HMPL-004 (Andrographis paniculata extract) Prevents Development of Murine Colitis by Inhibiting T-cell Proliferation and TH1/TH17 Responses

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Background: Extracts of the plant Andrographis paniculata have been used to treat inflammatory diseases in Asian countries. A recent double-blind, placebo-controlled trial of HMPL-004 (A. paniculata extract) has demonstrated its safety and effectiveness for induction of clinical response, remission, and mucosal healing in patients with mild to moderate ulcerative colitis (UC). We aimed to determine if HMPL-004 could prevent the development of T-cell-dependent murine colitis and to define its in vivo mechanism(s) of action.

Methods: CD4+CD45RBhigh T cells were transferred into Rag1−/− mice and gavaged daily with HMPL-004 or methyl cellulose (MC). Severity of colitis was evaluated by weight loss, histology, and cytokine expression.

Results: Mice treated with MC developed colitis within 4–7 weeks, as evaluated by weight loss, and severe intestinal inflammation. HMPL-004-treated mice did not lose weight and displayed only very mild intestinal inflammation. Tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, interferon-gamma (IFN-γ), and IL-22 expression were significantly decreased in HMPL-004-treated mice. We observed higher percentages of naïve CD4+ T cells in the lamina propria of HMPL-004-treated mice. At early timepoints HMPL-004-treated mice have significantly reduced splenic cell counts, reduced CD4+, and IL-17, and IFN-γ+ T cells. Furthermore, HMPL-004 inhibited the proliferation of CD4+ T cells and differentiation into TH1/TH17 cells in vitro.

Conclusions: HMPL-004 inhibits the development of chronic colitis by affecting early T-cell proliferation, differentiation, and TH1/TH17 responses in a T-cell-driven model of colitis, presenting a unique mechanism of action. Our data suggest that HMPL-004 could be an attractive herbal therapeutic for inflammatory bowel disease.

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(ERK)1/2 phosphorylation.\textsuperscript{15} Interestingly, andrographolides also exhibited antiapoptotic effects in T cells, suggesting that the observed inhibitory effects on cytokine production are not due to cytotoxicity of T cells. Extract of \textit{A. paniculata} has been shown to inhibit tumor necrosis factor alpha (TNF-\textalpha{}) and IL-1\beta{} secretion from lipopolysaccharide (LPS)-stimulated macrophage-like cell line RAW264.7 in vitro via the inhibition of NF-\kappa{}B signaling.\textsuperscript{5} These in vitro data suggest that extracts of \textit{A. paniculata} have inhibitory effects on multiple immune cells (DC, macrophages, T cells) implicated in the development and disease progression of ulcerative colitis (UC) and Crohn’s disease (CD).

Recently, a randomized, double-blind, placebo-controlled, multicenter Phase Ib clinical trial of HMPL-004 (a proprietary extract of \textit{A. paniculata}) was conducted in patients with mild to moderate UC.\textsuperscript{16} This clinical trial demonstrated similar efficacy of HMPL-004 compared to mesalazine for the treatment of UC with a good safety profile. Remission and response as evaluated by colonscopy was not significantly different between HMPL-004- and mesalazine-treated patients (28% vs. 24% remission for HMPL-004 and mesalazine, respectively), suggesting that HMPL-004 may be an efficacious alternative to mesalazine in UC patients. Clinical trials are currently under way to evaluate the efficacy of HMPL-004 in CD.

