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J Immunol 2020; 204:707-717; Prepublished online 27 December 2019;
doi: 10.4049/jimmunol.1900470
http://www.jimmunol.org/content/204/3/707

Supplementary Material
http://www.jimmunol.org/content/suppl/2019/12/26/jimmunol.1900470.DCSupplemental

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TLR2 Dimerization Blockade Allows Generation of Homeostatic Intestinal Macrophages under Acute Colitis Challenge

Mor Gross-Vered,*1 Liraz Shmuel-Galia,†1 Batya Zarmi,† Fiachra Humphries,‡ Christoph Thaiss,* Tomer-Meir Salame,§ Eyal David,* Louise Chappell-Maor,* Katherine A. Fitzgerald,§ Yechiel Shai,† and Steffen Jung*

Recruited blood monocytes contribute to the establishment, perpetuation, and resolution of tissue inflammation. Specifically, in the inflamed intestine, monocyte ablation was shown to ameliorate colitis scores in preclinical animal models. However, the majority of intestinal macrophages that seed the healthy gut are also monocyte derived. Monocyte ablation aimed to curb inflammation would therefore likely interfere with intestinal homeostasis. In this study, we used a TLR2 trans-membrane peptide that blocks TLR2 dimerization that is critical for TLR2/1 and TLR2/6 heterodimer signaling to blunt inflammation in a murine colitis model. We show that although the TLR2 peptide treatment ameliorated colitis, it allowed recruited monocytes to give rise to macrophages that lack the detrimental proinflammatory gene signature and reduced potentially damaging neutrophil infiltrates. Finally, we demonstrate TLR blocking activity of the peptide on in vitro cultured human monocyte-derived macrophages. Collectively, we provide a significantly improved anti-inflammatory TLR2 peptide and critical insights in its mechanism of action toward future potential use in the clinic. *The Journal of Immunology, 2020, 204: 707–717.

Blood monocytes are generated in the bone marrow (BM) through a series of developmental stages before their extravasation (1–3). In the circulation, short-lived classical monocytes are defined as CD14+ cells in humans (4) and Ly6C+ cells in mice (5). On demand, classical monocytes are recruited to sites of tissue damage where they contribute to the establishment and resolution of inflammation as monocyte-derived macrophages (MoDM) (6). In preclinical models, Ab-mediated ablation of circulating monocytes or interference with their recruitment by CCR2 knock-down has been shown to improve pathological conditions, ranging from gut inflammation (7) to atherosclerotic plaque and myocardial infarct size (8). However, classical monocytes also play an important role in maintaining selected tissue macrophage compartments under steady-state, including barrier tissues, such as the skin and intestine (9–12). In fact, a majority of murine intestinal macrophages are constantly replenished under homeostasis by blood monocytes, which replace the original embryo-derived macrophage population, secrete IL-12, IL-23, TNF-α, and inducible NO synthase (7). Their critical contribution to gut pathology...
is highlighted by the fact that monocyte in vivo ablation ameliorates dextran sulfate sodium (DSS)–induced colitis (7).

TLR2 is broadly expressed on immune and nonimmune cells, senses lipid-containing pathogen-associated patterns, and, for its function, has to undergo critical homo- and heterodimerization. TLR2 heterodimers with TLR1 are triggered by lipoteichoic acid (LTA) and di-acylated cysteine-containing lipopeptides, whereas TLR2/TLR6 heterodimers are stimulated by triacylated cysteine-containing lipopeptides (20, 21). TLR2 and the respective heterodimers are thought to play a major role in gut inflammation (7, 22). TLR2-deficient mice display reduced sensitivity to DSS-induced colitis (23), whereas TLR6 mutant animals were reported to resist the DSS challenge (24). In humans, TLR2 levels reportedly increase in the terminal ileum and ileal pouch mucosa of patients with ulcerative colitis (25, 26). Moreover, TLR2 up-regulation was observed in submucosal cells (27). Studies in inflammatory bowel disorder (IBD) patients suggest that infiltrating monocytes and inflammatory macrophages, as well as TLR2 signaling, are involved in the pathogenic IBD axis (27, 28). Of note, upregulation of TLR2 was also observed in other inflammation-related diseases, such as type 1 diabetes (29), renal ischemia/reperfusion injury (30), and sepsis (31), implicating a potential general role of this receptor in the generation of proinflammatory monocytes.

