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TLR Signaling Modulates Side Effects of Anticancer Therapy in the Small Intestine

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Intestinal mucositis represents the most common complication of intensive chemotherapy, which has a severe adverse impact on quality of life of cancer patients. However, the precise pathophysiology remains to be clarified, and there is so far no successful therapeutic intervention. In this study, we investigated the role of innate immunity through TLR signaling in modulating genotoxic chemotherapy-induced small intestinal injury in vitro and in vivo. Genetic deletion of TLR2, but not MD-2, in mice resulted in severe chemotherapy-induced intestinal mucositis in the proximal jejunum with villous atrophy, accumulation of damaged DNA, CD11b+ myeloid cell infiltration, and significant gene alterations in xenobiotic metabolism, including a decrease in ABCB1/multidrug resistance (MDR1) p-glycoprotein (p-gp) expression. Functionally, stimulation of TLR2 induced synthesis and drug efflux activity of ABCB1/MDR1 p-gp in murine and human CD11b+ myeloid cells, thus inhibiting chemotherapy-mediated cytotoxicity. Conversely, TLR2 activation failed to protect small intestinal tissues genetically deficient in MDR1A against DNA-damaging drug-induced apoptosis. Gut microbiota depletion by antibiotics led to increased susceptibility to chemotherapy-induced mucosal injury in wild-type mice, which was suppressed by administration of a TLR2 ligand, preserving ABCB1/MDR1 p-gp expression. Findings were confirmed in a preclinical model of human chemotherapy-induced intestinal mucositis using duodenal biopsies by demonstrating that TLR2 activation limited the toxic-inflammatory reaction and maintained assembly of the drug transporter p-gp. In conclusion, this study identifies a novel molecular link between innate immunity and xenobiotic metabolism. TLR2 acts as a central regulator of xenobiotic defense via the multidrug transporter ABCB1/MDR1 p-gp. Targeting TLR2 may represent a novel therapeutic approach in chemotherapy-induced intestinal mucositis. The Journal of Immunology, 2015, 194: 000–000.

Abbreviations used in this article: ABx, antibiotics; dKO, double knockout; DUSP-1, dual specificity phosphatase 1; FL, fluorescein; KO, knockout; MDR, multidrug resistance; MPO, myeloperoxidase; MTX, methotrexate; OTU, operational taxonomic unit; PARP, poly(ADP-ribose) polymerase; PCSK, Pam3CysSK4; p-gp, p-glycoprotein; PI, propidium iodide; WT, wild-type.

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Copyright © 2015 by The American Association of Immunologists, Inc. 0022-1767/15/$25.00 to 100% of patients undergoing high-dose chemotherapy with stem cell or bone marrow transplantation for hematological or solid tumors (1). Gastrointestinal toxicity is the most dose-limiting factor for anti-cancer drugs, such as methotrexate (MTX), an effective chemotherapeutic agent often used to treat many cancers in pediatric and adult patients (2). The condition may affect the entire gastrointestinal tract and causes severe pain, diarrhea, and weight loss. However, despite the severity and prevalence of chemotherapy-induced intestinal mucositis, there is so far no successful therapeutic intervention that prevents or treats all symptoms of disease (3).

The precise pathophysiology underlying chemotherapy-induced intestinal mucositis has not been clarified yet. Current understanding suggests the culmination of a dynamic sequence of complex inflammatory events that are initiated by direct chemotherapy-induced injury to crypt cells and cells in the underlying mucosal tissue (4). In addition, chemotherapy-induced diarrhea has been associated with potential alterations in microbiota composition (5, 6), which may contribute to dysregulated mucosal immune responses. The gut microbiota may modulate intestinal metabolism of xenobiotics and anticancer drugs (7), thus potentially inducing or inhibiting gut toxicity. For instance, β-glucuronidase produced by Escherichia coli generates SN-38, a toxic metabolite of irinotecan, which directly damages the intestinal mucosa (8). The indigenous microbiota may also influence endogenous xenobiotic detoxification (9), however, the innate immune mechanisms of host-chemotherapy drug interactions and their role in triggering adverse effects have not yet been delineated.

Commensal bacterial ligands are recognized by TLRs (10). Recent findings, mainly derived from murine colitis models, imply that this constant interaction ensures mucosal homeostasis through...
restrained inflammatory responses and accelerated restitution and healing in the healthy colon (11). Mice deficient in TLR2/4/5 or MyD88 exhibit delayed or diminished tissue repair responses during acute dextran sulfate sodium–induced colonic inflammation (12–16). The gut microbiota may differentially modulate mucosal TLR responsiveness in a susceptible host, thus subverting immune responses to a predominantly proinflammatory phenotype (11). It has been postulated that aberrant microbiota–TLR signaling may be involved in the pathogenesis of chemotherapy-induced mucositis (17, 18), but direct experimental evidence is so far lacking.

Transmembrane p-glycoprotein (p-gp) functions as an ATP-dependent efflux transporter pump to prevent cellular accumulation of numerous xenobiotics and drugs, including antineoplastics (19, 20). Induction of p-gp results from ABCB1/multidrug resistance (MDR1) transcriptional and translational activation. P-gp is expressed by many different cell types, including monocyes/macrophages and intestinal epithelial cells (21). As a central member of the superfamily of ABC transporters, p-gp interacts with many drug-metabolizing enzymes in a complex network and absence of p-gp can lead to severe drug toxicity (22). ABCB1/MDR1 gene polymorphisms (23) may alter drug levels and host susceptibility to diseases, such as inflammatory bowel diseases. Mice deficient in MDR1A show increased susceptibility to microbiota-induced colitis (24, 25) and radiation-mediated intestinal injury (26). Genetic disruption of MDR1A may contribute to intestinal dysbiosis (27). Furthermore, p-gp expression may be differentially modulated by various mediators, including intestinal bacteria (28, 29), yet the signaling mechanisms remain to be determined.