Although the effects of \textit{A. paniculata} extracts have been studied extensively in vitro in several immune cells, the exact mechanism(s) of action of HMPL-004 in experimental colitis has not been elucidated to date. Here we demonstrate that HMPL-004 prevents the development of chronic colitis in the CD4+CD45RB\textsuperscript{high} T-cell transfer model of colitis. We observed significantly reduced expression of the proinflammatory mediators TNF-\textalpha{}, IL-1\beta{}, IFN-\gamma{}, IL-22, and IL-6 in HMPL-004-treated mice. HMPL-004 had no effect on the expression of the antiinflammatory cytokine IL-10. HMPL-004 also prevented the conversion of naïve T cells into effector and memory T cells in this colitis model. At early timepoints during the development of colitis, HMPL-004-treated mice had significantly reduced cellularity, reduced CD4+ T cells, and IL-17\textsuperscript{a}, IFN-\gamma\textsuperscript{a}, and IL-17/IFN-\gamma double-positive cells in the spleens. These data suggest that HMPL-004 affects early T-cell proliferation and/or differentiation/in maturation. In vitro experiments confirmed that HMPL-004 inhibits the proliferation of CD4+ T cells and the differentiation of naïve CD4+ T cells into \textit{T}_{\text{H}1} and \textit{T}_{\text{H}17} cells. In conclusion, HMPL-004 inhibits the development of chronic colitis by affecting T-cell proliferation and \textit{T}_{\text{H}1}/\textit{T}_{\text{H}17} responses in a T-cell-driven model of chronic colitis, presenting a unique mechanism of action, and suggesting that HMPL-004 warrants further investigation as an herbal therapeutic for CD and UC.

**MATERIALS AND METHODS**

**Reagents**

Lyophilized HMPL-004 Hutchison MediPharma (Shanghai, China) was reconstituted in methyl cellulose (MC; 0.5% w/v; Sigma, St. Louis, MO).

**Mice**

C57BL/6, and Rag1\textsuperscript{−/−} mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were maintained under specific pathogen-free conditions in the Animal Care Facility at Cedars-Sinai Medical Center. The mice used in all experiments were handled according to the guidelines and approved protocols of the Cedars-Sinai Medical Center Animal Care and Use Committees.

**T-cell Transfer Model**

C57BL/6 mice were used as donors and B6 Rag1\textsuperscript{−/−} males (Jackson Laboratory) as recipients. Spleens were homogenized and the resulting cell suspension was passed through a 25G needle. CD4+ T cells were negatively selected using the EasySep Mouse CD4+ T Cell Enrichment Kit (STEMCELL Technologies, Vancouver, Canada). Cells were labeled with anti-CD4 and anti-CD45RB. Using the MoFlow cell sorter (Dako Cytomation, Carpinteria, CA), CD4+ CD45RB\textsuperscript{high} cells were purified by gating and sorting 40% of the highest fluorescing CD45RB cells. Each recipient mouse was injected intraperitoneally with 0.5 × 10\textsuperscript{6} cells in sterile phosphate-buffered saline (PBS). Mice were gavaged daily with 300 mg/kg HMPL-004 or MC. Mice were weighed and observed for signs of colitis over an 8-week period. Mice were sacrificed 1 or 2 weeks after T-cell transfer or when the majority of mice started to lose more than 10% of their peak weight.\textsuperscript{17} Histology was scored as described previously.\textsuperscript{18}

**Induction and Assessment of Chronic Dextran Sodium Sulfate (DSS) Colitis**

DSS-induced chronic colitis was induced by multicycle administration of DSS drinking water.\textsuperscript{19} Female mice of 8 weeks of age received 3% (w/v) DSS drinking water (MP Biomedicals, Irvine, CA) on days 1–5, 8–12, 15–19, and 22–26. Mice were checked daily for development of colitis by monitoring body weight, gross rectal bleeding, and stool consistency. Mice were gavaged daily with 300 mg/kg HMPL-004 or MC and sacrificed on day 29. Tissues were fixed in 10% formalin. Cross-sections were prepared and stained with hematoxylin and eosin (H&E). Histology was scored as described.\textsuperscript{19}

