolation Kit (STEMCELL Technologies, Cambridge, MA) and Multi Tissue Dissociation Kit (Miltenyi Biotec, Auburn, CA). The macrophages with >90% (CD11b+F4/80+) purity by flow cytometry were used for experiments.

Cell culture experiments

RAW 264.7 cells (ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. In some experiments, cells were incubated with 0.1 μg/mL R848 (Sigma-Aldrich, St. Louis, MO), a TLR7 agonist as indicated concentration and time. For inhibition of EGFR, cells were incubated with 10 or 20 μM of Gefitinib in addition to R848 for 16h. The cell lysates were collected for Western blotting and IL-6 in the supernatants were measured by ELISA kit.

Immunocytochemistry of p65

9×10^4 of RAW 264.7 cells were seeded in 8-well Millicell EZ glass chamber slides (MilliporeSigma, Burlington, MA) and incubated at 37°C for overnight. The cells were incubated with 0.1 μg/mL of R848 (Sigma-Aldrich, St. Louis, MO) with or without 1-hr pretreatment of 20 μM Gefitinib (Selleck Chemicals, Houston, TX). After fixation with 100% methanol for 5 min and blocking with 1% bovine serum albumin for 30 min, the cells were incubated with anti-NF-κB p65 (D14E12, #8242; Cell Signaling Technology, Danvers, MA) for 1 hr, and followed by the incubation with Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody Alexa Fluor 488 (ThermoFisher Scientific, Waltham, MA) for 1 hour at room temperature. The slides were mounted with ProLong™ Gold Antifade Mountant with DAPI (Life Technologies, Waltham, MA). Immunofluorescence images were randomly obtained with a fluorescence microscope BZ-X710 (Keyence, Osaka, Japan).

Western blotting and Co-immunoprecipitation assay

Western blotting was performed as described elsewhere (30). For co-immunoprecipitation, cells were lysed in buffer containing 150 mM NaCl, 20 mM HEPES [pH 7.4], 1.5 mM $MgCl_2$, 2 mM dithiothreitol, 2 mM EGTA, 10 mM NaF, 12.5 mM β-glycerophosphate, 1 mM Na_3VO_4, 0.5% (v/v) Triton X-100, and complete EDTA-free protease inhibitor (Roche Applied Science, Indianapolis, IN). The lysates were precleared with rabbit IgG agarose for 1 h, and incubated overnight with anti-EGFR Ab and Protein A/G plus agarose (Santa Cruz, Dallas, TX). After washing, the indicated coprecipitated proteins were detected by immunoblotting.

Wound-healing assay

Wound-healing assay has been described elsewhere (31). Briefly, confluent cell cultures were grown on 6-well plates. Wounds were scratched with a micropipette tip. After the cells were washed with warm media, they were maintained in regular media with 10% FBS, with or without R848 for indicated time. To analyze cell migration, a picture for each scratch was taken at the same area of cells at 0 h and the end of the incubation time. At least eight fields for each condition were taken, and the numbers of migrating cells into scratched fields were calculated. The number of migrating cells with R848 at 0.1 μg/ml was defined as 100.

Tyrosine phosphorylation of TLR7

Cell lysates were precleared with mouse IgG agarose for 1 h. The precleared lysates were incubated with anti-phosphotyrosine Ab (Platinum 4G10, MilliporeSigma, Burlington, MA) overnight. The beads were washed with cell lysis buffer containing 500 mM NaCl and 1% Triton X-100, boiled with SDS-PAGE buffer, and analyzed by SDS-PAGE and Western blot. Tyrosine-phosphorylated TLR7 was detected by Western blot with anti-TLR7 Ab (Cell Signaling Technology, Danvers, MA).

Statistical analyses

All statistical analyses were performed using Prism 7 (Graphpad Software, San Diego, CA). Data are presented as mean ± SEM. Comparison of 2 groups was performed using Mann-Whitney test. Differences among more than two groups were compared by one-way analysis of variance (ANOVA), followed by Tukey–Kramer tests. For all tests, a two-tailed $P<0.05$ was considered statistically significant.

RESULTS

Lacking TLR7 ameliorates glomerular injury in murine crescentic glomerulonephritis.

TLR7 is a sensor for endogenous RNA(32–35), a danger signaling, released from damaged tissue, and is known to play an important role in the pathogenesis of SLE(13, 14) and lupus nephritis(15, 16, 36). We examined first whether TLR7 is involved in the pathogenesis of nephrotoxic serum nephritis (NTN), a murine model of sever crescentic glomerulonephritis (GN) that shares the pathogenesis with human proliferative lupus nephritis and anti-glomerular basement membrane (GBM) GN. We immunized WT and $TLR7^{-/-}$ mice with 0.5 mg normal rabbit IgG emulsified with incomplete Freund's adjuvant (IFA) on Day

−5, and intravenously injected with 50 μl nephrotoxic serum on Day 0. TLR7$^{-/-}$ mice exhibited significantly reduced proteinuria than WT mice on Day 7 (Fig. 1A; urine protein creatinine ratio: 9.36 ± 2.267 versus 29.00 ± 4.995 of WT; $P < 0.005$). Next, on Day 7, we sacrificed mice to histologically evaluate glomerular injuries. Fibrinoid necrosis is a precursor lesion for crescent (3, 6, 22, 23) and characterized with glomerular basement membrane (GBM) rupture, fibrin deposition, and karyorrhexis (24). TLR7$^{-/-}$ mice showed significantly reduced numbers of glomerular fibrinoid necrosis compared to WT mice (Figs. 1B, 1E and 1F; 6.054 ± 2.585 versus 24.63 ± 6.41 of WT; $P < 0.050$). In addition, TLR7$^{-/-}$ mice developed less number of crescent formation than WT mice (Figs. 1C, 1D, and 1F; 0.100 ± 0.1000 versus 2.333 ± 0.8944 of WT; $P < 0.05$).

Leukocyte recruitment into the kidney is closely associated with the initiation and progression of glomerulonephritis (GN) (37–39). In NTN, neutrophils are recruited to glomeruli within few hours after nephrotoxic serum challenge (40) while MΦs infiltrate into glomeruli later (about Day 7) (41). We investigated the leukocyte infiltration in glomeruli in our model. Neutrophil glomerular infiltration, evaluated by esterase staining, was comparable between WT and TLR7$^{-/-}$ mice with NTN 2 hrs after NTS challenge (Figs. 1G and 1H; 0.3173 ± 0.02085 of WT vs. 0.3053 ± 0.02093 of TLR7$^{-/-}$; no significant difference). On the other hand, MΦs is known to express TLR7 (11), and its glomerular infiltration was significantly reduced in TLR7$^{-/-}$ NTN mice evaluated by immunohistochemistry of Mac-2 (42) 7 days after NTS challenge, compared to WT NTN mice (Figs. 1I and 1J; 0.849 ± 0.09267 versus 1.458 ± 0.1975 of WT; $P < 0.05$). In addition, no significant glomerular infiltration of lymphocytes (characterized with small nuclei with dense chromatin and unidentifiable cytoplasm in the capillary loops) was observed in the early and late time points (2 hrs and 7 days) in the WT and TLR7$^{-/-}$ NTN mice. These data clearly show that TLR7$^{-/-}$ mice show less severe glomerular injury in NTN, and suggest that endogenous TLR7 ligands and TLR7 signaling have the pathogenic role of NTN.