We previously reported the beneficial effect of a rationally designed TLR2 trans-membrane (TM) peptide that ameliorated murine DSS-induced colitis (32). Specifically, we established that the peptide inhibited critical TLR2 homo- and heterodimerization, prevented TLR signaling, and reduced colitis severity. In this study, we modified the original inhibitory TLR2 peptide to improve pharmacokinetics and establish a stabilized peptide superior in blocking TLR2 signaling in cultured murine but also human monocytes/macrophages. Moreover, we provide mechanistic insight into the TLR2 peptide’s action by establishing that in the DSS colitis model, the peptide prevents the generation of proinflammatory cells from acute monocyte infiltrates but enables monocytes to adopt gene expression signatures compatible with gut homeostasis and health.

Materials and Methods

Mice

C57BL/6 mice were kept in a specific pathogen-free, temperature-controlled (22°C ± 1°C) facility on a reverse 12 h light/dark cycle at the Weizmann Institute of Science and at UMass Medical School, Worcester, MA. Food and water were given ad libitum, unless otherwise indicated. Mice were fed regular chow diet (Harlan Biotech Israel, Rehovot, Israel). Animals were handled according to protocols approved by the Weizmann Institute Animal Care Committee as per international guidelines. Experiments at UMass Medical School were approved by the Institutional Animal Care and Use Committee.

Peptide synthesis and purification

For in vitro experiments, peptides were synthesized by a 9-fluorenlymethoxycarbonyl (Fmoc) solid-phase method on Rink Amide MBHA resin (Calbiochem-Novabiochem) by using Liberty Blue peptide synthesizer (CEM Corporation) as described previously (33). All peptides were cleaved from the resin by incubation for 2 h with 95% trifluoroacetic acid, 2.5% H2O, and 2.5% triethylsilane. Purification of the crude peptides was performed by reverse phase HPLC (>98%) on a Vydac C4 column (Grace Discovery Sciences, Deerfield, IL) using a linear gradient of 20–90% acetonitrile in 0.1% trifluoroacetic acid for 40 min. The m.w. of the peptides was confirmed by platform lathocholic acid electrospray mass spectrometry. Two lysine residues were added to the C termini and the N termini of the peptides to confer water solubility to the hydrophobic TM domains, as previously described (34). For in vivo studies, peptides were purchased from Peptide 2.0 and tested for their purity by reverse phase HPLC and platform lathocholic acid electrospray mass spectrometry.

Peptide stability assay

Peptides in a final concentration of 100 μM were incubated in 25% human serum for up to 80 h at 37°C. Following, aliquots of the incubations were taken at different time points and mixed with 95% cold ethanol for 15 min to precipitate serum proteins. The supernatants were collected after centrifugation at 10,000 × g for 10 min and analyzed on HPLC in a 20–90% gradient of acetonitrile for 40 min. Values are mean of two to three independent experiments.

Preparation of mouse BM-derived macrophages

 Femurs of WT C57BL6 mice were removed, and BM was flushed with cold PBS. BM was washed with cold PBS and filtered by 100-μm filter. Cells were suspended with PBS seeded on 24-well tissue culture plates: 3.5 × 10^6 cells per well in RPMI medium (Biological Industries) with 10% heat-inactivated FBS (Life Technologies), 1% Penicillin–Streptomycin–Amphotericin B Solution (Biological Industries), and 10 ng/ml M-CSF (Sigma-Aldrich). After 3 d, half the medium was renewed. Cells were analyzed on day 7.

Preparation of human MoDM

Blood was retrieved from healthy participants and added to 60% Dextran solution (6% Dextran T-500 [Sigma-Aldrich] in PBS+-, pH 7.4) and 40% citrate buffer (5% Na citrate + 1.6% citric acid in PBS+). To isolate PBMCs, mixture was left for 30 min at room temperature, and supernatant was collected and loaded on Ficoll. Tubes were centrifuged in 920 × g in 8°C for 25 min without breaks, and interphase was collected and washed with cold PBS. Pellet was suspended and filtered with a 70-μm filter. Cells were washed with MACS buffer (PBS with 0.5% BSA and 2 mM EDTA), counted, and incubated in MACS buffer with 1:10 CD14 microbeads (Miltenyi Biotech), 100 μl for 10^7 cells. CD14+ cells were isolated by MACS magnetic column (Miltenyi Biotech), according to manufacturer’s instructions. Cells were seeded on 24-well tissue culture plates: 1 × 10^6 cells per well in RPMI medium (Biological Industries) with 20% heat-inactivated FBS (Life Technologies), 1% Penicillin–Streptomycin–Amphotericin B Solution (Biological Industries), and 1% l-Glutamine (Biological Industries). After 3 d, half the medium was renewed. Cells were analyzed on day 7.