**Cell Isolation and Culture**

Mononuclear cells from mesenteric lymph nodes (MLNs) were isolated after gentle cell dispersion using 25G needles and passage through a 60-μm nylon membrane. Lamina propria mononuclear cells (LPMCs) were isolated from cecum and colon. Briefly, epithelial cells were removed by washing in 5 mM EDTA. Colon were cut into small pieces and digested with collagenase D (Roche Diagnostics, Mannheim, Germany), dispase II (Roche Diagnostics), and DNase I (Sigma). Next, LPMCs were purified by a 45%/72% Percoll (GE Healthcare, Piscataway, NJ) gradient. Single-cell suspensions of splenocytes were depleted of red blood cells by hypotonic lysis (RBC Lysis buffer, eBioscence, San Diego, CA) and passed through a 70-μm cell strainer. Cells were cultured in 96-well round-bottom plates at 1 × 10\textsuperscript{6} cells/mL of complete medium (RPMI 1640 supplemented...
with 10% fetal bovine serum [FBS], gentamicin, amphotericin B) for 3 days. Anti-CD3e (0.5 µg/mL, plate-bound) and anti-CD28 (1 µg/mL, soluble) mAb (BD Biosciences, San Jose, CA) were used for restimulations. Supernatants were harvested after 72 hours of stimulation. Cytokine concentrations in supernatants or serum were assayed by enzyme-linked immunosorbent assay (ELISA) using kits for IFN-γ, IL-17, IL-22, TNF-α, and IL-6 (eBioscience).

In Vitro T-cell Proliferation and Differentiation

Splenocytes were prepared as described above. CD4+ T cells were negatively selected using the EasySep Mouse CD4+ T Cell Enrichment Kit (STEMCELL Technologies). For T-cell proliferation assays cells were labeled with anti-CD4 and anti-CD25 for cell sorting. CD4+ CD25- cells were isolated by flow cytometry and labeled with carboxyfluorescein succinimidyl ester (CFSE; Invitrogen, Carlsbad, CA). Cells were stimulated with Dynabeads Mouse T-Activator CD3/CD28 (Invitrogen) and restimulated daily with either 300 mg/kg HMPL-004 or MC, and monitored for development of colitis. Mice treated with MC began to lose signiﬁcantly higher weight at sacrifice than those treated with HMPL-004. To assess the role of HMPL-004 in the development of chronic colitis, we transferred CD4+ T-cell-induced chronic colitis. Mice treated with MC began to lose weight at 3 to 5 weeks after T-cell transfer (Fig. 1A). In contrast, mice that received HMPL-004 did not lose significant weight (Fig. 1A). Analysis of body weight at the end of the study revealed a significantly higher weight at sacrifice in HMPL-004-treated mice (Fig. 1B). Macrophoscopically, we only observed minimal inflammation of the colon and rectum, macroscopic differences in the stool consistency (loose stool in MC-treated mice, and formed stool pellets in HMPL-004 prevented the development of chronic colitis.

Flow Cytometry

Cells were stained with antibodies against murine CD4 (RM4-5), CD45RB (16A), CD62L (MEL-14), CD44 (IM7). Cells were acquired using a CyAn ADP flow cytometer (Dako Cytomation) and analyzed with the Summit software package (Dako) or FlowJo (Tree Star, Ashland, OR). For intracellular staining, cells were restimulated with 50 ng/mL phorbol myristate acetate (PMA) and 500 ng/mL ionomycin in the presence of monensin for 4 hours. Cells were stained with anti-CD4 FITC, fixed, permeabilized with mouse Foxp3 staining buffer set (eBioscience), intracellular stained with antibodies against IFN-γ and IL-17 (eBioscience), and analyzed using a CyAn flow cytometer.

Real-time Polymerase Chain Reaction (PCR)