In this study, we identify a novel molecular link between innate immunity and xenobiotic metabolism and show its implication in host detoxification and protection against chemotherapy-induced side effects in the small intestine. We demonstrate that TLR2-mediated activation of ABCB1/MDR1-encoded p-gp in CD11b+myeloid cells critically controls the severity of MTX-associated gut toxicity. Our findings provide a rationale for developing new drugs for the management of cancer therapy–induced mucosal damage in the gastrointestinal tract based on commensal-mediated innate immune modulation via TLR2.

Materials and Methods

Reagents and Abs

The synthetic lipopeptide Pam3Cys-SKKKKx3HCl (Pam3CysSK4 [PCSK]; lot A15) was obtained from EMC Microcollections and dissolved in sterile water (vehicle control). MTX was obtained from Pfizer, and sterile saline was used as vehicle control; fluorescein (FL)-MTX was from Life Technologies, respectively. The specific p-gp inhibitor (C-4), a cell-permeable cinnamoyl compound that reversibly inhibits p-gp efflux function (30), was from Merck and dissolved in DMSO (vehicle control).

Murine and human cells

Primary murine myeloid cells flushed from femurs were isolated from female mice aged ~5 wk, passed through a 100-μm-cell strainer (BD Falcon) and purified using the EasySep CD11b Positive Selection Kit (Stemcell Technologies). Murine bone-marrow derived CD11b+-myeloid cells were cultured on films-coated (BD) culture plastic in Leibovitz’s L-15 medium (Life Technologies) supplemented with 10% v/v FBS (Thermo or PAA) and penicillin/streptomycin (Life Technologies) in an air incubator at 37˚C. Human CD11b+-myeloid (34) monocytic-like THP-1 cells (TIB-202; passages 13–19) were obtained from the American Type Culture Collection and maintained in RPMI 1640 medium (PAA or Life Technologies) supplemented with 10% v/v FBS, 2 mm l-glutamine (Life Technologies) and penicillin/streptomycin at 37˚C in 5% CO2. The intestinal epithelial cell line Caco-2 (HTB-37; passages 11–15) was purchased from the American Type Culture Collection and maintained as described previously (35).

Murine model of chemotherapy-induced intestinal mucositis

A common murine model of chemotherapy-induced mucositis was applied by i.p. injection of MTX (40 mg/kg body weight/d; for 4 d) in female mice aged ~9 wk. For microbiota depletion, mice were treated with antibiotics (vancomycin [ratiopharm] and imipenem [MD] [both 50 mg/kg body weight/d] in drinking water (36)) for 5 d prior to MTX treatment [40 mg/kg body weight/d; for 2 d]. Short intake of vancomycin and imipenem has been shown to notably reduce gut microbial load and diversity (37). Six hours after finishing antibiotic treatment, PCSK (150 μg/ml) was added to fresh drinking water starting 1 d prior to MTX, renewed every other day, and stopped on day 4, day 5. Mice were sacrificed on day 6 and histologic analysis performed as described. Mice were the desired genotype via standard genotyping techniques. For studies, all reagents were obtained from Sigma-Aldrich, unless otherwise specified.

Animals

TLR2 knockout (KO; B6.129-Tlr2^tm1Koi/J; The Jackson Laboratory) (31) and MD-2 KO (32) (provided by Dr. K. Miyake, University of Tokyo, Tokyo, Japan) mice (all C57BL6/J; > F10) were intercrossed for double KO (dKO) homozygotes (TLR2/MD-2 dKO; wild-type (WT) C57BL6/J mice were used as controls. WT and MDR1A KO (FVB/J29P2-Abcb1a^tm1Corex N7; originally developed by Dr. A. Schinkel, the Netherlands Cancer Institute, Amsterdam, the Netherlands) (20) mice (all FVB/N; > F7) were obtained under crossing agreement from Taconic Farms (Germantown, NY). All mice were bred and housed in the same temperature- and humidity-controlled room on a 12-h light-dark cycle under strict specific-pathogen-free conditions (Central Animal Facility, University Hospital of Essen, Essen, Germany). All animals were provided ad libitum with autoclaved tap water and autoclaved pelleted laboratory chow (ssniff M-Z, ssniff Spezialdiäten, Soest, Germany) containing folic acid (10 mg/kg).

Extensive animal health monitoring (criteria of the Federation for Laboratory Animal Science Associations) was conducted routinely on sentinels from this room and no pathogens were detected. Mice were confirmed to be the desired genotype via standard genotyping techniques. For studies, only age-matched female mice were used, as indicated. For microbiota analysis, successive litters of individual heterozygous breeding pairs were analyzed to control for maternal influence; offspring were housed on the same rack but individually in cages to avoid synchronization, as described previously (33). Protocols were in compliance with German law for use of live animals and reviewed and approved by the local animal protection officer at the University Hospital of Essen and the responsible district government.

Organ culture of mucosal biopsies of human small intestine

Small duodenal specimens from a total of seven Caucasian patients (median age: 64 y) undergoing screening esophagogastroduodenoscopy for gastro-esophageal reflux disease were freshly obtained at the Endoscopy Unit, Department of Gastroenterology and Metabolic Diseases, Klinikum Essen (an affiliate of the University Hospital Essen, Innsbruck). Tissue was obtained from all patients before the procedure, and the protocol was approved by the Human Studies Committee of the Medical Faculty, University of Duisburg-Essen. All patients underwent pre procedural fasting for at least 8 h (no medication). Macroscopically and microscopically, the morphology of the duodenal mucosa was normal in all cases. The samples were immediately washed in ice-cold HBSS (PAA or Life Technologies) supplemented with 10% v/v FBS, 1 mm l-glutamine (Life Technologies), and antibiotic/antimycotic solution (PAA), mixed into a thin layer of cold liquid Matrigel (BD Biosciences) with or without PCSK (20 μg/ml), which was allowed to form a gel at 37˚C within 30 min, covered with warm full L-15 medium.
Flow cytometry analysis of p-gp function

The fluorochromes rhodamine 123 and DiOC(3) represent substrates for transport mediated by p-gp (38). The functional efflux activity of p-gp was assessed using the Multidrug Resistance Direct Dye Efflux Assay kit (Mili-pore), according to the manufacturer’s instructions with minor modifications. In brief, after stimulation with or without PCSK (20 μg/ml), THP-1 cells were loaded with rhodamine 123 for 15 min on ice and washed, and dye efflux was initiated by incubating the cells for 1 h at 37˚C. To assess efflux of MTX, THP-1 cells were incubated with FL-MTX (10 μM) for 21 h, washed, and stimulated with or without PCSK (20 μg/ml) for 3 h or THP-1 cells were pretreated with the p-gp inhibitor (10 μM) or vehicle (DMSO) for 15 min, stimulated with or without PCSK (20 μg/ml) for 5 min, washed and incubated with FL-MTX (10 μM) for 21 h. After washing, cells (3 × 10⁶/sample) were analyzed using a FACSCan II (BD Biosciences). Dead cells were excluded by forward/side scatter, and flow cytometry data were analyzed using FlowJo software for PC (version 7.6.5; Tree Star).