DSS-induced colitis

Mice were given 1.5% DSS (TdB Consultancy) in the drinking water for 7 d. Peptides were dissolved in saline and injected every other day starting from day 1 of DSS. Control mice received regular water and were injected with saline, DSS-only mice were injected with saline.

Colonoscopy

To assess colitis severity, high-resolution murine video endoscopic system was used. The system was composed of a miniature probe (1.9-mm outer diameter), a xenon light source, a triple chip high-definition camera, and an air pump (Coloview, Karl Storz) to inflate the colon. Grading of colitis scores was performed as previously described (35) and conducted in a blind experimental setup.

Histology

Ten percent of 10% neutral buffered formalin was used to fix the tissues for 24 h before being processed and paraffin embedded. Five-micrometer-thin sections and tissues were embedded in paraffin, sectioned, and stained with H&E in an automated stainer (Leica Autostainer XL). Slides were evaluated using an Olympus BX40 microscope, and image acquisition was conducted with Olympus cellsSens Entry Software at indicated magnifications. Grading of histology scores was done by Applied Pathology Systems and conducted as follows. Inflammatory cell infiltrate (leukocyte) was evaluated by the number of leukocyte foci: 0, no significant change; 1, mild, infiltrated leukocytes in focal or occasional; 2, moderate, infiltrated leukocytes with more than one focus (multiple foci); and 3, severe, infiltrated leukocytes diffuse or continuous. Epithelial changes in goblet cell loss were evaluated by the reduction of goblet cell numbers relative to baseline goblet cell numbers per crypt: 0, no significant change; 1, goblet cell loss < 10% of normal amount; 2, goblet cell loss of ~10–50%; and 3, goblet cell loss > 50%. Crypt abscesses were evaluated by the neutrophils in crypt lumen: 0, no crypt abscesses; 1, rare crypt abscesses; 2, multiple crypt abscesses; and 3, continuous crypt abscesses. Erosion was evaluated by the loss of surface epithelium: 0, no erosion; 1, one focus; 2, multiple foci; and 3, continuous surface loss. Hyperplasia (epithelial cells) was evaluated by the increase in crypt number or length relative to baseline, visible as crypt elongation or thickened crypt layers: 0, no
hyperplasia; 1, mild, increase <50%; 2, moderate, increase of ~50–100%; and 3, marked, increase >100%. Ulceration was evaluated by crypt loss reaching beyond muscularis mucosae: 0, no ulceration; 1, one focus; 2, multiple foci; and 3, continuous. Lymphoplasmacytic aggregates were evaluated by a score based on the location of lymphoplasmacytic aggregates: 0, no lymphoplasmacytic aggregates; 1, lymphoplasmacytic aggregates reaching beyond muscularis mucosae; 2, lymphoplasmacytic aggregates reach mucosa; 2, lymphoplasmacytic aggregates reach submucosa; and 3, lymphoplasmacytic aggregates reach muscularis propria or subserosa.

**ELISA**

For cell culture, cells were incubated with the peptides at 37°C. After 2 h, LTA (5 μg/ml for mouse BM-derived macrophage [BMDM], 0.5 μg/ml for human MoDM) or 1 mM LPS was added for additional 5 h.

For in vivo, colons were flushed with cold PBS and opened longitudinally. Three-millimeter-squared punch biopsies were obtained from the distal end of the colon and incubated for 24 h in RPMI 1640 including 10% FBS and 1% penicillin/streptomycin (100 μg/ml for mouse BM-derived macrophage [BMDM], 0.5 μg/ml for human MoDM) or 1 mM EDTA containing fluorescent Abs. Following incubation, the DNA template was reverse transcribed with a second strand primer complementary to the ligated adapter.