No significant defects in B cell and T cell immunity for IgG priming in TLR7$^{-/-}$ mice

Both T cell function (43, 44) and B cell function to produce autologous IgG are necessary to develop severe glomerular injury in NTN. Since TLR7$^{-/-}$ mice exhibited less severe glomerular injury than WT, we examined humoral immune reaction in TLR7$^{-/-}$ mice. We analyzed autologous murine anti-rabbit antibody levels in serum by ELISA on Day 7 of NTN. TLR7$^{-/-}$ mice showed comparable level of serum IgG against rabbit IgG (Fig. 2A). Recall assay using spleen cells from the primed WT or TLR7$^{-/-}$ mice revealed no significant difference in the production of IFN-γ and IL-6, T cell cytokines, between two strains (Fig. 2B). Finally, we examined the deposition of rabbit IgG and mouse IgG in the glomeruli by immunofluorescence (IF) study, showing comparable levels of rabbit and mouse IgG staining in both linear and fine granular pattern (Figs. 1C and 1D). Similarly, no significant difference in C3 deposition was observed between WT and TLR7$^{-/-}$ mice with NTN (Supplemental Figs. A **and** B). These results were confirmed by ultrastructural study: WT mice and TLR7$^{-/-}$ mice showed the similar levels of electron dense immune complex deposits in the subendothelial spaces (Fig. 2E) and the mesangial areas (Fig. 2F), corresponding to fine granular IgG staining by IF. These data clearly showed that TLR7$^{-/-}$ mice in NTN have abundant glomerular IgG deposition without significant defects of

humoral immunity at least for IgG priming, and suggested that immunological events after glomerular IC deposition were altered in TLR7$^{-/-}$ mice, leading to less severe glomerular injury.

TLR7 is expressed predominantly in macrophages

Neutrophils and macrophages are major inflammatory cells recruiting to glomeruli in NTN, in the early phase and the late phase, respectively (5, 45). Our NTN model has abundant deposition of mouse IgG and rabbit IgG (Fig. 2C) in glomeruli, and it is not practical to analyze TLR7 positive cells by immunohistochemistry. Therefore, we took the different approaches. First, we analyzed what kinds of cells express TLR7 in NTN glomeruli by Western Blot. Mouse mesangial cells and podocytes expressed little amount of TLR7 (Fig. 3A), while Raw 264.7 cells (a mouse MΦ cell line) and mouse bone marrow-derived MΦs expressed a high level of TLR7. The similar result was observed in human cells (Fig. 3B). Regarding its function, it is known that mesangial cells do not express a functional level of TLR7 (31). We tested that the TLR7 function on mouse podocytes by cell culture experiment, showing that TLR7 stimulation did not produce IL-6 while TLR4 activation secreted robust IL-6 (Fig. 3C). In addition, neutrophils are one of the earliest leukocyte subsets to be recruited to deposited autoantibodies and are known to promote glomerular injury (46). We collected neutrophils and peritoneal MΦs from WT or TLR7$^{-/-}$ mice and confirmed that MΦs did, but neutrophils did not express TLR7, consistent with the literatures (47, 48). Lastly, we tried to eliminate the possibility of TLR7 expression in glomerular endothelial cells. There are no reliable well-differentiated glomerular endothelial cell lines available. Therefore, we isolated glomeruli from WT and TLR7$^{-/-}$ mice, and examined TLR7 expression by Western Blot. We could not detect substantial TLR7 protein. These data were confirmed by immunohistochemistry (Fig. 3F). These data clearly showed that only MΦs express robust TLR7, but not neutrophils or glomerular resident cells.

EGFR inhibitor blocks TLR7-mediated gene induction

The series of experiments suggested that 1) endogenous ligand(s) released from the damaged tissue activates TLR7 in NTN, 2) TLR7 in MΦs exacerbates glomerular injury in NTN. These results prompt us to test the possibility of TLR7 inhibition for an excellent therapeutic target. However, there are no specific TLR7 antagonists available. Therefore, we took the different approach to block TLR7 signaling.

Recently we reported that epidermal growth factor receptor (EGFR) is essential for the signaling of TLR3 (30), TLR4 (49), and TLR9 (50). NF-κB is a major transcription factor that regulates inflammatory responses to TLR7, like other TLRs. We examined the effects of EGFR blockage on TLR7-mediated NF-κB activation, p65 nuclear translocation in mouse MΦs by immunocytochemistry (Fig. 4A). Without TLR7 stimulation, the MΦs exhibited cytoplasmic pattern of p65. Upon TLR7 stimulation with R848, p65 immediately translocated to the nuclei in the majority of the cells. However, the preincubation with gefitinib, an EGFR kinase inhibitor, significantly blocked such a nuclear translocation of p65. IκBα is the major regulatory inhibitor of NF-kB, and its phosphorylation at serine 32 leads to proteasomal degradation of IκBα, releasing p65 for the nuclear translocation. One-hour incubation with a TLR7 ligand immediately caused IκBα phosphorylation. Such

a phosphorylation, however, was significantly inhibited by an EGFR kinase inhibitor (Fig. 4D). Next, we tested IL-6 production, a downstream event in NF-κB pathway. The stimulation with R848 at 0.1 μg/ml strongly induced IL-6 production in RAW 264.7 cells (Supplemental Figs. C **and** D). Gefitinib near completely blocked IL-6 production upon TLR7 stimulation in RAW 264.7 cells (Fig. 4B) and bone marrow-derived MΦs (Fig. 4C), likewise the complete inhibition of TLR3 and TLR9 signaling stimulated by poly (I:C) and CpG, respectively. Importantly, IFN-γ mediated IL-6 production in MΦs was not affected by gefitinib treatment (Fig. 4B), suggested that the effect of gefitinib is specific to TLR7 signaling, not mediated by non-specific or toxic effects. TLR7 signaling activates type I IFN pathway in some types of cells (51). P56 is one of the interferon-stimulated gene (ISG) products of TLRs(52), including TLR7(53). The stimulation of TLR7 by R848 at 0.1 μg/ml for 8–16 hours strongly induced ISGs in RAW 264.7 cells (Supplemental Figs. E **and** F). The p56 induction was near completely blocked by gefitinib in MΦs (Fig. 4E). These data clearly showed that TLR7 signaling requires EGFR kinase activity.

EGFR is necessary for TLR7-mediated cell function

It is well known that TLR7 activation induces inflammatory cytokines and type I IFN production (54). However, other cell functional effects by TLR7 have not been well understood. Here, we tested the cell migration effect of TLR7. TLR7 signaling stimulated cell migration of MΦs (RAW264.7 cells) after 24-hour incubation with R848 in would healing assay, and this effect is in a dose-dependent manner (Fig. 5A). EGFR kinase inhibitor significantly inhibited the TLR7-mediated cell migration effect (Fig. 5D). Src and ERK are major signaling molecules of EGFR (55), and regulate many important cellular functions such as migration, adhesion, and differentiation (56, 57). Src was immediately and transiently phosphorylated at its tyrosine 416 (Fig. 5B), and this phosphorylation is completely blocked by EGFR kinase inhibitor (Fig. 5E). ERK1/2 were also quickly phosphorylated at threonine 202 and tyrosine 204 (Fig. 5C), which is partially blocked by EGFR inhibitor (Fig. 5F). These data show that not only inflammatory cytokines, but also cellular function, such as cell migration was regulated by TLR7 signaling at the upper level than Src and ERK activation.

