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Toll-like receptor-4 (TLR4) promotes the development of colitis-associated colorectal tumors

Running Title: TLR4 promotes colitis-associated cancer

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Methods

Real-time PCR

The primers and probes used in this study are as follows (5' to 3' direction), for mouse Cox-2: sense primer, AAG GAA CTC AGC ACT GCA TCC, anti-sense primer, ACA GGG ATT GGA ACA GCA AGG A, and probe, ACC GCC ACC ACT ACT GCC ACC TCC; for mouse TLR2: sense primer, TTC AAC AAG ATC ACC TAC ATT GGC, anti-sense primer, GGC GTC TCC CTC TAT TGT ATT GAT, and probe, TGA CCT CCG AGC GTG TGC GAA CCT; for mouse TLR3: sense primer, GGG TGT TTC CAG ACA ATT GGC A, anti-sense primer, GTT CCC AGC AAA GCT TCT CTG TG, and probe, CGC CCT CCT CTT GAA CAA CGC CCA; for mouse TLR4: sense primer, CAG CAG AGG AAG AAC AAG AA, anti-sense primer, TGC AAA CAG ACT GGG TTT AG,

and probe, CGG CAA CTT GGA CCT GAG GA; for mouse TLR5: sense primer, GCT CGC TTA GAC CTA TCT GGC, anti-sense primer, TAC GTC GCT TAA GGA ATT CAG TTC, and probe, ACC AGA TTC ACA GCC TCC GCC TCC; for mouse TLR9: sense primer, CAC CAC CTG CAC AAC TCC GA, anti-sense primer, GCA ATG GAA AGG ACT GTC CAC TTT GTG, and probe, TCG TCC ACC TGT CCA ACC TGC GGC; for human amphiregulin: sense primer, CGT GTC CCA GAG ACC GAG TT, anti-sense primer, AGG TCC AAT CCA GCA GCA TAA TG, and probe, AGA CCG AGA CGC CGC CGC TGC; for mouse amphiregulin: sense primer, TGT CAC TAT CTT TGT CTC TGC CAT, anti-sense primer, AGC CTC CTT CTT TCT TCT GTT TCT, and probe, TCC TCG CAG CTA TTG GCA TCG GCA; for human β-actin: sense primer, CAT CCT CAC CCT GAA GTA CC, anti-sense primer, GCT CAT TGT AGA AGG TGT GG, and probe, CAC GGC ATC GTC ACC AAC TG; for mouse β-actin: sense primer, ATG ACC CAG ATC ATG TTT G, anti-sense primer, TAC GAC CAG AGG CAT ACA, and probe, CGT AGC CAT CCA GGC TGT GC. All TaqMan probes and primers were designed using Beacon Designer 3.0 software (Premier Biosoft International, Palo Alto, CA). The cDNA was amplified using TaqMan universal PCR Master Mix (Roche, Indianapolis, IN) on an ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA), programmed for 95°C for 10 minutes, then 40 cycles of: 95 °C for 15 seconds, 60 °C for 1 minute. The amplification results were analyzed using SDS 2.2.1 software (Applied Biosystems, Foster City, CA) and the gene of interest was normalized to the corresponding β-actin results. Data were expressed as fold induction relative to the lowest gene product amplified.

Immunofluorescent and immunohistochemical studies

Human paraffin-embedded human colectomy specimens were stained with biotinylated mouse monoclonal anti-human TLR4 (1:500, eBioscience, San Diego, CA) overnight at 4°C, followed by streptavidin-FITC (10 μg/ml, eBioscience, San Diego, CA) for 1 hour at room temperature. Antigen retrieval by microwave was performed prior to the staining procedure. Nonspecific binding of the primary antibody was blocked by normal rabbit serum and 5% skim milk. Avidin/biotin blocking reagent (Zymed Laboratories, South San Francisco, CA) was used to block nonspecific binding of streptavidin-FITC. The specificity of staining was confirmed by omitting the primary antibody.

