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MTG16 contributes to murine colonic epithelial integrity after injury and has reduced expression in ulcerative colitis

Supplementary Methods:

DSS Protocol

Dextran Sodium Sulfate (DSS), MW 40,000-50,000, was obtained from USB Corporation (Cleveland, OH). A single lot was used for all experiments described in this publication. A DSS solution was produced at the indicated w/v percentage in water then filtered through 0.22 mm cellulose acetate filters[1]. This solution, or non-DSS containing water, was then substituted for animal drinking water and barrier tips were placed on the automatic watering spigots in each cage. Animals were treated with DSS for the indicated time periods and then either allowed to recover for 3 days on water, or sacrificed immediately. Animals were weighed on day 0, day 4, and day 7. Stools were examined for consistency and the presence of blood. At sacrifice, colons were excised, flushed with PBS and opened longitudinally then rolled as "Swiss Rolls"[2]. The tissue was fixed in formalin overnight and then switched to 70% ethanol prior to embedding, sectioning and staining with H+E for histologic analysis.

Histologic Assessment of Injury

M. B. P. reviewed and scored the *C. rodentium* slides and M. K. W. analyzed the DSS slides. For *C. rodentium*-induced colitis both acute (neutrophilic) and chronic (lymphocytic) inflammation, extent of inflammation, and epithelial damage were each scored on 0–3 scale[3]. The aggregate histologic injury score was the sum of acute and chronic inflammation multiplied by the extent of inflammation, plus the epithelial injury (0 –21). For the DSS injury model severity of injury was assessed in a blinded fashion

and damage was quantified on a multi-component scale based on the Dieleman method[4].

Enterocyte Proliferation Determination

BrdU (5-bromo-2'-deoxyuridine) was injected intraperitoneally (IP) at a dose of 16.7 μg/gram of body weight 2 hours prior to sacrifice. Actively replicating cells were visualized by immunohistochemical labeling using anti-BrdU[5]. Five-micron sections of paraffin-embedded tissues were placed on charged slides and the paraffin removed. The sections were rehydrated and placed in heated Target Retrieval Solution (Dakocytomation, Carpinteria, CA) for 20 minutes. Endogenous peroxidase was neutralized with 0.03% hydrogen peroxide and samples were treated with diluted rabbit serum prior to primary antibody addition. Tissues were incubated with monoclonal rat anti-BrdU (Accurate Labs, Edison, NJ) diluted 1:2000 for 45 minutes. Sections without primary antibody served as negative controls. The Vectastain ABC Elite (Vector Laboratories, Burlingame, CA) system and DAB+ (Dakocytomation) was used to produce localized, visible staining. Slides were lightly counterstained with Mayer's hematoxylin, dehydrated and cover-slipped. A distal colon Proliferation Index was obtained by counting the number of positive staining cells in 100 contiguous full crypts[6].

Assessment of Apoptosis

Apoptosis rates were measured via indexing selectively labeled apoptotic cells using an *in situ* TUNEL assay per the manufacturers protocol (ApopTag, #S7100, Chemicon, Billerica, Massachusetts) In brief, 5 μm sections were cut from formalin fixed, paraffin embedded colon. The sections were de-parafinized and rehydrated through

washes with graded concentrations of ethanols. Tissue was pretreated with Proteinase K (20 mg/ml) for 15 minutes at room temperature, followed by incubation in 3.0% H_2O_2 in PBS for 5 minutes at room temperature to quench endogenous peroxidase activity. Apoptosis analysis was performed using terminal deoxynucleotidyl-transferase (Td-mediated) dUTP nick end-labeling (TUNEL) assay, per the manufacturers protocol. Control stains were obtained by processing in parallel, duplicate sections, omitting only the TnT enzyme. Sections were counterstained in 0.5% (w:v) methyl green for 15 seconds, dehydrated with n-butanol and xylenes and mounted with permamount. Non-specific staining was not observed in the control reactions. An apoptosis index was generated by counting the number of TUNEL positive cells per crypt, examining each crypt sequentially proceeding retrograde from the rectum until 100 full crypts were counted.

Caspase 3 staining

Following antigen retrieval in 10 mM sodium citrate for 20 minutes at 100° C, and inactivation of peroxidases with 3% H2O2 for 5 minutes, slides were blocked with TNT blocking buffer (Perkin Elmer) for 30 minutes. Slides were incubated with anti-active caspase 3 (Cell signaling, 1:200) for 2 hours, and anti-rabbit HRP (Amersham, 1:1000) for one hour. Slides were washed and incubate with Tyramide Signal Amplifying reagent (Perkin Elmer) (1:50 in amplification diluent, according to the manufacturer's instructions) for 10 minutes. Slides were washed, mounted in Vectashield H1000 (Vector laboratories), and visualized by Fluorescence microscopy using a Leica DM IRBE inverted wide-field microscope (Bannockburn, IL USA) in the Vanderbilt University Cell Imaging Core.

Bone Marrow Transplantation

Bone marrow was harvested from the femurs and tibias of WT mice as previously described[7], and 1 x 10^6 cells were injected via the tail vein into lethally irradiated (single dose of 900 rads) 7 weeks old syngeneic recipient *Mtg16*$^{-/-}$ mice. The mice were then fed acidified water (0.015% HCl in autoclaved water) supplemented with 1.1 g/L neomycin sulfate and 125 mg/L polymyxin B sulfate for two weeks post transplantation (modified from [8]). Three mice were not transplanted and served as lethal irradiation controls. Eight weeks post-transplantation the mice were treated with 3% DSS for 4 days (as described previously), allowed to recover on water and then euthanized on day 7. Wild type and *Mtg16*$^{-/-}$ and wild type-marrow recipients were given regular water as controls.