Immunofluorescence

Frozen sections of tissues were cut (7 or 10 μm) and mounted on Superfrost Plus Gold slides (Thermo). Dependent on primary Abs, sections were fixed with either acetone (100%) for 5-10 min at −20 °C or paraformaldehyde (3–4%) for 15 min at room temperature. Sections were blocked with normal goat serum (1:10–1:20 in PBS: 2%–3% TX-100 (Thermo)) for 60 min at room temperature and incubated with primary Abs (1:50–1:1000; for anti-p-gp: 5 μg/ml) overnight at 4°C. Fluorescent-labeled Abs were used as secondary Abs (1:50–1:100, 60 min, room temperature). After mounting with Vectashield Mounting Medium with DAPI or propidium iodide (PI), sections were assessed by using optical sectioning with confocal microscopes (AxioObserver.Z1 with ApoTome) microscopes (Zeiss). The multitrack option and sequential scanning for each channel were used to eliminate any cross-talk of the fluorophores and to ensure reliable colocalization results with the combination of Alexa Fluor dyes 530 and 488. Control experiments were performed with isotype control IgG (Santa Cruz Biotechnology).

Analysis of apoptosis in murine small intestinal tissues

Murine small intestinal tissues from female mice aged ∼9 wk were cultured short-term ex vivo, as previously described (14), except that samples were stimulated with or without PCSK (20 μg/ml) for 30 min and then exposed to MTX (10 μM) for 4 h. Matrigel-attached small intestinal tissues were immediately processed for analysis of apoptosis (in situ cell death detection kit, FL; Roche) by quantifying TUNEL-positive cells in relation to the total number of PI-labeled nuclei (>1000 cells/condition) by using ImageJ (National Institutes of Health) in a random collection of captured ≥40 images by epifluorescence microscopy.

Myeloperoxidase assay

Myeloperoxidase (MPO) activity was determined by commercially available MPO assay (Mouse MPO ELISA kit; Hycult Biotech), according to the manufacturer’s instructions. Briefly, proximal jejunal tissue harvested from mice was lysed (5 mg/100 μl) in ice-cold lysis buffer (10% glycercin, 200 mM NaCl, 10 mM Tris·HCl [pH 7.4]), and 5 mM EDTA; supplemented with PhosSTOP Phosphatase and complete Mini protease inhibitor mixture tablets (Roche), and 1 mM PMSF), followed by gently mixed in a bead mixer mill (MM301, Retsch, Haan, Germany). Protein content in the supernatant was determined by Bradford protein assay (Bio-Rad).

Protein analysis by immuno blotting

For preparation of whole-cell lysates, cells were rinsed once in cold PBS (without Ca²⁺/Mg²⁺) with 100 μM Na₂VO₃ and then lysed in ice-cold lysis buffer (1% TX-100, 150 mM NaCl, 20 mM Tris·HCl [pH 7.5], 2 mM EDTA, supplemented with PhosSTOP Phosphatase and complete Mini protease inhibitor mixture tablets, and 1 mM PMSF). Immuno blotting was performed as described previously (39). To confirm equal protein loading, blots were reprobed with anti-β-actin. Representative blots of at least two independent experiments are shown.

RNA/DNA extraction

Total RNA from cells or tissues was extracted (RiboPure; Ambion) and purified including the RNeasy-free DNase digestion step (RNeasy Mini Kit; Qiagen). Genomic DNA from full thickness proximal jejunal samples containing liquid stool was isolated (FastDNA SPIN Kit for Soil; MP Biomedicals) and processed by the BIO101/Savant Fast Prep FP120 homogenizer, according to the manufacturer’s instructions.

Microarray analysis

All RNA samples from murine proximal jejunum (n = 3/condition) were analyzed independently and further processed at MFT Services (Tübingen, Germany) using GeneChip Mouse Gene 1.1 ST arrays (Affymetrix). Hybridization, washing, staining and scanning was performed automatically in a ScanArrayetch (Affymetrix). Data were normalized with RMA (Robust Multichip Average) to yield log-transformed signal values that were then averaged for the individual subgroups. Probe sets with low variance over all samples were regarded as noninformative and removed from the data set. Data have been deposited in the Gene Expression Omnibus (GSE56426). Data were further analyzed, as previously described (40), through 1) hierarchical clustering analysis and plotting as a heatmap map using the ArrayStar software (DNASTar) and 2) Ingenuity Pathways Analysis (Ingenuity Systems, www.ingenuity.com).

Real-time PCR for gene expression analysis

QuantiTect Primer Assays (Qiagen) were used as the gene-specific murine or human primer pairs. Quantitative real-time RT-PCR was performed using the one-step QuantFast SYBR Green RT-PCR kit (Qiagen) on the Mastercycler ep realplex (Eppendorf) real-time amplification system. Copy numbers of individual transcripts were related to Gapdh or endogenous control (×100,000 copies Gapdh) and normalized as indicated.