Total RNA was isolated from tissues using RNaseasy kits (Qiagen, Valencia, CA) according to the manufacturer’s protocol and reverse-transcribed into cDNA with the Omniscript RT kit (Qiagen). Gene expression was quantified by real-time PCR using iCycler Thermal cycler (Bio-Rad Laboratories, Hercules, CA). Real-time RT-PCR was performed with TaqMan probes and primers designed using Beacon Design 4.0 (Premier Biosoft International, Palo Alto, CA): for IFN-γ; forward 5′-CCA AGC GGC TGA CTG AAC TC-3′; reverse 5′-TGG CCC GGA GTG TAG ACA TC-3′; probe 5′-FAM-CCT CCC ATC AGC AGC ACC TCC CG-BHQ-3′; for IL-17; forward 5′-ATG CTG TTG CTG CTG AG-3′; reverse 5′-TTG GAC ACG CTG AGT CCT GAG-3′; probe 5′-FAM-GGC GCT ACA GTC AAG GCA GCA GCG-BHQ-3′; for TNF-α; forward 5′-GAC AAG GCT GCC CCG ACT AC-3′; reverse 5′-ACG GCA GAG AGG AGG TTG AG-3′; probe 5′-FAM-CTC TTC ACC CAC ACC GTC AGC AGC-3′, for b-actin; forward 5′-ATG AGC ATC ATG TTT GA-3′; reverse 5′-TAC GAC CAT CAG AGT ACA G-3′; probe 5′-FAM-CGT AGC CAT CCA GGC TGT GC-BHQ-3′, for IL-22; forward 5′-AGC TTG AGG TGT CCA ACT TCC-3′; reverse 5′-GTA GCA CTG ATC TTT AGC ACT GAC-3′; probe 5′-FAM-AGC GTG ACA TTC TCA ACC GCA CCT-BHQ-3′, for IL-10, forward 5′-GCC CAG AAA TCA AGG AGC ATT TG-3′; reverse 5′-CAG GGG AGA AAT CGA TGA CAG C-3′; probe 5′-FAM-AGC CGC ATC CTG AGC TTC AGC-BHQ-3′, for IL-1β; forward 5′-CAC TAC AGC CTC CGA GAT GAA-3′; reverse 5′-ATT TTG TCG TTG OGT TCT CC-3′; probe 5′-FAM-AAA GCC TCG TGC TGT CGG ACC CAT-BHQ-3′. Murine IL-6 TaqMan Gene Expression Assay was purchased from Applied Biosystems (Carlsbad, CA).

Statistical Analysis

Statistical signiﬁcance was determined by two-tailed Student’s t-test or Mann–Whitney U-test. Differences were considered signiﬁcant at P < 0.05 or as indicated.

Ethical Considerations

The mice used in all experiments were handled according to the guidelines and approved protocols of the Cedars-Sinai Medical Center Animal Care and Use Committees.

RESULTS

HMPL-004 Prevents the Development of CD4+CD45RBhigh T-cell-induced Chronic Colitis

To assess the role of HMPL-004 in the development of chronic colitis, we transferred CD4+ CD45RBhigh naïve T cells isolated from wildtype mice into Rag1−/− mice, gavaged mice daily with either 300 mg/kg HMPL-004 or MC, and monitored for development of colitis. Mice treated with MC began to lose weight at 3 to 5 weeks after T-cell transfer (Fig. 1A). In contrast, mice that received HMPL-004 did not lose significant weight (Fig. 1A). Analysis of body weight at the end of the study revealed a signiﬁcantly higher weight at sacrifice in HMPL-004-treated mice (Fig. 1B). Macrophoscopically, we only observed minimal inflammation of the colon and rectum, macroscopic differences in the stool consistency (loose stool in MC-treated mice, and formed stool pellets in...
HMPL-004-treated mice), and colonic thickness/rigidness in the distal colon of MC-treated mice compared to normal appearance in HMPL-004-treated mice (Fig. 1C). Microscopically, in HMPL-004-treated mice we observed minimal intestinal inflammation with little cellular infiltrate or epithelial hyperplasia, in particular in the rectum and colon, consistent with a significantly reduced histological score in HMPL-004- compared to MC-treated mice (Fig. 1D,E). Furthermore, we observed a significant reduction in the cellular counts in the lamina propria (LP), MLN, and spleens of HMPL-004-treated mice (Fig. 2A–C). We did not observe a therapeutic effect of HMPL-004 when mice were treated with a lower dose of 100 mg/kg/day HMPL-004, suggesting a dose–response effect (data not shown). Real-time PCR analysis of tissues from the colon and cecum revealed a significant reduction of the expression of the proinflammatory cytokines TNF-α, IFN-γ, IL-1β, and IL-22 in HMPL-004-treated mice compared to MC-treated mice (Fig. 3A). We did not observe differences in the expression of the antiinflammatory cytokine IL-10.
between the treatment groups (Fig. 3A). The reduced intestinal inflammation in HMPL-004-treated mice was also reflected in reduced serum concentrations of IL-6 and TNF-α in HMPL-004-treated mice (Fig. 3B,C).