**RNA sequencing and analysis**

RNA sequencing (RNASeq) of populations was performed as described previously (36). Cells were sorted into 100 μl of lysis/binding buffer (Life Technologies) and stored at 80°C. mRNA was captured using Dynabeads oligo(dT) (Life Technologies) according to manufacturer’s guidelines. We used a derivation of massively parallel single cell RNA-seq (MARS-seq) (37) to prepare libraries for RNASeq. RNA was reverse transcribed with MARS-seq Barcoded RT primer in a 10 μl volume with the Affinity Script kit (Agilent). Reverse transcription was analyzed by quantitative real-time PCR, and samples with a similar cycle threshold were pooled (up to eight samples per pool). Each pool was treated with Exonuclease I (New England BioLabs [NEB]) for 30 min at 37°C and subsequently cleaned by 1.2× vol of SPRI beads (Beckman Coulter). Subsequently, the cDNA was converted to dsDNA with a second strand synthesis kit (NEB) in a 20-ml reaction, incubating for 2 h at 16°C. The product was purified with 1.4× vol of SPRI beads, eluted in 8 μl, and in vitro transcribed (with the beads) at 37°C overnight for linear amplification using the T7 High Yield RNA polymerase IVT kit (NEB). Following in vitro transcription, the DNA template was removed with Turbo DNase I (Ambion) for 15 min at 37°C, and the amplified RNA (aRNA) was purified with 1.2× vol of SPRI beads. The aRNA was ligated to the MARS-seq ligation adapter with T4 RNA Ligase I (NEB). The reaction was incubated at 22°C for 2 h. After 1.5× SPRI cleanup, the ligated product was reverse transcribed using Affinity Script RT enzyme (Agilent) and a primer complementary to the ligated adapter. The reaction was incubated for 2 min at 42°C, 45 min at 50°C, and 5 min at 85°C. The cDNA was purified with 1.5× vol of SPRI beads. The linearization was completed and amplified through a nested PCR with 0.5 mM P5_Rd1 and P7_Rd2 primers and PCR ready mix (Kappa Biosystems). The amplified pooled library was purified with 0.7× vol of SPRI beads to remove primer leftovers. Library concentration was measured with a Qubit fluorometer (Life Technologies), and mean molecule size was determined with a 2200 TapeStation instrument. RNASeq libraries were sequenced using the Illumina NextSeq 500. Raw reads were mapped to the genome (NCBI37/mm9) using HISAT (version 0.1.6). Only reads with unique mapping were considered for further analysis. Gene expression levels were calculated and normalized using the HOMER software package (analyzeRepeats.pl rna mm9 -d<tagDir>-count exons -condenseGenes -strand + -raw) (38). Gene expression matrix was clustered using k-means algorithm (MATLAB function kmeans) with correlation as the distance metric. Principal component analysis was performed by MATLAB function pca. Gene ontology was performed by DAVID (https://david.ncifcrf.gov).

**Cytometry time-of-flight**

Cells were stained for cytometry time-of-flight (CyTOF) analysis according to manufacturer’s instructions. Briefly, isolated cells were washed with PBS and incubated with 1:4000 Cell-ID Cisplatin for 5 min, then washed with MaxPar Cell Staining Buffer and incubated with MaxPar Metal Conjugated Abs for 30 min. Cells were then washed with Cell Staining Buffer and incubated overnight with MaxPar Fix and Perm Buffer with 1:1000 Cell-ID Interカルカー-1r (125 μM). All reagents are products of Fluidigm. Results were analyzed on Helios (Fluidigm), in FlowJo software (Tree Star), and in the Cytobank platform.

**Table I. TLR2 dimer inhibitory peptides**

<table>
<thead>
<tr>
<th>Designation</th>
<th>Peptide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1 TLR2 Cys to Ala (TLR2-p)</td>
<td>kkLILLVGALAHFHFGLWkk</td>
</tr>
<tr>
<td>M2 TLR2 Cys to Ala partial D (TLR2-p parD)</td>
<td>kkLILLVGALAHFHFGLWkk</td>
</tr>
<tr>
<td>M3 TLR2 Cys to Ala TMD D (TLR2-p TMD D)</td>
<td>kkLILLVGALAHFHFGLWkk</td>
</tr>
<tr>
<td>M4 TLR2 Cys to Ala termini Lys D (TLR2-p K-D)</td>
<td>kkLILLVGALAHFHFGLWkk</td>
</tr>
<tr>
<td>H1 TLR2 (hTLR2-P)</td>
<td>kkLILLTGVCLCHRHFGLWkk</td>
</tr>
<tr>
<td>H2 TLR2 Cys to Ala (hTLR2-p A)</td>
<td>kkLILLTGVCLCHRHFGLWkk</td>
</tr>
<tr>
<td>H3 TLR2 partial D (hTLR2-p parD)</td>
<td>kkLILLTGVCLCHRHFGLWkk</td>
</tr>
<tr>
<td>H4 TLR2 Cys to Ala partial D (hTLR2-p A parD)</td>
<td>kkLILLTGVCLCHRHFGLWkk</td>
</tr>
</tbody>
</table>

List of TLR2 dimer inhibitory peptides and their sequences. Bold black letters within sequences indicate differences between mouse and human sequences. Underlined regions indicate replacement of L-amino acids with D-amino acids. Gray letters indicate cysteine (Cys) residues that were replaced by alanine (Ala).