Analysis of microbiota composition

DNA extracted from murine proximal jejunum samples was used as a template for PCR amplification using barcoded fusion primers MiSeq-27F (5’-AATGATACGGCGACCACCGAGATCTACACTATGGTAATTCCAG-3’), MiSeq-538R (5’-CAAGCAGAACGACGACGAGATACGGGATCATGATCGMTTGGATYMTGCTAG-3’) and MiSeq-388R (5’-CAAGCAGAACGACGACGAGATACGGGATCATGATCGMTTGGATYMTGCTAG-3’), which target the V1-V2 regions of the bacterial 16S rRNA gene and contain adaptors for downstream Illumina MiSeq sequencing. A unique barcoded reverse primer was used for each of the individual proximal jejunal samples. PCR was carried out using Q5 Taq polymerase (New England Biolabs) and cycling conditions were: 98°C for 2 min, then 20 cycles of 98°C for 30 s, 55°C for 30 s, 72°C for 90 s, and then a final 72°C extension step for 5 min. Four reactions were carried out per sample. Pooled amplicons were then ethanol precipitated to concentrate to 25–50 μl volumes/sample, which were then quantified using a Qubit 2.0 Fluorometer (Life Technologies) and equimolar concentrations of each sample were added together to a sequencing MasterMix. Sequencing was done at the Wellcome Trust Sanger Institute on an Illumina MiSeq machine using 2 × 250 bp read length. Sequence data from all mice (numbers 1–16) have been deposited in the European Nucleotide Archive (http://www.ebi.ac.uk/ena; mouse number, accession number: 1, ERS563915; 2, ERS563916; 3, ERS563898; 4, ERS563909; 5, ERS563897; 6, ERS563899; 7, ERS563900; 8, ERS563901; 9, ERS563903; 10, ERS563904; 11, ERS563906; 12, ERS563907; 13, ERS563905; 14, ERS563908; 15, ERS563910). The Illumina sequence data were processed using the mothur software package (41), closely following their MiSeq SOP (42). To summarize the steps involved: forward and reverse read MiSeq data were assembled into paired-read contigs, and then, all resulting contigs that were shorter than 260 bp, longer than 450 bp, contained any ambiguous bases, or contained homopolymeric stretches longer than 7 bases were removed. Putative chimeric sequences were removed using Perseus software (43) incorporated into mothur. Remaining sequences were also removed that were derived from chloroplasts, mitochondria, eukarya, or archaea. Following these quality control steps 102,373 sequences remained in the final dataset (range of 31–19,716 sequences/sample). Three mice (numbers 4, 12, and 14) had to be excluded from final analysis because there were not enough sequence data to give reliable results. Samples from 13 mice remained (range of 1,223–19,716 sequences/sample).

A preclustering step (diss = 3) was carried out to reduce the impact of sequencing errors and then operational taxonomic units (OTUs) were generated at a 97% similarity cutoff level. Each OTU was given a taxonomic classification using the Ribosomal Database Project reference taxonomic database, the dataset was first randomly subsampled to 1084 sequences/sample to ensure equal sequencing depth among each of the
samples. Following this step, observed diversity (i.e., the number of observed OTUs) and Shannon diversity indices for each subsample were calculated in mothur. To test whether there were any significant differences in diversity between the mouse cohorts, Kruskal–Wallis and Mann–Whitney U tests were carried out using Minitab (version 16). To assess whether there were significant microbiota clustering patterns associated with chemotherapy, the subsampled dataset was then used to create a cluster dendrogram, using the Bray Curtis calculator, in mothur. The resulting dendrogram was visualized using the iTOL online software tool (45).

**Statistical analysis**

The unpaired, two-tailed t-test was used to calculate differences between means (GraphPad Prism version 5.04; GraphPad Software), if not indicated otherwise. Survival data were analyzed using the log-rank test. p < 0.05 was considered as significant. All data are expressed as the means ± SEM.

**Results**

**Loss of TLR2 results in severe chemotherapy-induced intestinal mucositis in mice**

To investigate the functional role of central innate immune regulators in modulating side effects of anti-cancer therapy in the small intestine, mice lacking TLR2 or MD-2 (an essential coreceptor of TLR4 signaling) were exposed to a common model of chemotherapy-induced intestinal mucositis by systemically administering high-dose MTX, as described in Materials and Methods. In contrast to MTX-WT or MTX-MD-2 KO, all MTX-TLR2 KO mice were highly susceptible to chemotherapy-induced small intestinal injury (Fig. 1). Microscopic inflammation in the small intestine from MTX-TLR2 KO mice on day 7 was characterized by villous atrophy, crypt ablation, intestinal epithelial integrity was emphasized by dramatic depolarization of phospho-H2A.X and abnormal mitoses (phosphorylated histone H2A.X) and abnormal mitoses (phosphorylated histone H3). Disruption of intestinal epithelial barrier integrity was emphasized by dramatic depolarization of phosphorylated β-catenin and goblet cell depletion during MTX-induced genotoxic stress (Fig. 2C).

**Chemotherapy induces genotoxicity and alterations in xenobiotic metabolism in the small intestine of TLR2 KO mice**

We further characterized the extensive mucosal barrier injury seen in severe chemotherapy-induced mucositis in TLR2 deficiency. Immunofluorescent staining of MTX-TLR2 KO proximal jejunum demonstrated aberrant accumulation of CD11b+ myeloid cells, but not CD4+ T cells, in the inflamed lamina propria (Fig. 2A). These early cell infiltrates showed fulminant genotoxic stress in response to MTX (Fig. 2B), as evidenced by the presence of dsDNA breaks (phosphorylated histone H2A.X) and abnormal mitoses (phosphorylated histone H3). Disruption of intestinal epithelial barrier integrity was emphasized by dramatic depolarization of phosphorylated β-catenin and goblet cell depletion during MTX-induced genotoxic stress (Fig. 2C).

**Next, we analyzed the impact of chemotherapy on the overall microbial composition and structure in the proximal jejunum by Illumina MiSeq-based analysis of bacterial 16S rRNA genes.** There
were no statistically significant differences in bacterial diversity between MTX-TLR2 KO and MTX-WT proximal jejunum samples on day 7, or when compared with healthy controls, respectively (Supplemental Fig. 1A, 1B). Compositional analysis of the microbiota indicated that there was a high degree of variation between mice. One TLR2 KO mouse (number 13) showed overgrowth with Enterobacteriaceae in response to MTX. Nevertheless, individual samples of the same group did not show any distinct clustering patterns and no broad-scale compositional differences and signatures were identified between groups (Fig. 3). These data suggest that MTX-induced intestinal mucositis in combination with or without genetic deletion of TLR2 may not provoke consistent shifts in microbiota community structure in the proximal jejunum.