EGFR is required for TLR7 tyrosine phosphorylation

All endosomal TLRs contain tyrosine residues in the cytoplasmic domains and we reported that EGFR is required for the tyrosine phosphorylation in TLR3(30) and TLR9(50), which are essential for the signaling. Upon TLR7 stimulation, endogenous EGFR was immediately and transiently recruited to TLR7 after 15 min in (Fig. 6A) in MΦs. TLR7 has 10 tyrosine residues in the cytoplasmic domain(58), and the tyrosine residue(s) were phosphorylated only after TLR7 stimulation (Fig. 6B). Interestingly, the TLR7 tyrosine phosphorylation was significantly blocked by an EGFR kinase inhibitor in RAW 264.7 cells (Fig. 6C) and bone marrow-derived MΦs (Fig. 6D). These data show that EGFR physically interacts with TLR7 in a signal-dependent manner, and that EGFR is needed for TLR7 tyrosine phosphorylation, taken together suggesting that EGFR inhibitor efficiently ameliorates TLR7-mediated glomerular injury by TLR7-positive MΦs in crescentic GN.

Renoprotective effects of EGFR inhibitor on glomerular injury is mainly through TLR7 signaling

The series of experiments revealed that our model of crescentic GN is TLR7-positive MΦs dependent because TLR7$^{-/-}$ mice are resistant to the NTN and because only MΦs express substantial and functional TLR7 in glomeruli. We, now, examined that EGFR inhibitor had any additional protective effects on glomerular injury in TLR7$^{-/-}$ mice with NTN. Surprisingly, we did not observe significant additional protective effects of gefitinib on proteinuria in TLR7$^{-/-}$ mice (group: TLR7$^{-/-}$, Gefitinib +) compared to TLR7−/− mice without gefitinib (group: TLR7$^{-/-}$, Gefitinib -) (Fig. 7A). Likewise, no significant difference was seen among three groups (TLR7$^{-/-}$ mice with or without gefitinib, and TLR7$^{+/+}$ mice with gefitinib) histologically in fibrinoid necrosis (Fig. 7B), crescent formation (Fig. 7C), and MΦ infiltration in glomeruli (Fig. 7D). Finally, we employed mice lacking EGFR only in MΦs (EKOMΦ mice) to test the effect of EGFR in MΦs on the TLR7-dependent glomerular injury. The EKOMΦ mice are resistant to NTN: proteinuria was significantly reduced in NTN EKOMΦ mice compared to WT mice (group: TLR7$^{+/+}$, Gefitinib -), but not reduced to the level of the three groups (TLR7$^{-/-}$ mice with or without gefitinib, and TLR7$^{+/+}$ mice with gefitinib) (Fig. 7A). Other histological findings were improved in the EKOMΦ mice to the similar level of the three groups statistically (Figs. 7B–D). Finally, we confirmed that the induction of p56, a TLR7-downstrem molecule in purified glomerular macrophages was significantly diminished in TLR7−/− mice, TLR7+/+ mice with an EGFR inhibitor, and EKOMΦ mice (Fig. 7E) , and that GFR impairment was improved in these mouse groups (Fig. 7F). These data clearly showed that renoprotective effects of EGFR inhibitor on glomerular injury is mainly through TLR7 signaling in MΦs.

DISCUSSION

In this study, we provide support for the paradigm that endogenous danger signaling exacerbates glomerular injury in crescentic GN via TLR7-EGFR axis in MΦs (Fig. 7E). NTN is a murine severe crescentic GN that manifests the pathological features of human proliferative lupus nephritis and anti-GBM GN. (1) Nephrotoxic serum induces injuries in glomerular resident cells and inflammatory cells, releasing endogenous TLR7 ligands. (2) TLR7 is exclusively expressed in MΦs in glomeruli of NTN. TLR7 ligands activate TLR7 in macrophages, which requires EGFR kinase activity to phosphorylates TLR7 tyrosine residues. Upon EGFR-mediated TLR7 activation, macrophages exhibit cell migration enhancement (3) and inflammatory cytokine production (4), leading to crescent formation, a malignant glomerular lesion. (5) EGFR inhibition efficiently blocks TLR7 tyrosine phosphorylation, TLR7 signaling, and subsequent glomerular damages.

In the most common protocol of NTN(41, 59, 60), mice are primed with normal IgG emulsified with complete Freund’s adjuvant (CFA). However, we employed incomplete Freund’s adjuvant (IFA) because CFA, but not IFA, contains heat-killed mycobacterium tuberculosis, known TLR2, 4, and 9 agonists(61) and these TLRs shares in part the downstream signaling with TLR7. We eliminated the effects of mycobacterium tuberculosis through TLR2, 4, and 9 on our experiments.

We showed that TLR7 is not expressed in glomerular resident cells or neutrophils, but confined in the MΦs in GN. MΦs/monocytes are the major effector cells in crescentic GN (62–65), and observed in the early lesion of GN such as endocapillary inflammation (Fig. 1G–J), and within crescents (66–69). On the other hand, EGFR is more widely expressed in the glomeruli (70). Bollee et al. reported that EGFR promotes glomerular injury in a CFA-primed NTN via heparin-binding epidermal growth factor-like growth factor (HB-EGF) - EGFR pathway in podocytes(71). Conditional deletion of the *Egfr* gene from podocytes of mice alleviated proteinuria and crescent formation, but only by 40% compared to wild type mice (71). This evidence suggested that EGFR has the pathogenic role of crescentic GN in other cell types than podocytes. According to our results, EGFR is required for TLR7 signaling in MΦs, responding to endogenous RNAs released from injured cells, likely glomerular endothelial cells and/or MΦs, and would exacerbate glomerular injury. Several endogenous ligands for TLR7 have been identified in general, including microRNAs (miR-21 and miR29a(34); miR-34a, −122, −133a, −142, −146a, and −208a(35)), self-RNA-antimicrobial peptide (72), and small nuclear ribonucleoprotein (32).

In addition to glomerular lesions, EGFR has an impact on tubulointerstitial injury and renal fibrosis in an ischemia-reperfusion injury (IRI) model (73, 74). EGFR has a protective role of promoting the recovery of damaged tubular epithelial cells in the early stage (75–77), and however sustained activation of EGFR in proximal tubular cells enhances renal fibrosis in the late stage(78–80). In our study, we focused on glomerular injury in the relatively early phase of crescentic GN on Day 7. We believe the effects of EGFR-mediated tubulointerstitial damage on our assays is minimal because our readouts are mainly histological evaluation of glomeruli and proteinuria in the early stage (within 7 days).

NTN has been commonly used as a murine model of human anti-GBM GN (5, 81) because of the linear staining of IgG and high frequency of crescent formation. However, our NTN model clearly shows the pathological features of lupus nephritis as well: fine granular IgG staining in mesangial area and capillary loops by immunofluorescence study (Fig. 2C) and electron dense deposits in ultrastructural study (Figs. 2E and 2F). Nephrotoxic serum (NTS) is generated by immunizing rabbit with whole glomeruli(82, 83), containing not only GBMs but also glomerular cell components, and composed of rabbit IgG against not only GBM but also various cellular antigens in glomeruli. In our study, $TLR7^{-/-}$ mice showed comparable levels of linear and fine granular deposition of IgG to WT mice, supporting the attenuated glomerular injury is not due to decreased IC deposition, rather MΦ reaction.