Lys, lysine.

**Cytometry time-of-flight**

Cells were stained for cytometry time-of-flight (CyTOF) analysis according to manufacturer’s instructions. Briefly, isolated cells were washed with PBS and incubated with 1:4000 Cell-ID Cisplatin for 5 min, then washed with MaxPar Cell Staining Buffer and incubated with MaxPar Metal Conjugated Abs for 30 min. Cells were then washed with Cell Staining Buffer and incubated overnight with MaxPar Fix and Perm Buffer with 1:1000 Cell-ID Interカルカー-1r (125 μM). All reagents are products of Fluidigm. Results were analyzed on Helios (Fluidigm), in FlowJo software (Tree Star), and in the Cytobank platform.

**Data and materials availability.** The RNASeq datasets reported in this article can be found at the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE140788).
FIGURE 1. Human and murine anti-TLR2 peptides inhibit TLR2-specific secretion of IL-6 from mouse BMDM and human MoDM and do not inhibit TLR4 activation. (A) Mouse BMDM or human CD14+ primary peripheral monocytes (human PBMC) were seeded on cell culture plates for 7 d then treated with the indicated concentration of TLR2 inhibitory peptides for 2 h. Cells were then stimulated with either LTA (5 μg/ml for BMDM and 0.5 μg/ml for PBMC) or 1 μg/ml LPS for 5 h and tested for the secretion levels of IL-6 by ELISA. Bars indicate IL-6 secretion percentage compared with IL-6 secretion levels in cells incubated with LTA/LPS only. Error bars indicate SE. Circle, p value, 0.05 between LTA only and 2.5 μM peptide; triangle, p value, 0.05 between LTA only and 5 μM peptide; square, p value, 0.05 between LTA only and 10 μM peptide. Experiments performed (Figure legend continues).
Results

Characterization of species specificity and stability of TLR2 inhibitory peptides

We recently established that a TLR2 TM peptide that impairs TLR2 dimerization can inhibit the development of DSS-induced colitis by reducing IL-6, IL-12, and IL-23 expression in gut-infiltrating proinflammatory Ly6C+ monocytes (32). In this study, we set out to improve the original, mouse sequence-based TLR2-p peptide for future clinical use, test it on human cells, and investigate the underlying mechanism of the TLR2-based inhibition on intestinal cells, particularly monocytes and macrophages. Eight TLR2 peptide variants were designed, including the original peptide (M1): four were based on mouse and four were based on human TLR2 TM sequence (Table I). Modifications were chosen to ease synthesis and improve solubility and stability of the peptides. To measure the inhibitory activity of the peptides, we assayed IL-6 secretion by stimulated mouse BMDM and human macrophages derived from peripheral blood CD14+ monocytes (MoDM). Cells were incubated with increasing concentrations of the peptides and activated by either the TLR2 ligand LTA or the TLR4 ligand LPS (Fig. 1A, 1B). Mouse peptides affected IL-6 secretion of both murine and human cells. Human TLR2 peptides at all concentrations inhibited 20–40% of IL-6 secretion in LTA-stimulated BMDCMs but were particularly efficient in inhibiting IL-6 secretion by human MoDM, with over 80% inhibition at the highest dose. TLR2-p (M1) and TLR2-p K-D (M4) showed the best inhibition even at low peptide concentrations (Fig. 1A, 1B). Minor inhibition of IL-6 secretion following LPS activation was observed with low or absent dose dependency. Of note, inhibition following LPS activation was higher in human MoDM compared with mouse BMDCM, and TLR2-p ParD (M2) and TLR2-p TMD D (M3) had the least effect on IL-6 secretion in MoDM activated by LPS. We next assessed the stability of the peptides by measuring their degradation in human serum. The mouse peptides TLR2-p ParD (M2) and TLR2-p K-D (M4) showed highly improved stability over the original peptide, TLR2-p (M1) (Fig. 1C). Among the human peptides, hTLR2-p A ParD (H4) was the most stable. Based on the above results, we chose the mouse peptide TLR2-p K-D (M4) and human peptide hTLR2-p A ParD (H4) as lead peptides for further in vitro and in vivo experiments.