To gain further insight into the underlying host mechanisms responsible for the severity of chemotherapy-induced genotoxic injury in the context of TLR2 deficiency, we performed a broad gene expression profiling analysis to identify potential target genes. We compared mRNA expression levels in proximal jejunal tissues between healthy and MTX-exposed WT versus TLR2 KO mice. Through hierarchical clustering of the microarray data sets (Fig. 4A, Supplemental Table II), we identified 409 genes significantly regulated with ≥8-fold differential expression (MTX-TLR2 KO versus WT control). Within this set, 140 genes were most differentially regulated between MTX-TLR2 KO and MTX-WT (log2 ratio cutoff, 4.0) and belonged predominantly to canonical pathways associated with xenobiotic metabolism (Fig. 4B, Supplemental Table IIIA, IIIB), as annotated by the Ingenuity knowledge base. Real-time RT-PCR analysis of a selection of representative genes validated the gene expression changes observed by the array analysis. Levels of mRNA expression of inflammation and tissue injury markers (including matrix metalloproteinases) were significantly increased in MTX-TLR2 KO mice compared with MTX-WT or healthy controls (Fig. 4C). Importantly, MTX-TLR2 KO proximal jejunum exhibited upregulation of TLR4 mRNA expression. Several drug transporters and metabolizing enzymes (ABCA and CYP450) were altered in MTX-TLR2 KO (Fig. 4D), but not in MTX-WT, confirming specific defects in xenobiotic metabolism in response to chemotherapy in the context of TLR2 deficiency.

**TLR2 modulates p-gp synthesis and function**

Our data demonstrate that mRNA expression of Abcb1a was significantly decreased in the inflamed proximal jejunum of MTX-TLR2 KO mice but not in MTX-WT (Fig. 4D). Basal mRNA expression of Abcb1a remained unchanged in all untreated KO models when compared with WT (Fig. 4D; data not shown). Double immunofluorescence labeling of tissue sections determined membrane expression of ABCB1/MDR1-encoded p-gp to colocalize with CD11b+ staining in the lamina propria of healthy WT controls (Fig. 5A). Expression of p-gp was also weakly
detectable at the apical pole of the intestinal epithelium (Fig. 5B). Downregulation of expression was confirmed at protein level for \( \text{ABCB}1/\text{MDR}1 \)-encoded p-gp in the lamina propria of inflamed proximal jejunum of MTX-TLR2 KO mice compared with MTX-WT (Fig. 5B).

Next, we examined whether p-gp may represent a direct target of TLR2 activation in human and murine CD11b\(^+\)-myeloid cells. A short-term time-course analysis showed that stimulation with the synthetic TLR2 ligand PCSK of THP-1 human mononuclear cells promptly (i.e., within 5 min) yielded increased p-gp protein expression levels (Fig. 5C) followed concomitantly by a maximal 6-fold induction of \( \text{ABCB}1 \) mRNA synthesis within 6 h of stimulation (Fig. 5D). In contrast, p-gp protein or \( \text{ABCB}1 \) mRNA expression levels were not modulated by TLR2 stimulation with PCSK in Caco-2 cells (Fig. 5C, 5D), an intestinal epithelial cell line that exhibits high p-gp expression at baseline (46). Of note, we observed a decrease in \( \text{Cyp1a1} \) mRNA expression in the TLR2 KO proximal jejunum (Fig. 4D), as previously shown (47), but we
were not able to detect any modulation of CYP1A1 mRNA by direct TLR2 stimulation of THP-1 cells (data not shown).

Treatment with MTX induced loss of p-gp expression in THP-1 cells, which was associated with increased myeloid cell death, as evidenced by PARP cleavage (Fig. 5E). However, prestimulation with the TLR2 ligand PCSK inhibited MTX-induced loss of p-gp expression and associated cytotoxicity. Despite MTX, prestimulation with PCSK significantly induced phosphorylation of the MKK3/6→p38 MAPK-pathway (Fig. 5E). MTX-induced loss of p-gp coincided with decreased protein expression of p38 MAPK and DUSP-1, which was blocked by pretreatment with the TLR2 ligand. Finally, PCSK did not modulate the level of p-gp expression in MTX-exposed CD11b⁺-myeloid cells from TLR2 KO mice, confirming that induction of p-gp expression requires TLR2 signaling (Fig. 5F).

To analyze p-gp function in response to TLR2 activation, we performed efflux assays to determine the ability of myeloid cells to pump out known MDR1 substrates (rhodamine 123, DiOC₂(3)) with or without PCSK treatment. As shown in Fig. 6A, stimulation with PCSK significantly enhanced efflux of both fluorescent labeled p-gp–transported substrates from THP-1 cells (as evidenced by a significantly higher portion of cells with decreased intracellular dye concentration). We also investigated whether TLR2 activation may directly modulate the efflux of MTX (Fig. 6B). THP-1 cells were loaded (~80%) with green-fluorescent MTX for 21 h (baseline). Subsequent stimulation with the TLR2 ligand, but not vehicle control, markedly induced extracellular transport of MTX. However, pretreatment with a specific p-gp inhibitor significantly attenuated PCSK-mediated efflux of MTX (Fig. 6C), implying p-gp–mediated transport of MTX by TLR2 signaling.

TLR2 fails to protect small intestinal tissues genetically deficient in MDR1A against DNA-damaging drug-induced apoptosis

We found that mice deficient in MDR1A developed severe acute mucosal toxicity in response to systemic MTX exposure (Fig. 7A), which was even more progressive than in mice deficient in TLR2 (Fig. 1A), and experiments had to be terminated on day 6 because of wasting disease. In contrast, control WT mice on a FVB/N background showed normal intestinal morphology after MTX treatment, similar to results for the WT mice on a C57BL/6 background (Fig. 1A). Of note, baseline levels of TLR2 mRNA expression were not altered in MDR1A-deficient proximal jejunum (data not shown).