HMPL-004 Inhibits the Production of IL-17 and IL-22 from LPMC

Next, we analyzed the potential of HMPL-004 to inhibit the differentiation of Th1 and Th17 cells in vivo. LPMC were isolated from HMPL-004- or MC-treated mice and restimulated with anti-CD3ε and anti-CD28. HMPL-004 treatment led to significantly reduced IL-17 and IL-22 production in restimulated LPMC (Fig. 4A,B). We also observed a trend toward reduced production of IFN-γ in LPMC, and reduced IL-17, IL-22, and IFN-γ in restimulated MLN cell isolated from HMPL-004-treated mice (data not shown).

HMPL-004 Inhibits the Development of Colitis During Early T-cell Proliferation

To further characterize the protective effect of HMPL-004 on the development of colitis and to determine if HMPL-004 could inhibit the development of colitis when given at later timepoints after T-cell transfer we started the treatment with HMPL-004 or MC 2 weeks after CD4+ CD45RBhi naive T-cell transfer. HMPL-004 treatment could not prevent weight loss when mice were gavaged daily starting on day 14 after T-cell transfer (Fig. A, Supplemental Digital Content 1, http://links.lww.com/IBD/A2). In contrast to our observation that HMPL-004 prevented the development of colitis, we did not observe any significant differences in colon length, cellularity in LPMC, MLN, spleens, or histology score between HMPL-004- and MC-treated mice when treatment was started 2 weeks after T-cell transfer (Fig. B-D, Supplemental Digital Content 1, http://links.lww.com/IBD/A2). In addition, LPMC or MLN cells isolated from HMPL-004- or MC-treated mice and restimulated with anti-CD3ε and anti-CD28 produced similar amounts of IL-17 and IFN-γ (Fig. E, Supplemental Digital Content 1, http://links.lww.com/IBD/A2). These data suggest that HMPL-004 inhibits early events during the development of CD4+ CD45RBhi naive T-cell transfer colitis such as homeostatic T-cell expansion and/or the differentiation of naïve T cells into effector T cells.

HMPL-004 Inhibits T-cell Accumulation in the Intestine Through Effects on Early T-cell Proliferation

To confirm that HMPL-004 inhibits early events during the development of CD4+ CD45RBhi naive T-cell transfer colitis such as homeostatic T-cell expansion and/or the differentiation of naïve T cells into effector T cells, we analyzed T-cell expansion 1 and 2 weeks after T-cell transfer. One week after T-cell transfer we

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**FIGURE 2.** HMPL-004 treatment decreases cellularity in LP, MLN, and spleen. (A) Total numbers of mononuclear cells in LP of HMPL-004- and MC-treated mice. (B) Total numbers of mononuclear cells in MLN of HMPL-004- and MC-treated mice. (C) Total numbers of mononuclear cells in spleens of HMPL-004- and MC-treated mice. All data represent the mean ± SD. n = 5 for MC, n = 5 for HMPL-004. One representative experiment out of four independent experiments is shown. Statistical significance was determined by Student’s t-test. **P < 0.01, ***P < 0.005.
FIGURE 3. HMPL-004 treatment inhibits the expression of proinflammatory mediators but has no effect on antiinflammatory cytokines. (A) TNF-α, IFN-γ, IL-22, IL-1β, and IL-10 mRNA expression was determined in cecum and colon of HMPL-004- and MC-treated mice by real-time PCR. Data were normalized to the expression of β-actin mRNA (n = 5/group). (B) IL-6 serum concentration of HMPL-004- and MC-treated mice at day of sacrifice (n = 15–16/group). (C) TNF-α serum concentration of HMPL-004- and MC-treated mice at day of sacrifice (n = 13–15/group). All data represent the mean ± SD. Statistical significance was determined by Student’s t-test. *P < 0.05, **P < 0.01.
observed significantly reduced cellularity in spleens but not LP of HMPL-004-treated mice (Fig. 5A). The percentage of CD4+ splenocytes was not significantly different between HMPL-004- and MC-treated mice (Fig. 5B). However, we observed significantly reduced percentages of CD4+ IL-17+ T cells in the spleens of HMPL-004-treated mice 1 week after T-cell transfer (Fig. 5C, D).