TLR2-p peptides ameliorate DSS-induced colitis

The in vivo potential of the stabilized peptides to inhibit TLR2 signaling and therefore neutralize proinflammatory infiltrating gut monocytes was tested in a colitis model induced by oral DSS delivery (39). Mice were given 1.5% DSS in the drinking water for 7 d and injected i.p. every other day with 5 μg/g bodyweight of M4, H4, or scrambled TLR2-p (scrambled peptide [Scram]) for control. At day 7, mice were sacrificed for analysis (Fig. 2A). Scram did not affect the colonoscopy score compared with mice challenged with DSS only, nor did the human peptide H4 (Fig. 2B, 2C, Supplemental Fig. 1A). In contrast, M4 significantly ameliorated inflammation according to all five tested parameters (i.e., feces consistency, cell wall translucency, vasculature structure, tissue granularity, and presence of fibrin) (Fig. 2B, 2C, Supplemental Fig. 1B). ELISA analysis of whole-tissue colon extracts for the inflammatory cytokines IL-6 and TNF confirmed that treatment with M4 reduced the levels of these factors compared with the DSS-only group (Fig. 2D).

To substantiate our finding, we decided to test the efficacy of M4 in a different animal facility, predicted to harbor distinct microbiota. Also in this experiment, the M4 peptide significantly ameliorated colitis, as seen from the histological analysis (Fig. 2E, 2F) and weight measurements (Fig. 2G). Collectively this establishes that the TLR2 membrane peptide has the potential to curb the acutely induced microbiota-driven gut inflammation in the DSS model.

The TLR2 TM peptide prevents DSS-induced changes in the colonic myeloid composition of challenged animals

Monocyte infiltrates are established inducers of gut inflammation (7, 19, 40), and their ablation has been shown to ameliorate DSS-induced colitis (7). However, other cell populations are also affected by the DSS regimen and the resulting breach of the epithelial barrier. To explore the global effect of DSS administration and the TLR2p K-D treatment on the colonic immune cell compartment, we used CyTOF that allows simultaneous analysis of all major lymphoid and myeloid subpopulations, including their activation state (Supplemental Fig. 2A, 2B). As expected, DSS administration induced significant immune cell infiltrates, defined by the pan CD45 marker (Fig. 3A). Adaptive immune cells, namely T and B cells, do not play a major role in the acute stage of DSS-induced colitis (41) and displayed minor numeric changes; analysis was hence focused on the myeloid lineage (Fig. 3B). As we had reported earlier for the TLR2-p (M1) peptide (32), monocyte infiltrates were observed both in mice treated with DSS and mice treated with DSS and the peptide, albeit reduced following M4 treatment (Fig. 3B, 3C). A similar trend was observed for neutrophils. In contrast, relative abundance of macrophages was reduced as a result of the acute infiltrates (42). Also, selected cell surface markers exhibited expression changes following DSS that were altered by the peptide treatment, particularly on monocytes and macrophages (Fig. 3D-F). This included Ly6a/Sca-1, which was reported to be expressed on monocytes under pathological conditions (43) (Fig. 3D). Collectively, this establishes that the M4 TLR2 peptide treatment ameliorates colitis despite significant intestinal monocyte and neutrophil infiltrates.

The TLR2 membrane peptide M4 prevents the induction of proinflammatory signatures in infiltrating monocytes and resident macrophages