To avoid the high degree of systemic morbidity caused by in vivo treatment of MDR1A-deficient mice with MTX, we cultured small
**FIGURE 6.** Functionally, stimulation of TLR2 mediates transport activity of ABCB1/MDR1 p-gp and efflux of MTX. (A) p-gp function measured by rhodamine 123 or DiOC<sub>2</sub>(3) efflux assays. THP-1 cells were left untreated for 24 h or stimulated with PCSK (20 μg/ml) for 5 min or 24 h, loaded with rhodamine 123 for 1 h or DiOC<sub>2</sub>(3) for 15 min on ice, followed by efflux for 1 h, after which the immunofluorescence of the cell population was measured by flow cytometry. Left panel, Efflux was quantified in terms of the fraction of fluorescent cells in the M1 region of the plot (low fluorescence in light gray) as percentage of the total cells. Representative plots show the level of fluorescence versus cell counts; light gray: unloaded control cells. Right panel, Cumulative data representing means ± SEM (n = 3 independent experiments/dye assay; *p < 0.05; **p < 0.01; ***p < 0.001) versus untreated controls. (B) Cellular efflux of MTX in response to TLR2 stimulation. THP-1 cells were directly loaded with FL-MTX (10 μM) for 21 h (baseline), washed, and treated with or without PCSK (20 μg/ml) for 3 h and subjected to FACS analysis; percentages of FITC-positive cells are indicated. (C) Blockage of TLR2-mediated efflux of MTX by p-gp inhibition. THP-1 cells were pretreated with the p-gp inhibitor (10 μM) or vehicle (DMSO) for 15 min, stimulated with or without PCSK (20 μg/ml) for 5 min, washed, and incubated with FL-MTX (10 μM) for 21 h. (B and C) Data are presented as the means ± SEM (n = 2–3 independent experiments; **p < 0.01; ***p < 0.001).

**Gut microbiota depletion results in increased chemotherapy-induced toxicity in WT mice, which is alleviated by TLR2 ligand supplementation**

The gut microbiota contains TLR2 agonists (49). As shown in Fig. 8A–C, depletion of gut commensals by broad-spectrum antibiotics (ABx) led to increased susceptibility of WT mice to chemotherapy-induced small intestinal damage, similar to that seen in TLR2-deficient mice (Figs. 1, 2). ABx-treated WT mice exhibited severe mortality and morbidity during MTX-induced intestinal mucositis. Overall mortality rate was ~60% in ABx-treated MTX-WT mice versus 0% in untreated MTX-WT controls (Fig. 8A). ABx-treated MTX-WT mice showed significant inflammatory stress-induced injury of the proximal jejunum with villous atrophy, CD11b<sup>+</sup>-myeloid cell infiltration and barrier dys-integrity with DNA damage, which was not evident in untreated MTX-WT controls (Fig. 8B, 8C). These results point to a specific association between gut commensal microbiota and protection against mucosal toxicity induced by chemotherapy treatment.

We next assessed the effect of oral supplementation with the TLR2 agonist PCSK on MTX-toxicity in gut microbiota-depleted WT mice. PCSK-supplemented ABx/MTX-WT mice demonstrated markedly decreased lethality (10%; Fig. 8A) and showed architecturally preserved proximal small intestinal mucosa without evidence of severe inflammation and DNA damage (Fig. 8B, C). These data imply that supplementation of a TLR2 ligand can rescue WT mice from exacerbated DNA damage–associated mucosal inflammation and subsequent death induced by chemotherapy after antibiotic treatment. Of note, reduced protection against chemotherapy-induced small intestinal mucositis in ABx-treated MTX-WT mice coincided with loss of p-gp expression in the lamina propria (Fig. 8C). However, supplementation with the TLR2 agonist PCSK after antibiotic therapy markedly attenuated intestinal tissues ex vivo from WT and MDR1A KO mice and assessed cell death in response to MTX stimulation. As shown in Fig. 7B, TUNEL assay revealed excessive DNA-damaging drug-induced apoptosis in small intestines from MDR1A KO mice in response to MTX stimulation, which persisted despite PCSK pretreatment. In contrast, small intestinal tissues from WT mice showed no increase in apoptotic cell death, confirming WT resistance to MTX-induced toxicity (Fig. 7A). Of note, cell death was slightly elevated in control small intestinal tissues from MDR1A KO mice cultured ex vivo when compared with control WT, which is consistent with previous findings that loss of p-gp function results in increased baseline sensitivity to stress-induced injury (48). These data confirm functional dependence between ABCB1/MDR1-encoded p-gp and TLR2 signaling in MTX-mediated intestinal mucosal damage.

**Treatment with a TLR2 ligand is effective in a preclinical model of human chemotherapy–induced mucositis**

Finally, to validate that TLR2 activation also protects against chemotherapy-induced cellular DNA damage in the human small intestine, we set up a proof-of-principle experiment by culturing human duodenal pinch biopsies ex vivo in the presence or absence of PCSK followed by exposure to MTX in a three-dimensional Matrigel-based model. MTX alone resulted in a destructive inflammatory response (Fig. 9A), because most of the epithelial cells

**Figure 6.**
were lost and the lamina propria mononuclear cells within the remaining mucosal tissue showed signs of a strong DNA damage response with intense phospho-histone H2A.X staining (Fig. 9B) and loss of p-gp expression (Fig. 9C). In contrast, treatment with PCSK markedly reduced the severity of MTX-associated toxicity, as evidenced by the significantly improved biopsy pathology with a relatively high number of surviving epithelial cells (Fig. 9A) and low levels of DNA damage in the lamina propria (Fig. 9B), comparable to controls. PCSK-mediated prevention of genotoxicity was associated with inhibition of MTX-induced p-gp downregulation (Fig. 9C). These data suggest that TLR2 activation limits the toxic-inflammatory reaction and preserves assembly of the drug transporter p-gp in a preclinical model of human chemotherapy-induced intestinal mucositis.

**Discussion**

Intestinal mucositis is the most common complication of cancer chemotherapy. Here, we identify a previously unappreciated link between the innate immune system and chemotherapy toxicity in the gastrointestinal tract. Our results provide evidence that xenobiotic metabolism via the ABC transporter p-gp is part of the innate host defense to protect intestinal mucosal homeostasis. We show that TLR2 functions as a modulator of ABCB1/MDR1 p-gp synthesis and activity in myeloid derived cells, driving chemotherapy drug efflux, which reduces cytotoxicity.