Immunophenotyping

The colon was cut and weighed. After weighing, it was rinsed gently in PBS, and cut with scissors into 2-mm pieces. Colon pieces were digested for 20 min with 1 mg/ml dispase, 0.25 mg/ml collagenase A, and 25 U/ml DNase (Roche Diagnostics, Indianapolis, IN) at 37°C. The suspension was passed through a 70-μm cell strainer (BD Biosciences, San Diego, CA). Cells were harvested by centrifugation and washed with PBS containing 4% fetal bovine serum. Cells were resuspended in cold IMag buffer (0.05% bovine serum albumin and 2 mM EDTA in PBS), and viable cells were counted with trypan blue.

Next, 1 x 10^7 cells were labeled with the following mouse polyclonal antibodies: anti CD11b conjugated with fluorescein isothiocyanate (FITC) (dilution 1: 200); anti

CD11c conjugated with allophycocyanin (APC) – cyanine (Cy)-7 (dilution 1:100); anti Gr-1 conjugated with phycoerythrin (PE) (dilution 1:100); anti F4/80 conjugated with APC (dilution 1: 200); anti MHC II conjugated with pacific blue (dilution 1: 200). Cells isolated from other mice were incubated with Golgi blocker (BD Biosciences, San Jose, CA) according to manufacturer instruction for 2 hours. Cells were fixed, permeabilized, and labeled with the following antibodies: anti CD-3 conjugated with FITC (dilution 1: 100); anti CD-19 conjugated with APC-Cy7 (1:100); anti CD-4 conjugated with PE-Cy5.5 (dilution 1: 250); anti IFN-γ conjugated with PE (dilution 1: 200). Cells were washed with 1 ml of IMag buffer. Cells were analyzed by multi color flow cytometry to determine the percentage of positive cells using a BD LSRII system (BD Biosciences). All antibodies used in immunophenotyping were purchased from BD Biosciences.

Cytokine profiling

Fresh excised colon was rinsed in PBS and colon tissue was homogenized in cell Lytic MT Mammalian Tissue Lysis /Extraction buffer (Sigma, St Louis, MO). Thirteen cytokines (IFN-γ, IL-1β, IL-4, IL-6, IL-10, IL-12 p40, TNFα, IL-12 p70, IL-13, KC, MCP-1, MIP1α, MIP-1β) analytes were measured in tissue lysate using MILLIPLEX MAP Mouse Cytokine/Chemokine Panel kit according to the manufacturers instruction (Millipore, Billerica, MA). In brief, 25 μl of colon lysate were incubated with 25 μl of mixture of capture antibodies overnight at 40°C. After washing, captured analytes were incubated with detection antibodies mixture for 1 hour at room temperature. After incubation, 25 μl of streptavidin-phycoerythrin was added and analytes were analyzed on Luminex 100 (Millipore) on settings recommended by the manufacturer. Standard was also prepared for all 14 cytokines analytes according to manufacturers' instruction.

Protein concentration was measured using Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules,CA). Concentration of each cytokine was presented as pg/mg colon protein.

Gene Name	**Forward Primer (5' – 3')**	**Reverse Primer (5' – 3')**
iNOS	CACCTTGGAGTTCACCCAGT	ACCACTCGTACTTGGGATGC
IL-17	GCTCCAGAAGGCCCTCAGA	CTTTCCCTCCGCATTGACA
KC	GCTGGGATTCACCTCAAGAA	CTTGGGGACACCTTTTAGCA
IFN-γ	GCCACGGCACAGTCATTGAA	CGCCTTGCTGTTGCTGAAGA
TNF-α	CTGTGAAGGGAATGGGTGTT	GGTCACTGTCCCAGCATCTT
β-actin	CCAGAGCAAGAGAGGTATCC	CTGTGGTGGTGAAGCTGTAG

Supplemental Bibliography

1 Okayasu I, Hatakeyama S, Yamada M, Ohkusa T, Inagaki Y, Nakaya R. A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. Gastroenterology 1990;**98**:694-702.

2 Whittem CG, Williams AD, Williams CS. Murine Colitis modeling using Dextran Sulfate Sodium (DSS). J Vis Exp 2010.

3 Gobert AP, Cheng Y, Akhtar M, Mersey BD, Blumberg DR, Cross RK*, et al.* Protective role of arginase in a mouse model of colitis. J Immunol 2004;**173**:2109-17.

4 Dieleman LA, Palmen MJ, Akol H, Bloemena E, Peña AS, Meuwissen SG*, et al.* Chronic experimental colitis induced by dextran sulphate sodium (DSS) is characterized by Th1 and Th2 cytokines. Clin Exp Immunol 1998;**114**:385-91.

5 Mizoguchi E, Xavier RJ, Reinecker H-C, Uchino H, Bhan AK, Podolsky DK*, et al.* Colonic epithelial functional phenotype varies with type and phase of experimental colitis. Gastroenterology 2003;**125**:148-61.

6 Vetuschi A, Latella G, Sferra R, Caprilli R, Gaudio E. Increased proliferation and apoptosis of colonic epithelial cells in dextran sulfate sodium-induced colitis in rats. Digestive diseases and sciences 2002;**47**:1447-57.

7 Chyla B, Moreno-Miralles I, Steapleton M, Thompson M, Bhaskara S, Engel M*, et al.* Deletion of Mtg16, a target of the t(16;21), alters hematopoietic progenitor cell proliferation and lineage allocation. Mol Cell Biol 2008.

8 Cotta-de-Almeida V, Bonomo A, Mendes-da-Cruz DA, Riederer I, De Meis J, Lima-Quaresma KR*, et al.* Trypanosoma cruzi infection modulates intrathymic contents of extracellular matrix ligands and receptors and alters thymocyte migration. Eur J Immunol 2003;**33**:2439-48.