Frozen sections of mouse specimens were fixed in cold acetone for 5 minutes. After blocking with 10% normal goat serum for 1 hour, sections were incubated with rabbit anti-TLR4 antibody (1:100, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. FITC-conjugated anti-rabbit IgG (1:200, Sigma, St. Louis, MO) was used as a secondary antibody.

Double immunofluorescent staining of CD68 or a fibroblast marker and Cox-2 was performed using acetone fixed OCT sections. Sections were incubated with 0.1% Trypsin (Sigma, St. Louis, MO) $CaCl_2$ dissolved in 0.05M Tris-HCl pH 7.6 for 15 minutes at 37 °C. Subsequently sections were blocked in a 5% skim milk for 1 hour and then incubated with the rat anti-CD68 antibody (1:20, MCA1957S, Serotec Ltd., Raleigh, NC) or rat monoclonal anti-fibroblast antibody (ER-TR7; Biogenesis, Poole, United Kingdom) overnight at 4°C. After washing in PBS, sections were incubated with TRITC-conjugated rabbit anti-rat IgG (1:200, Sigma, St. Louis, MO) for 1 hour at room temperature. Then

sections were re-incubated with 5% skim milk and stained with anti-murine Cox-2 antibody (1:200, Cayman, Ann Arbor, MI) overnight at 4°C, followed by FITC-conjugated goat anti- rabbit IgG (1:200, Sigma, St. Louis, MO) for 1 hour at room temperature.

Double stained tissue slides were examined using a Leica TCS-SP (UV) confocal microscope. Other slides were viewed on a Nikon eclipse E600 immunofluorescence microscope and photographs were taken with a digital camera using the Spot Advanced software program (Diagnostic Instruments Inc, Sterling Heights, MI).

Western blots

Tissue lysates were prepared from either human or mouse colon samples or SW480 cells using a lysis buffer containing 50mM Tris HCl, 50mM NaF, 1% Triton X100, 2mM EDTA, and 100mM NaCl, with a proteinase inhibitor cocktail (Calbiochem, San Diego, CA). Protein concentration was determined by the Bradford method using Bio-Rad Protein Assay Dye and SmartSpec^{TM} 3000 (Bio-Rad Laboratories, Hercules, CA). Twenty-five μg of the lysates were subjected to 10% SDS-PAGE and transferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA). The membrane was blocked in 5% skim milk and was immunoblotted with the primary antibodies for 1 hour, followed by incubation with HRP-conjugated secondary Ab. The membrane was exposed on an x-ray film using an enhanced chemiluminescent substrate SuperSignal West Pico Trial Kit (Pierce Biotechnology, Rockford, IL). For human TLR4 Western blots, we used biotinylated monoclonal anti-human TLR4 from eBioscience (San Diego, CA), in combination with streptavidin-HRP (Zymed Laboratories, South San Francisco, CA). For mouse TLR4 Western blots, tissue samples were excised from OCT-embedded frozen tissues after staining slides from same blocks with hematoxylin and

eosin for histologic assessment to distinguish neoplasia from non-dypslastic mucosa. Anti-mouse TLR4 Ab was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and HRP-conjugated goat anti-rabbit IgG was from Jackson ImmunoResearch Laboratories, Inc (West Grove, PA). Antibodies against phospho-EGFR (Tyr 1068, mAb) for human cells was purchased from Cell Signaling (Beverly, MA). Anti-phospho-EGFR (Tyr 1173, goat polyclonal) for mouse samples and anti-EGFR (rabbit polyclonal) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA) for mouse and Upstate (Lake Placid, NY) for human, respectively. Corresponding secondary Abs, anti-goat and anti-rabbit -HRP were both from Jackson ImmunoResearch Laboratories, Inc (West Grove, PA). β-actin (both human and mouse anti-β-actin from Sigma, St. Louis, MO) was used as an internal control for protein loading. For experiments using amphiregulin Ab, cells were pre-incubated with an anti-human amphiregulin neutralizing antibody (R&D Systems, Minneapolis, MN) or anti-EGFR neutralizing antibody (Upstate, Lake Placid, NY) at a concentration of 10μg/ml two hours prior to LPS stimulation. Normal mouse IgG (10μg/ml) (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a negative control.