Our data from in vitro studies indicate that TLR2 activation induces sustained ABCB1/MDR1 p-gp synthesis and activity in murine and human myeloid cells. Presence of TLR2 was essential for PCSK-mediated induction of p-gp during chemotherapeutic DNA damage. ABCB1/MDR1 transcriptional activation occurs through a complex regulatory network, including the MKK3/6→p38 MAPK-pathway (50), which is shared by TLR2 signaling (51). p38 signaling has recently been implicated in cellular protection against genotoxic insults (52) and interacts with DUSP1 in a tight balance to avoid excessive inflammatory activities in macrophages (53). Loss of DUSP1 may increase cell susceptibility to injury (54). Future studies will need to determine whether TLR2-induced transcription factors, such as p38-mediated AP-1, may bind directly to the promoter region of the ABCB1/MDR1 gene that may distinctly drive the expression of this key ABC transporter.

Functionally, TLR2-induced p-gp mediated efflux of specific ABCB1/MDR1 substrates. The anticancer drug MTX represents one such substrate for p-gp (55). In response to TLR2 stimulation with PCSK, MTX was actively transported out of myeloid cells, reducing cellular drug accumulation and thus inhibiting drug-mediated cell death. Furthermore, our ex vivo and in vivo findings demonstrate that TLR2 activation distinctly modulates ABCB1/MDR1 p-gp function. Absence of TLR2 signaling (by either genetic deletion or depletion of the indigenous gut microbiota by antibiotics) aggrandized chemotherapy-induced mucositis, which correlated with loss of p-gp in the inflamed mucosa. Remarkably, supplementation with the TLR2 ligand PCSK during chemotherapy in ABx-treated WT mice preserved p-gp expression in lamina propria mononuclear cells, which limited genotoxic stress-induced damage in the small intestine and decreased morbidity and mortality. TLR2-induced suppression of chemotoxicity in the small intestinal mucosa was essentially regulated through ABCB1/MDR1 because lack of MDR1A abolished TLR2-mediated inhibition of DNA-damaging drug-induced cytotoxicity. ABCB1/MDR1 p-gp has been shown to transport a wide range of chemotherapy drugs. Furthermore, ABCB1/MDR1 p-gp cooperates with many xenobiotic mediators because of its overlapping substrate specificity. It is possible that TLR2 activation may broadly reduce cellular accumulation of other drugs that are p-gp substrates, such as other antineoplastics or immunosuppressive agents. Future studies will need to determine whether TLR2 signaling can also be exploited for the treatment of intestinal mucositis induced by chemotherapy drugs other than MTX. We observed significant alterations of a number of xenobiotic metabolism-associated genes in MTX-TLR2 KO proximal jejunal samples, whose products are responsible for many different steps of detoxification, including phase I and phase II drug-metabolizing enzymes and transporters. Besides ABCB1/MDR1 p-gp (55), gene expression levels of ABCC1-4 and ABCG2 were distinctly deregulated in the inflamed proximal jejum of MTX-TLR2 KO mice. Future studies will need to test the possible impact of TLR2 signaling on these specific ATP-dependent drug transporters, which may also be involved in mediating MTX efflux (56), potentially contributing to genotoxic stress in the small intestinal mucosa.

We cannot exclude the possibility that additional cell-specific protective mechanisms of TLR2 may be involved in decreasing chemotoxicity in the small intestine—beyond modulation of the multidrug transporter ABCB1/MDR1 p-gp in CD11b⁺-myeloid.
cells. The anti-inflammatory effects of commensal-induced TLR2 signaling in colonic epithelial cells have previously been demonstrated. TLR2 plays a key role in maintaining tight- and gap-junction-associated barrier integrity of the colonic epithelium (14, 57). Activation of TLR2 signaling may lead to repositioning of constitutive cyclooxygenase-2–expressing mesenchymal stem cells to the crypt, thus enhancing intestinal epithelial survival during radiation therapy (58). In addition, TLR2 controls goblet

FIGURE 8. Gut microbiota depletion results in increased small intestinal chemotoxicity in WT mice, which is alleviated by TLR2 ligand supplementation. After gut decontamination with oral broad-spectrum antibiotics (vancomycin/imipenem; 50 mg/kg body weight) for 5 d, WT mice were administered i.p. MTX (40 mg/kg body weight) once daily for 2 d; from day −1 to maximal day 5, the mice were treated with or without oral PCSK [150 μg/ml], as described in Materials and Methods. Pooled data from at least two independent experiments are shown. (A) Survival analysis. Mice were monitored for survival up to day 7 post-MTX start (n = 10–12/group). Data are plotted on a Kaplan–Meier curve. *p < 0.05; **p < 0.01 (log-rank test versus “+ABx/+PCSK/+MTX”-control). (B) Representative histology of the proximal jejunum (n = 3–4/group) on day 3 (H&E; scale bar, 50 μm). (C) Representative immunofluorescent staining with markers (green) for inflammatory infiltration (CD11b), DNA damage (p-histone H2A.X), intestinal epithelial cell barrier integrity (p-β-catenin), and central efflux transporter (p-gp) of proximal jejunum on day 3 (n = 2/group), as assessed by optical sectioning microscopy (scale bar, 100 μm). Green arrows indicate examples of positive cells. Nuclei were counterstained with DAPI or TO-PRO-3 (blue). (D) Representative colocalization of CD11b (green) and p-gp (red) in the lamina propria of “+ABx/+PCSK/+MTX”-proximal jejunum on day 3 (n = 2/group), as assessed by optical sectioning microscopy (scale bar, 100 μm).