To directly test the impact of the M4 peptide on resident and acutely recruited myeloid cells in the gut, we isolated colonic infiltrating monocytes (CD45+CD11b+Ly6C+MHCII+) and colonic resident macrophages (CD45+CD11b+CD64+Ly6C−MHCII+) from DSS-challenged animals that were treated or not with peptide by i.p. injection (Fig. 4A, Supplemental Fig. 3). The sorted macrophages were then subjected to RNASeq. Of note, monocytes were detected also in the colon of control mice, albeit in much smaller numbers compared with all other groups (Fig. 4A). These cells likely represent recent tissue immigrant cells that entered to maintain the steady-state macrophage pool of the intestine, before differentiating along the “monocyte waterfall” (42). Transcriptomes in duplicates, three to four mice/donors in each condition. (B) IC50 values calculated by the results from (A). (C) Stability of the peptides in human serum. Fourteen micrograms of TLR2-p, TLR2-p ParD, TLR2-p TMD D, M4, hTLR2-P A, and H4 were incubated with human serum for up to 80 h, and samples were injected and analyzed on HPLC in a 20–90% gradient of acetonitrile for 40 min. Graphs represent the degradation profile for each peptide. Values are mean of two to three independent experiments. Because of technical constraints, the stability of the human peptides hTLR2-p (H1) and hTLR2-p ParD (H3) could not be measured.
FIGURE 2. The M4 TLR2 peptide reduces severity of DSS-induced colitis. (A) Scheme of the DSS model and injection regimen. (B) Representative images from the colonoscopy of control mice injected with saline (no DSS) and mice challenged with DSS and injected i.p. with saline (DSS only) or 5 mg/kg M4 (DSS + M4), hTLR-2p A ParD (DSS + hTLR-2p A ParD), or Scram (DSS + Scram). (C) Colonoscopy score of all 5 scoring parameters (feces, translucency, vascularity, granularity, and fibrin) combined. (D) Levels of murine IL-6 secretion from colons of indicated samples as determined by ELISA. Experiment was performed five times, 20–40 mice total in each group. Error bars indicate SE. (E) Representative H&E staining histology images of colons of mice with indicated treatments, original magnification ×10. (F) Histology scores of colons of mice with indicated treatments stained with H&E. For scoring, see Materials and Methods section. For treated groups, n = 8; for untreated control, n = 3. (G) Weight measurements. For treated groups, n = 8; for untreated control, n = 3. Error bars indicate SE. *p < 0.05, **p < 0.01, ***p < 0.001, Student t test.
of both monocytes and resident macrophages displayed prominent changes upon DSS challenge, as highlighted by heat map analysis (Fig. 4B). Specifically, in both cell types, the challenge induced the expression of genes encoding proinflammatory cytokines (Il-23a, Il-1b, Tnf) and chemokines, including neutrophil attractants (Cxc11, Cc12, Cc14), TLRs (Tlr2, Tlr4), and IRFs (Irf1, Irf4) (Fig. 4B, 4C). Likewise, expression of a large number of genes was silenced in the cells retrieved from DSS-challenged animals. This included genes encoding proteins related to cellular respiration, metabolism, and other steady-state functions (Pparg, Bcl2l2, Aldh2) (Fig. 4B, 4D). Treatment with M4 peptide, but not the scrambled control peptide (Scram), prevented the acquisition of these robust responses, including both induced and silenced genes. Importantly, the prominent monocyte infiltrate that arose in response to the DSS challenge adopted an expression profile identical to the rare “homeostatic” monocytes that were found in the gut in absence of...
FIGURE 4. The M4 TLR2 peptide shifts transcriptomes of infiltrating monocytes and resident colonic macrophages to steady-state profiles. (A) Representative flow cytometry analysis of colonic myeloid cells in mice treated with DSS and DSS and peptides. (B) Heat maps for the 5359 genes (in infiltrating monocytes) and 4782 (in resident macrophages) that change in at least 2-fold across all samples, including representative genes and gene ontology (GO) terms associated with genes in each cluster. Clusters were calculated by the K-means algorithm (MATLAB), results visualized in Gene-E. GO pathways were predicted by DAVID (david.ncifcrf.gov/tools.jsp). Bars indicate mean and SE. (C) Representative genes upregulated following DSS administration and downregulated following peptide treatment. (D) Representative genes downregulated following DSS administration and upregulated following peptide treatment. *p < 0.05, **p < 0.01, ***p < 0.001.
Collectively, these data highlight the potency of the TLR2 TM peptide to prevent acute colon inflammation by blunting the proinflammatory activity of infiltrating monocytes and resident macrophages (Supplemental Fig. 3B). Of note, rather than perpetuating inflammation, monocyte infiltrates display signatures similar to rare monocytes that can be seen in healthy gut and to resident macrophages of unchallenged animals.

M4 TLR2 peptide impairs LTA-induced global activation of primary human MoDM

To test the global effect of the TLR2 TM peptides on human TLR2 activation, human monocytes were isolated by magnetic bead separation based on CD14 expression, cultured, and incubated either with LTA, medium only, or LTA and different doses of M4. PCA analysis of global transcriptomes obtained by RNASeq analysis of these cells showed that M4 peptide incubation prevented the response of these cells to LTA in a dose-dependent manner (Fig. 5A). Unbiased K-means clustering of the 4180 genes that change more than 2-fold across all samples. Clusters were calculated by the K-means algorithm (MATLAB), results visualized in Gene-E. Gene ontology (GO) pathways were predicted by DAVID (david.ncifcrf.gov/tools.jsp). (C) RNA levels of TNF, IL-6, IL-1β, and IDH2. Bars indicate mean and SE. *p < 0.05, **p < 0.01, ***p < 0.001, Student t test, compared with no LTA.

TNF, IL-6, TLR2, and NFκB, were related to the inflammatory response, TLR-mediated signaling, apoptosis, and other functions, which indicate activation of the immune system (Fig. 5B, 5C). Clusters IV and V comprised genes expressed in nonactivated cells incubated in medium only and whose expression was silenced by LTA, which are related to steady-state functions (e.g., metabolism and respiration). This gene list included IDH2, which participates in mitochondrial ATP production; phagocytosis-related genes FCGR1A and MERTK; and NDUF1, which is an essential factor for cellular respiration (Fig. 2B, 2C). Collectively, these data show that the mouse peptide M4 efficiently blocks LTA-induced TLR2-mediated global activation of human monocytes.