Lastly, EGFR is a member of the ErbB family of receptor tyrosine kinase, and regulates various cell functions(84–86). We provided a mechanistic evidence that EGFR is needed for TLR7 signaling. Upon TLR7 stimulation with a TLR7 ligand, EGFR is recruited and physically interacted with TLR7 (Fig. 6A). TLR7 has 10 tyrosine residues in the cytoplasmic domain (58). One or more of these tyrosine residues were phosphorylated upon TLR7 stimulation dependent on EGFR kinase activity (Fig. 6B–D), clearly showing that EGFR is necessary for TLR7 tyrosine phosphorylation. Our group previously reported that EGFR plays pivotal roles in the signaling of TLR3 (30), TLR4 (49), and TLR9 (50). EGFR directly phosphorylates TLR3 tyrosine 858 (30), essential for TLR3 signaling; EGFR is required only for TRIF branch of TLR4 without physical interaction of EGFR-TLR4 (49);

and EGFR is constitutively bound to TLR9 and required for TLR9 tyrosine phosphorylation and its signaling (50). Binding of EGF to EGFR on the cell surface results in its dimerization and subsequent endocytosis to endosomes(85), where TLR7 is located. Endosomal EGFR is autophosphorylated and catalytically active(86). Although further research is needed, we speculate, similar to the TLR3-EGFR axis, that transiently recruited EGFR directly phosphorylates TLR7 tyrosine residue(s), and such tyrosine phosphorylation is required for the recruitment of adaptor molecule, MyD88, essential for TLR7 signaling and leading to severe glomerular damage.

In summary (Fig. 7G), we have demonstrated that danger signaling, likely endogenous RNA, exacerbates glomerular injury via EGFR-dependent TLR7 signaling in MΦs in crescentic GN. While EGFR is broadly expressed in kidney tissue, TLR7 is exclusively expressed in MΦs in glomeruli of crescentic GN, and such a detrimental TLR7 signaling is effectively blocked by the depletion of EGFR kinase activity, pharmacologically and genetically. Our findings could stimulate evaluation of EGFR-TLR7 axis in MΦs as a therapeutic target in crescentic GN if could avoid EGFR-depletion effects on tubular cell regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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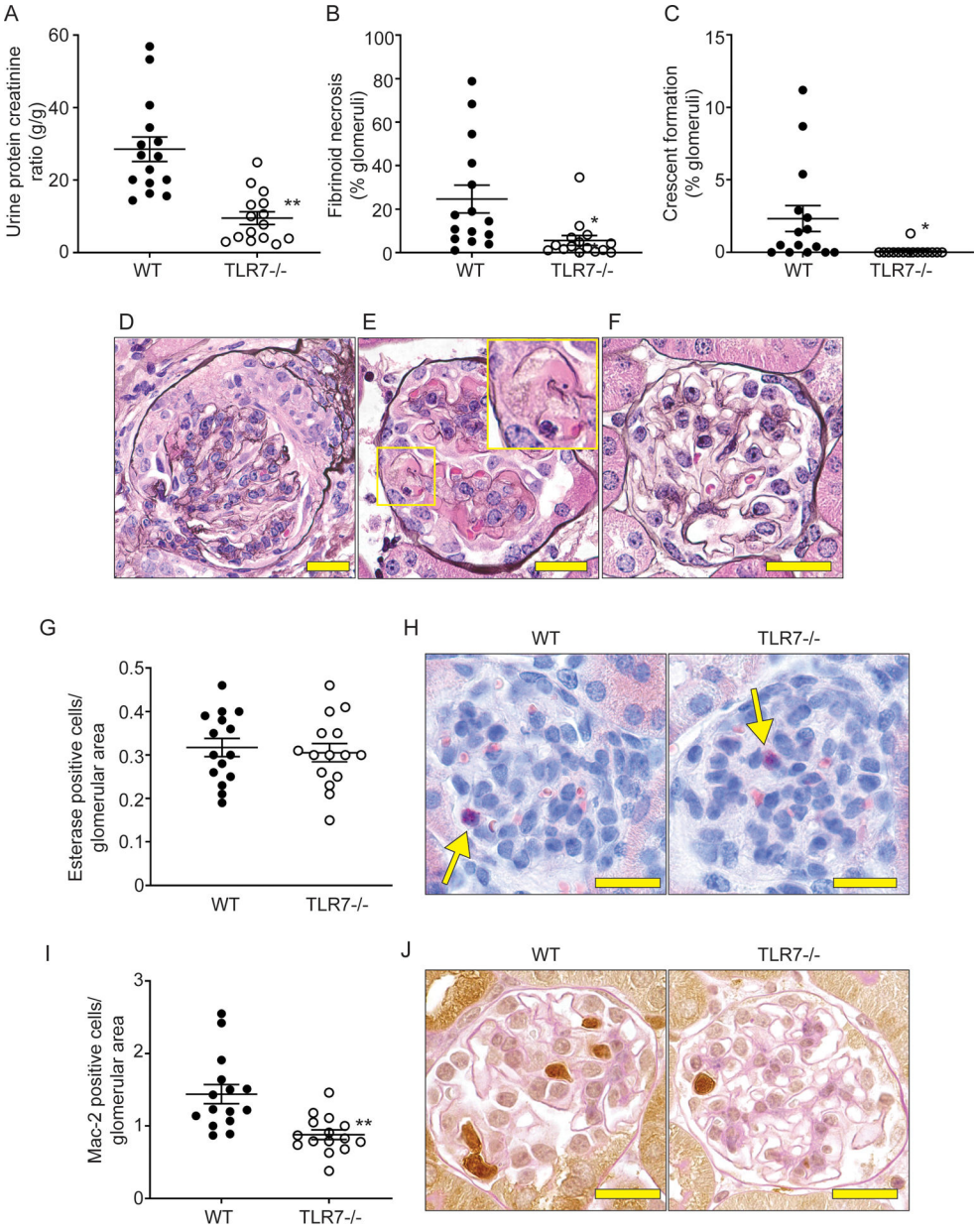

Figure 1. TLR7 knock-out mice are resistant to nephrotoxic serum nephritis (NTN).

(A) Wild type (WT) or TLR7$^{-/-}$ mice were primed with 0.5 mg of normal rabbit IgG emulsified with Incomplete Freund's Adjuvant (IFA) on Day −5, followed by intravenous injection with 50 ul of nephrotoxic serum on Day 0. Spot urine was collected on Day 7 to measure urine protein creatinine ratio. Kidneys were harvested on Day 7 to evaluate fibrinoid necrosis **(B)** and crescent formation **(C)**. **(D)** A representative image of crescent formation in glomerulus from a WT mouse. Periodic acid-methenamine silver staining. **(E)** A representative image of fibrinoid necrosis (glomerular basement membrane rupture, fibrin deposits, and karyorrhexis) in glomerulus from a WT mouse. Periodic acid-methenamine silver staining. **(F)** A representative image of unremarkable glomerulus from a TLR7$^{-/-}$ mouse. Periodic acid-methenamine silver staining. **(G)** Neutrophils were stained with

esterase on the kidney tissue sections from WT mice and TLR7$^{-/-}$ mice 2 hrs after NTS challenge. The number of esterase positive cells were counted in all glomeruli. **(H)** A representative image of esterase positive cells in glomeruli of WT mice and TLR7$^{-/-}$ mice. **(I)** Macrophages were stained with anti-Mac-2 Ab on the kidney tissue sections from WT mice and TLR7$^{-/-}$ mice 7 days after NTS challenge. The number of Mac-2 positive cells were counted in all glomeruli. **(J)** A representative image of Mac-2 positive cells in glomeruli of WT mice and TLR7$^{-/-}$ mice. All yellow bars: 25 μm; *: P<0.05; **: P<0.005.