FIGURE 9. Treatment with the TLR2 agonist PCSK prevents chemotherapy-induced cytotoxic damage in human duodenal lamina propria mononuclear cells of patients. Human small intestine tissues were exposed to PCSK (20 μg/ml) and/or MTX (10 μM) in an ex vivo Matrigel-based culture model of duodenal pinch biopsies, as described in Materials and Methods. (A) Representative histology (H&E; scale bar, 100 μm). (B and C) Representative immunofluorescent staining with markers (green) for DNA damage (p-histone H2A.X) (B) and central efflux transporter (p-gp) (C), as assessed by optical sectioning microscopy. Green/yellow arrows indicate examples of positive cells in the lamina propria. Nuclei were counterstained with DAPI (red or blue). Scale bar, 50 μm.
cell differentiation by selectively upregulating TFF3 expression (59). Deficient TLR2 signaling may imbalance microbiota-dependent intestinal epithelial barrier defense, facilitating mucosal injury and leading to increased susceptibility of acute and chronic colitis (14, 36). In this study, we demonstrate that chemotherapy-induced mucositis in the small intestine is also associated with intestinal epithelial barrier dysfunction in TLR2 deficiency, as evidenced by disruption of cell–cell adhesion via phosphorylated β-catenin and lack of goblet-cell specific TFF3 in the proximal jejunum, which both may contribute to exacerbation of DNA damage–induced inflammatory and apoptotic destruction and impaired wound healing of the gastrointestinal tract (60, 61).

LPS exposure has been shown to aggravate MTX-induced intestinal mucositis in C3H/HeN but not in TLR4-defective C3H/HeJ mice (62). In this study, loss of ABCB1/MDR1 p-gp in the inflamed mucosa of MTX-TLR2 KO was associated with increased expression of TLR4. Mice deficient in MD-2 were resistant to MTX-induced damage. Deletion of MD-2 in TLR2 KO mice blocked disease exacerbation, implying that MD-2/TLR4 signaling is required for the severity of chemotherapy-induced intestinal damage in mice deficient in TLR2. We have previously demonstrated that combined loss of MDR1A and TLR2 results in increased MD-2 activation and LPS hypersensitivity, which accelerates colitis progression through ROS-mediated lysosomal damage and pyroptosis via IL-1β (36). Others have shown that lack of signaling via Pregnane X receptor, a transcriptional regulator of ABCB1/MDR1 p-gp induction, leads to mucosal hyper-responsiveness to LPS (63). Collectively, these results imply that a distinct defect in xenobiotic defense signaling via ABCB1/MDR1 p-gp may result in compromised tolerance to LPS.

We considered that abnormal microbiota composition under chemotherapy and/or defined genetic deletion may have subverted the mucosal innate immune system toward LPS hypersensitivity. Using high-throughput 16S rRNA gene sequencing, we did not detect consistent differences in the microbiota between groups. Despite careful comparison of WT and TLR2 KO littermates, in which offspring resulted from controlled crosses of heterozygous mice, interindividual variation of proximal jejunal samples was high, which is consistent with previous observations along the murine jejunum (64, 65). Similarly, previous work has suggested that the impact of TLR2 deficiency on the composition of the intestinal microbiota may be minimal (66). It is possible that we could have detected subtle changes or effects in considerably larger sample groups and at other time points. However, in our study, all TLR2 KO mice consistently showed the same degree of severity of genotoxic injury of the proximal jejunum in response to chemotherapy—regardless of interindividual heterogeneity of bacterial communities, suggesting that unique microbiota changes may not represent the critical determinant of chemotherapy-induced mucosal damage.

Antimicrobial agents are routinely administered to patients during anticancer therapy. The present study shows that antibiotic medication may have a detrimental impact on chemotherapy-elicted toxicity in the small intestine. Antibiotic pretreatment promoted chemosensitivity in WT mice that were otherwise chemosensitivity resistant. Two recent studies suggested that microbiota depletion by antibiotics decreases the antitumor efficacy of chemotherapy (67, 68). Taken together, antibiotics may put patients who are being treated for cancer at increased risk of gastrointestinal toxicity and chemotherapy inefficacy. The frequent use of prophylactic antibiotics in clinical practice should therefore be reconsidered. Our results suggest that specifically targeting TLR2 may offer a potential solution to prevent toxic side effects of chemotherapy in conjunction with antibiotics. Probiotic strains may constitute an alternative to reduce chemotoxicity (69); however, large-scale and randomized, placebo-controlled clinical trials are currently lacking. In a small observational study, supplementation with \textit{Lactobacillus rhamnosus} GG showed a trend toward reducing severe diarrhea related to 5-FU-based chemotherapy in colorectal cancer patients (70), but its mechanisms of action (possibly via TLR2) were not studied.

Finally, to demonstrate the plausibility that TLR2 may represent a pharmacological target against chemotherapy-induced damage in the human gastrointestinal tract, we used an ex vivo three-dimensional model system of human duodenal biopsies cultured short term with or without PCSK and MTX. Our results show that TLR2 stimulation protected against chemotherapy-induced DNA damage, which correlated with preservation of p-gp expression. Future studies will need to assess whether single nucleotide polymorphisms within the TLR2 gene (71) may enhance individual susceptibility to chemotherapy-related gastrointestinal toxicity.

In conclusion, these studies provide the first evidence, to our knowledge, that TLR2 acts as a central regulator of xenobiotic defense via the multidrug transporter ABCB1/MDR1 p-gp. This novel mechanism may allow the innate immune system to respond to harmful xenobiotics and instruct their immediate clearance, thus efficiently protecting the host from the genotoxicity of high-dose chemotherapy and its adverse effects in the intestinal mucosa.

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\section*{Disclosures}

The authors have no financial interests of interest.

References


Suppl. Figure 1:
A. Observed OTU diversity and B. Shannon diversity index scores, at a sequencing depth of 1084 sequences per sample, in proximal jejunum samples. White boxes indicate WT mice, grey boxes indicate TLR2 KO mice. Centre lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by dots; crosses represent sample means; data points are plotted as open circles. $n = 3, 3, 4, 3$ sample points. Visualised using BoxPlotR (Spitzer M, Wildenhain J, Rappsilber J, Tyers M. Boxplotr: A web tool for generation of box plots. Nat Methods 2014;11:121-2). Significance was tested using the Kruskall-Wallis and Mann-Whitney U tests in Minitab (v16); $ns =$ not statistically significant.