Discussion

Intestinal macrophages and blood monocytes, their progenitors, play major roles in the maintenance of intestinal homeostasis as well as in the development of IBD. Imbalance of homeostasis, caused by damage to the epithelial monolayer, pathogen invasion, or inflammation, induces prominent infiltration of monocytes to the injured site (7, 40). The infiltrate gives rise to activated macrophages, which initially promote the inflammation but eventually...
also contribute to the restoration of homeostasis (7). In the intestine, the monocyte–macrophage axis is also critical in steady-state because unlike most other tissue macrophages, a large fraction of relatively short-lived intestinal macrophages are continuously replenished from the blood monocyte pool (9–11, 44).

Colitis development has been linked with TLR expression, supported by the fact that the proinflammatory monocyte infiltrate was shown to express TLR2 (7, 22, 25–28). In line with this notion, we had previously established that inhibiting TLR2 by peptides that bind its TM region and thereby block TLR2 homo- and heterodimerization could ameliorate colitis (32); in this study, we extended these studies to provide additional critical information on the detailed mechanisms and gene expression changes of infiltrating monocytes and resident macrophages in the colon during colitis.

We first modified the original TLR2 peptide to achieve better stability and, hence, efficacy by transforming some amino acids from L to D conformation (45, 46). Most peptides tolerated the L to D transition and retained activity, probably because the peptide is inserted into the membrane, which stabilizes its structure. Based on the in vitro studies, two peptides were chosen for the in vivo experiments, one of which, M4, displayed strong potency to ameliorate DSS-induced colitis in animals housed in two distinct animal facilities.

In line with earlier studies reporting DSS-induced colitis in T– and B cell–deficient animals (47), CyTOF analysis revealed that the lymphoid compartment was hardly effected by either DSS T– and B cell–deficient animals (47). CyTOF analysis revealed distinct animal facilities. Based on the in vitro studies, two peptides were chosen for the in vivo experiments, one of which, M4, displayed strong potency to ameliorate DSS-induced colitis in animals housed in two distinct animal facilities.

Upon tissue infiltration under DSS challenge, monocytes acquire a proinflammatory signature and give rise to proinflammatory macrophages (7, 40). Peptide treatment reversed this phenomenon, although classical monocytes were still prominently recruited to the intestine under DSS administration, presumably because of the damage to epithelial monolayer. Rather, the blockade of TLR2 homo- or TLR1/2 or TLR6/2 heterodimer signaling prevented acquisition of proinflammatory gene expression signatures and silencing of genes associated with gut homeostasis. Despite the proinflammatory surroundings induced by the DSS (e.g., the breach of the epithelial barrier), inhibition of TLR2 was sufficient to curb the proinflammatory switch of the recruited monocytes and alleviate colitis development.

Toward a future potential use of TLR2p K-D as a therapeutic, we performed a comprehensive RNASeq analysis to probe the global impact of the peptide on human MoDM. M4 efficiently prevented the activation of human MoDM following TLR2 activation by LTA. The inhibitory effect was dose dependent and characterized by prevention of expression of proinflammatory genes as well as retained expression of genes related to metabolism, respiration, and other cell maintenance functions. A number of interesting issues remain to be addressed, including the question of whether the peptide acts on monocytes in the circulation (i.e., before the cells enter the tissue) or only in the gut.

In conclusion, our results show that the specific targeting of TLR2 and its respective heterodimers can prevent the acquisition of proinflammatory activities by infiltrating monocytes and resident macrophages and thereby reduce colitis severity. This corroborates the earlier notion that TLR2 is a key regulator of intestinal inflammation but also an attractive target for IBD treatment. The rationally designed, stabilized peptide we report inhibits TLR2 signaling in both mouse cells and cultured human MoDM and might thus offer a novel therapeutic agent.

Acknowledgments

We thank all members of the Shai and Jung laboratory for helpful discussions. We further thank the staff of the Weizmann Animal facility, FACS facility, and Israel National Center for Personalized Medicine for expert advice as well as Drs. Udi Zigmond Dan Blat and Ran AfiK for help in the colitis study, Dr. Reinit Nevo for help with colitis analysis, and Dr. Ron Rotkopf for performing the statistics.

Disclosures

The authors have no financial conflicts of interest.

References