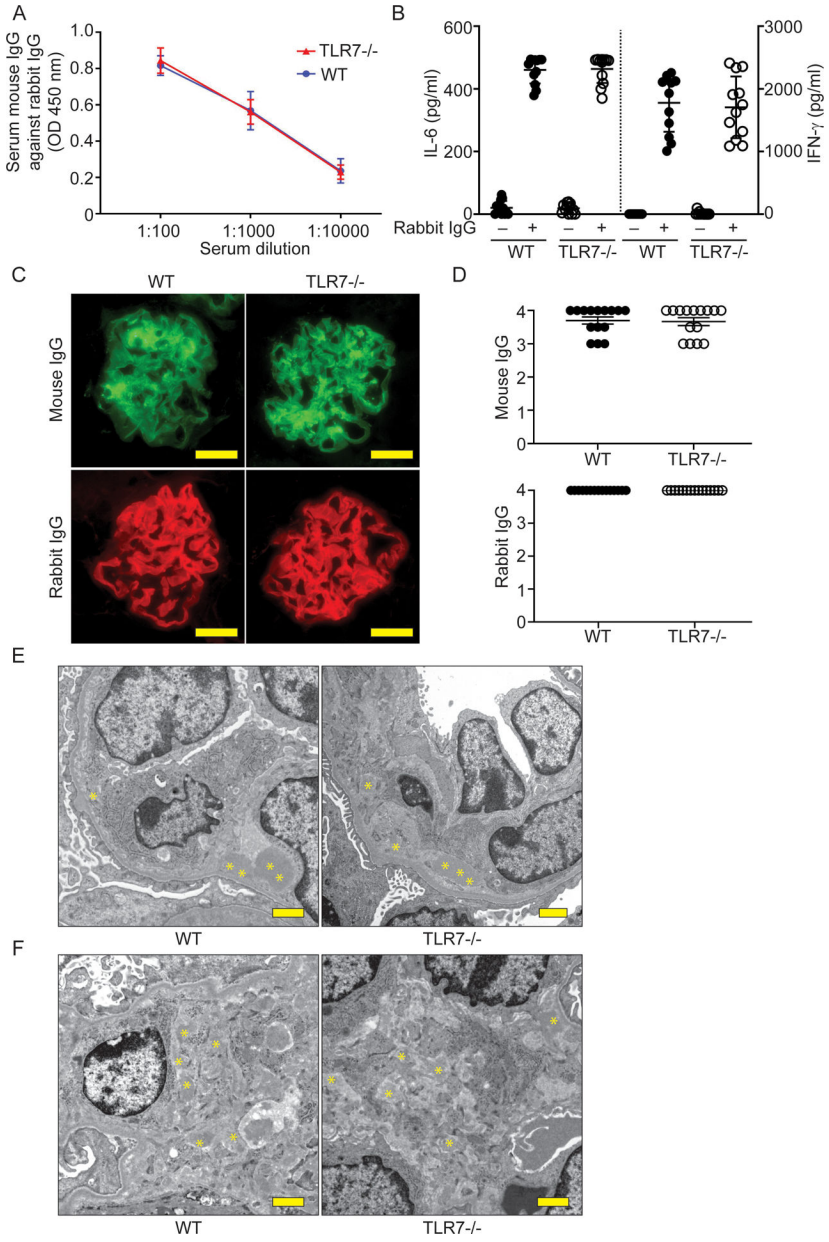

Figure 2. No significant defects in humoral immunity for IgG priming.

(A) Autologous murine anti-rabbit antibody levels were determined by ELISA from serum samples from WT (n=8) or TLR7$^{-/-}$ (n=8) mice on Day 7 of NTN. Titers in serial dilutions of serum are expressed in arbitrary units (OD 450 nm). **(B)** Splenocytes were isolated from WT or TLR7$^{-/-}$ mice after 14 days immunization of rabbit IgG. Cells were incubated with or without rabbit IgG for 24 h. Supernatants were collected to measure IL-6 and IFN-γ by ELISA. **(C)** Representative immunofluorescence images of glomerular mouse IgG (upper panels) and rabbit IgG (lower panels) depositions in WT (left panels) or TLR7$^{-/-}$ (right panels) mice on day 7 of NTN. Scale bars = 25 μm. **(D)** Semi-quantitative analysis of mouse IgG (upper panel) and rabbit IgG (lower panel) depositions in glomeruli of WT or TLR7$^{-/-}$ mice on day 7 of NTN. Representative Electron microscopy images of glomerular

capillary loops **(E)** and mesangial areas **(F)** from WT mice and TLR7$^{-/-}$ mice with abundant subendothelial electron dense immune-complex deposits and swollen endothelial cells, and numerous electron dense deposits in mesangial areas. Yellow asterisks indicate deposits. [uranyl acetate and lead citrate stain, scale bars: 1 μm].