Next, we analyzed T-cell expansion 2 weeks after T-cell transfer. We observed significantly reduced cellularity in spleens and MLN, but not LP of HMPL-004-treated mice (Fig. 6A). The percentage of CD4+ splenocytes and the total numbers of CD4+ splenocytes were significantly reduced in HMPL-004- compared to MC-treated mice (Fig. 6B, right and left panels). Although, there was not a significant difference we did observe a trend toward reduced percentages and total cell counts of CD4+ LPMC in HMPL-004-treated mice 2 weeks after T-cell transfer (Fig. 6C, right and left panels). One possible explanation might be that at this early timepoint (2 weeks after T-cell transfer) the differences are not apparent yet, whereas at later timepoints there are significant differences in the LP cellularity in HMPL-004- vs. MC-treated mice (Fig. 2; 8 weeks after T-cell transfer). Further analysis of IL-17 and IFN-γ-producing cells revealed a significant reduction of splenic CD4+ IFN-γ+ T cells (Fig. 6D, right panel) and significantly reduced total numbers of splenic CD4+ IL-17+ and CD4+ IFN-γ+ T cells of HMPL-004-treated mice compared to MC-treated mice 2 weeks after T-cell transfer (Fig. 6E, right and left panels). To address the possibility that HMPL-004 inhibits the proliferation of CD4+ T cells in vivo, we assessed the ability of CFSE-labeled CD4+ CD45RBhi naïve T cells to proliferate in Rag1−/− mice treated with either HMPL-004 or MC. We examined the proliferation of CD4+ T cells in the spleens and LPMC and found that there was a significant reduction in percentage of LP cells that underwent multiple cell divisions in HMPL-004-treated mice compared to MC-treated mice on day 4 after T-cell transfer (Fig. 6F). Thus, HMPL-004 inhibits the rate of proliferation of CD4+ T cells and as a result fewer T_{h1} and T_{h17} cells accumulate in tissues especially at earlier timepoints (Figs. 5, 6).

**HMPL-004 Inhibits the Differentiation into and/or Expansion of Effector T Cells In Vivo**

Next, we determined if HMPL-004 inhibits the differentiation of effector T cells in vivo. The percentage of LP CD4+ CD44low CD62Lhi naïve T cells was significantly higher in HMPL-004-treated mice compared to MC-treated mice 8 weeks after T-cell transfer, suggesting that HMPL-004 inhibits the differentiation into effector T cells in the LP (Fig. 7). The reduced numbers of T_{h1}/T_{h17} as well as higher percentages of CD4+ CD62Lhi naïve T cells in HMPL-004-treated mice could be due to an inhibition of proliferation and/or differentiation by HMPL-004.