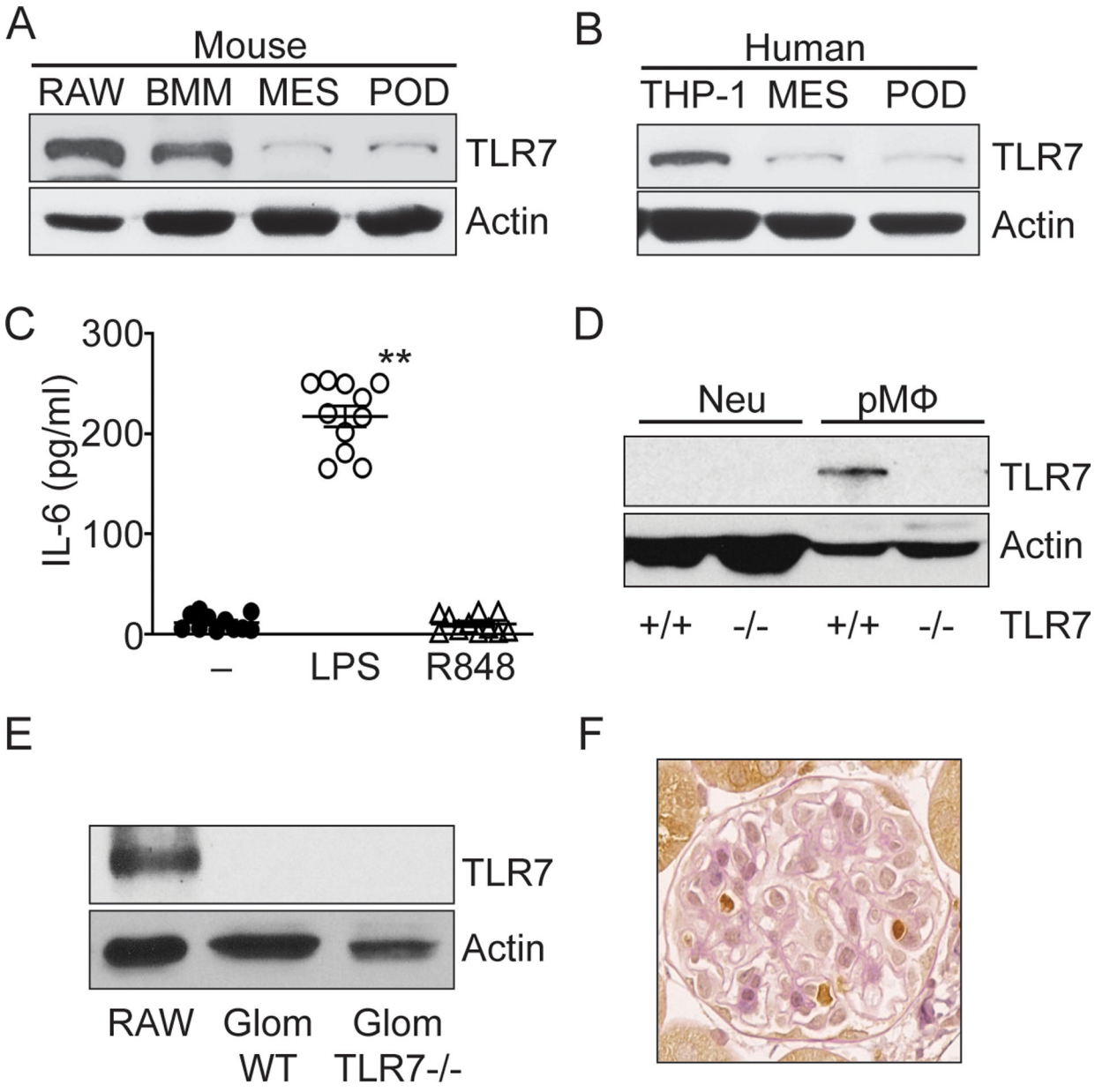

Figure 3. TLR7 is expressed predominantly in macrophages, but not in glomerular resident cells.

(A) TLR7 expression level was examined by Western Blot in various mouse cells. RAW: RAW 264.7 cells (a macrophage cell line), BMM: bone marrow-derived macrophages, MES: primary mesangial cells, and POD: temperature-sensitive SV40 T antigen-expressing podocytes. **(B)** TLR7 expression level was tested by Western Blot in human cells. THP-1: a macrophage cell line, MES: primary mesangial cells, and POD: temperature-sensitive SV40 T antigen-expressing podocytes. **(C)** Mouse podocytes were incubated with LPS (0.01 μg/ml), R848 (10 μg/ml), or left untreated for 24 hrs. The supernatants were collected to measure IL-6 by ELISA. **: $P<0.005$. **(D)** Neutrophils (Neu) and peritoneal macrophages (pMΦ) were collected from WT mice or TLR7$^{-/-}$ mice. TLR7 expression level was tested by Western Blot. **(E)** Expression of TLR7 in RAW 264.7 cells and isolated glomeruli from

WT or TLR7$^{-/-}$ mice were analyzed by Western blot. **(F)** Immunohistochemistry of TLR7 in glomerular infiltrates from a WT NTN mouse.

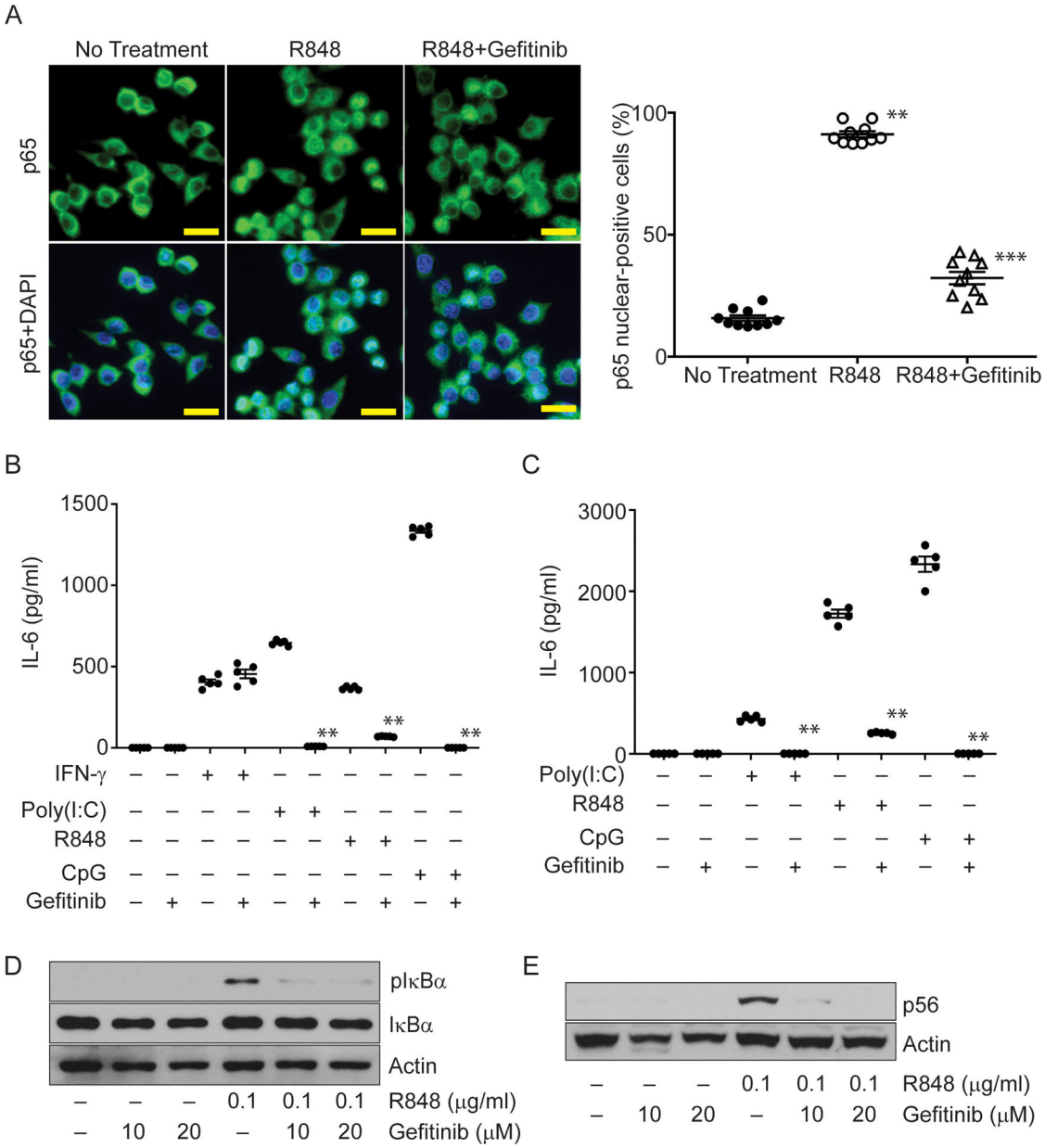

Figure 4. EGFR inhibitor blocks TLR7-mediated gene induction.

(A) RAW 264.7 cells were stimulated with R848 (0.1 μg/ml) for 60 min with or without Gefitinib (20 μM) preincubation for 60 min, or left untreated. p65 was stained to identify p65 localization (scale bars: 20 μm), and the nuclear positive cells for p65 were counted. **(B)** RAW 264.7 cells were incubated with IFN-γ (1000 U/ml), a TLR3 ligand (poly (I:C), 10 μg/ml), a TLR7 ligand (R848, 0.1 μg/ml) or a TLR9 ligand (CpGB, 0.1μM) in the presence or absence of an EGFR inhibitor (Gefitinib, 20 μM) for 24 hrs. IL-6 in the culture media was measured by ELISA. **(C)** Bone marrow-derived macrophages were incubated with poly(I:C), R848 or CpGB in the presence or absence of Gefitinib for 24 hrs. IL-6 in the culture media was measured by ELISA. **(D)** RAW 264.7 cells were stimulated by R848 (0.1 μg/mL) for 1 hour with or without pre-treatment of Gefitinib (10 or 20 μM). Expressions

of phospho-IκBα, total IκBα, and actin were tested by Western blot. **(E)** RAW 264.7 cells were stimulated by R848 (0.1 μg/mL) for 8 hours with or without pre-treatment of Gefitinib (10 or 20 μM). Expressions of p56 and Actin were tested by Western blot.

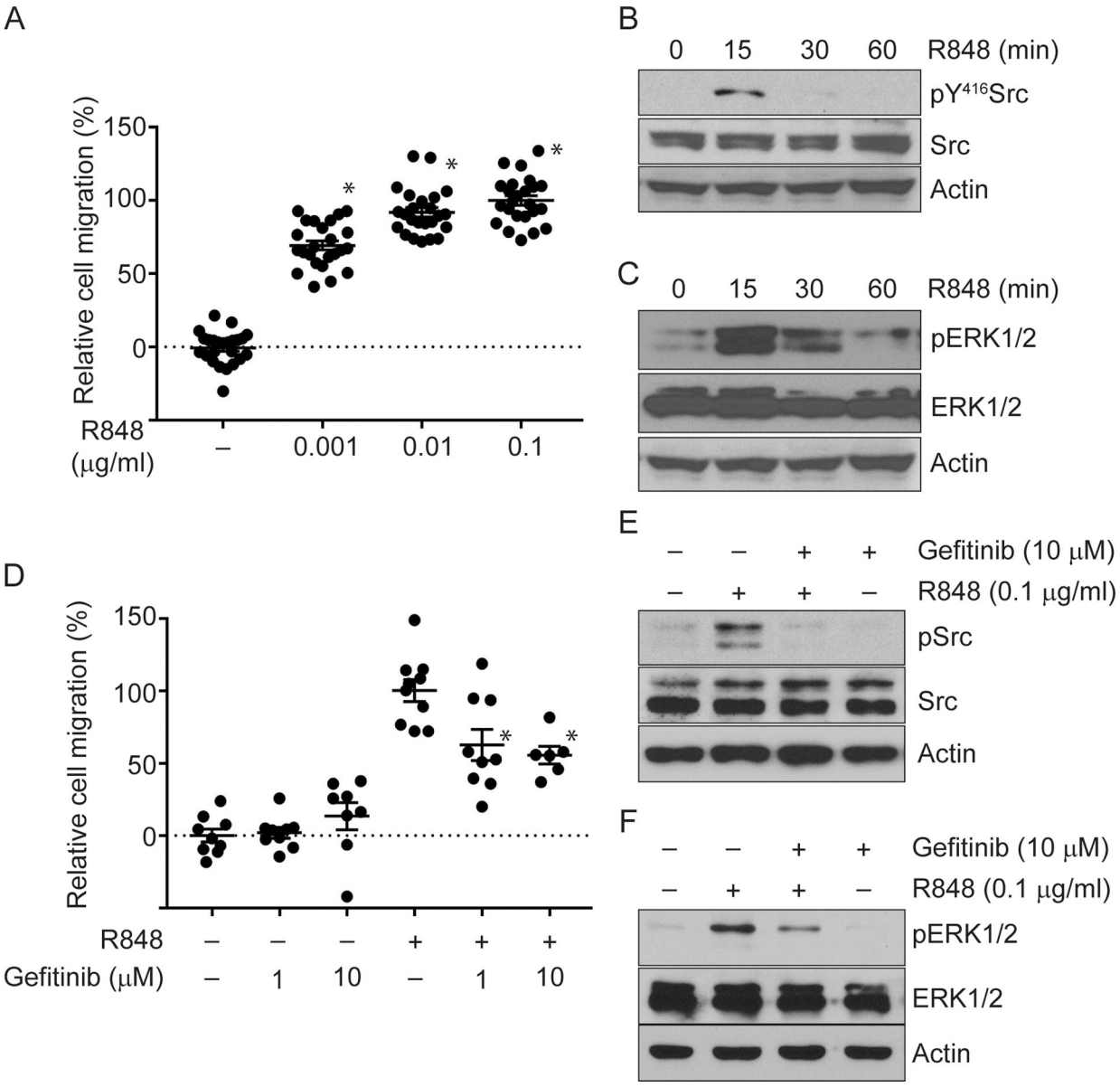

Figure 5. EGFR is required for TLR7-mediated cell migration.

(A) RAW 264.7 cells were treated with R848 at indicated concentration and their migration over 24 h was measured by wound healing assay. For each time point, the closed areas by the migrated cells were evaluated, and the area of the condition with R848 (0.1 μg/mL) was defined as 100 and all other values were normalized to this. **(B)** RAW 264.7 cells were stimulated by R848 (0.1 μg/mL) for the indicated time. pSrc, Src or actin was detected by Western blot. **(C)** RAW 264.7 cells were incubated with R848 (0.1 μg/mL) for the indicated time and pERK1/2, ERK1/2, or actin was examined by Western blot. **(D)** RAW 264.7 cells were treated with R848 and/or gefitinib as indicated combination and concentrations their migration over 24 h was measured by wound healing assay as described in (A). **(E)** RAW 264.7 cells were stimulated with R848 (0.1 μg/mL) for 15 min with or without gefitinib (10 μM). pSrc, Src or actin was tested by Western blot. **(F)** RAW 264.7 cells were stimulated by

R848 (0.1 μg/mL) for 15 min with or without gefitinib (10 μM). pERK1/2, ERK1/2, or actin was examined by Western blot.

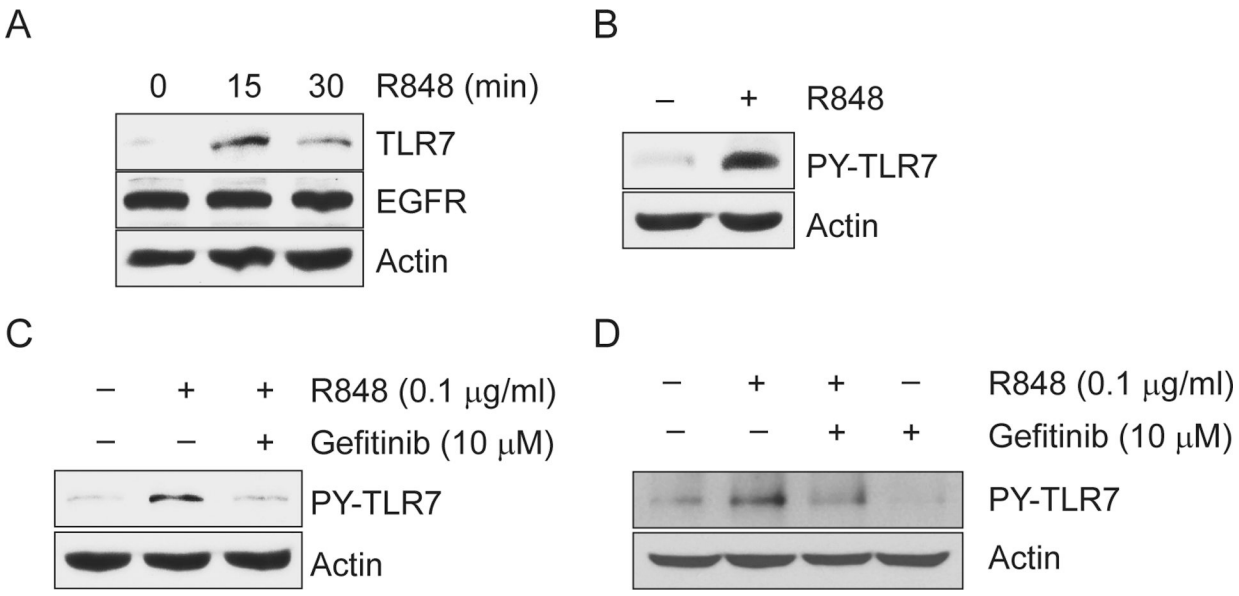

Figure 6. EGFR is required for TLR7 tyrosine phosphorylation.

(A) Raw 264.7 cells were incubated with R848 at 1.0 μg/ml for indicated time, and cell lysates were immunoprecipitated with anti-EGFR Ab. TLR7-EGFR interaction was detected with anti-TLR7 Ab Western Blot. The expression of EGFR or actin in original cell lysates was analyzed by Western Blot. **(B)** Raw 264.7 cells were incubated with R848 at 1.0 μg/ml for 30 min. The lysates were immunoprecipitated with anti-phosphotyrosine Ab. Tyrosine phosphorylation of TLR7 and actin (loading control) were detected by anti-TLR7 Ab and anti-actin Ab, respectively, by Western Blot. **(C)** Raw 264.7 cells were incubated with R848 ± gefitinib (20 μM), and cell lysates were immunoprecipitated with anti-phosphotyrosine Ab, followed by Western Blot with anti-TLR7 Ab to detect tyrosine phosphorylation of TLR7. Actin was used for loading control. **(D)** Bone marrow-derived macrophages were used for the same experiment as in (C).

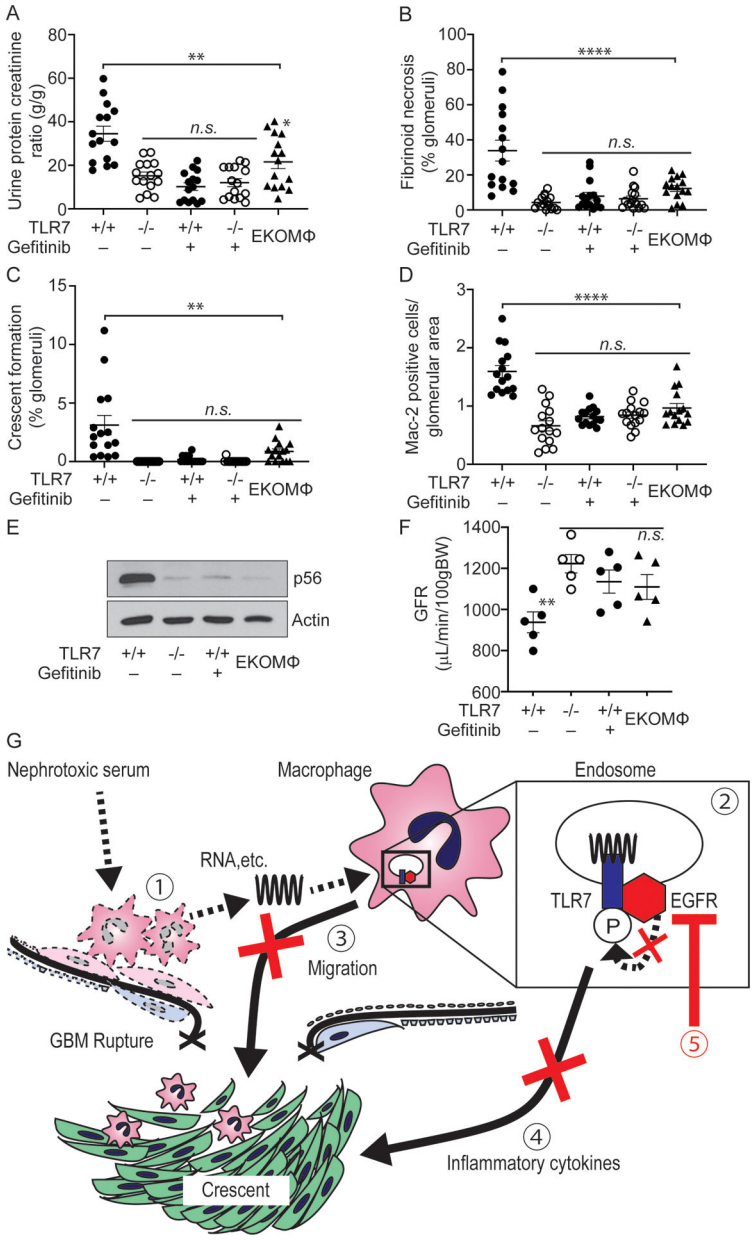

Figure 7. Renoprotective effects of EGFR inhibitor for glomerular injury is mainly through TLR7 signaling in macrophages.

Nephrotoxic serum nephritis was induced in wild type mice, TLR7$^{-/-}$ mice, or mice lacking EGFR in macrophages. Twelve hours before injection of nephrotoxic serum, some mice were treated with vehicle or 2.4 g/day of gefitinib by oral gavage (Day −1 to 7). Spot urine was collected on Day 7 to measure urine protein creatinine ratio **(A)**. Kidneys were harvested on Day 7, and glomeruli with fibrinoid necrosis **(B)** and crescents **(C)** and glomerular Mac-2 positive MΦs **(D)** were evaluated. n.s.: no significant difference; **(A)** *: P<0.01 for TLR7+/+ without gefitinib vs. EKOMΦ, and P<0.05 for TLR7−/− without gefitinib vs. EKOMΦ. **: P = 0.0044; (B) and (D) ****: P < 0.0001; (C) **: P = 0.0010 **(E)** Glomerular macrophages were purified from several different strains and conditions as indicated, on Day 7. P56 induction was measured by Western Blot (n = 5, each) **(F)** GFR

was measured in the same groups of mice on Day 7. **: $P = 0.0078$, TLR7+/+ vs. TLR7-/−. **(G)** Role of TLR7-EGFR axis in macrophages in crescentic GN. (1) Nephrotoxic serum induces injuries in glomerular resident cells and inflammatory cells, releasing endogenous TLR7 ligands. (2) TLR7 ligands activate TLR7 in macrophages, which requires EGFR kinase activity to phosphorylates TLR7 tyrosine residues. Upon EGFR-mediated TLR7 activation, macrophages exhibit cell migration enhancement (3) and inflammatory cytokine production (4), leading to crescent formation, a malignant histological lesion. (5) EGFR inhibition efficiently blocks TLR7 signaling, and subsequent glomerular damages.