**HMPL-004 Inhibits the Differentiation and Proliferation of T_{h1} and T_{h17} Cells In Vitro**

Next, we determined if HMPL-004 inhibits the proliferation of CD4+ T cells in vitro. First, CFSE-labeled CD4+CD25− T cells were activated with anti-CD3/CD28 beads in the presence or absence of increasing concentrations of HMPL-004 (1–10 μg/mL). The proliferation in the presence of HMPL-004 was detected by CFSE dilution and analyzed using flow cytometry and was compared to MC-treated cells. HMPL-004 inhibited the proliferation of CD4+ T cells in a dose-dependent manner (Fig. 8A). Ten μg/mL HMPL-004 led to a complete inhibition of proliferation of CD4+ T cells, while MC had no effect on the proliferation of CD4+ T cells. Furthermore, HMPL-004 also inhibited the secretion of IFN-γ from CD4+ T cells in a dose-dependent manner (Fig. 8B). HMPL-004 did not induce T-cell death at...
any of the doses tested (data not shown). Next, we determined if HMPL-004 treatment has an effect on in vitro T-cell differentiation. Naïve CD4+ T cells were isolated, CFSE-labeled, and stimulated under Th0, Th1, and Th17 conditions for 5 days in the presence of HMPL-004 or MC. We observed a dose-dependent inhibition of Th0, Th1, and Th17 proliferation by HMPL-004 (Fig. 9A). Fifteen μg/mL HMPL-004 led to a complete inhibition of proliferation of CD4+ gated Th0, Th1, and Th17 cells while MC had no effect on the proliferation of these cells (Fig. 9A). The inhibition of proliferation was accompanied by inhibition of IFN-γ and IL-17 secretion from Th1 and Th17 cells, respectively (Fig. 9B). Further analysis of the IFN-γ+ and IL-17+ CD4+ T-cell population revealed that HMPL-004 completely blocks the proliferation of these specific subsets (Fig. 9C,D). HMPL-004 did not induce T-cell toxicity at any of the doses tested (data not shown).
HMPL-004 Has No Effect on the Development of Chronic DSS Colitis

To further characterize the protective effect of HMPL-004 on the development of colitis we used a second model of chronic colitis, i.e., the chronic DSS model of colitis. HMPL-004 treatment could not prevent weight loss when mice were gavaged daily starting on day 1 of DSS treatment (Fig. A, Supplemental Digital Content 2, http://links.lww.com/IBD/A3). In contrast to our observation that HMPL-004 prevented the development of T-cell transfer colitis we also did not observe any significant differences in colon length, cellularity in LPMC, MLN, histology score, or cytokine secretion of restimulated LPMC or MLN between HMPL-004- and MC-treated mice (Fig. B-E, Supplemental Digital Content 2, http://links.lww.com/IBD/A3). In addition, pretreatment of mice with HMPL-004 1 week prior to starting DSS colitis did not have any effect on the development of chronic DSS colitis (Fig., Supplemental Digital Content 3, http://links.lww.com/IBD/A4). These data further support that HMPL-004 inhibits T-cell function during T-cell transfer colitis rather than an inhibition of macrophage function during chronic DSS colitis.

DISCUSSION

Our results indicate that HMPL-004, a proprietary extract from the herb *Andrographis paniculata*, prevents early proliferation of CD4+ T cells in vivo and concomitant differentiation into $T_{H17}$ effector T cells leading to reduced intestinal inflammation and expression of proinflammatory cytokines in a murine model of chronic colitis.

It was previously reported that HMPL-004 is effective in patients with mild to moderate UC. A randomized, double-blinded, placebo-controlled, multicenter Phase Ib clinical trial of HMPL-004 demonstrated similar efficacy of HMPL-004 compared to mesalazine for the treatment of UC, with a good safety profile. Further clinical trials are currently under way to evaluate the efficacy of
HMPL-004 in CD. However, precisely how HMPL-004 exerts its antiinflammatory effects in experimental colitis is not known. Using the T-cell transfer model of colitis we demonstrated that HMPL-004 inhibits the early proliferation of CD4\(^+\) T cells and the differentiation into T\(_{H}1\) and T\(_{H}17\) cells. Surprisingly, HMPL-004 treatment was not effective when treatment started 2 weeks after the transfer of T cells into Rag1\(^{-/-}\) mice. These data further support the prominent inhibitory effect of HMPL-004 on the early expansion of CD4\(^+\) T cells in lymphopenic Rag1\(^{-/-}\) recipient mice. An alternative explanation might be that the dosage of HMPL-004 used in the treatment experiments was not high enough to observe an inhibitory effect. Due to volume limitations of oral gavages in mice the maximal dose of HMPL-004 that could be administered was 300 mg/kg/day. We speculate that a higher dosage (greater than 300 mg/kg/day) and/or higher frequency (daily vs. twice/three times per day) might be required to treat murine colitis compared to preventing the development of colitis. Along those lines, we have shown that in vitro different T-cell subsets have a different sensitivity to the treatment with HMPL-004 (Fig. 9A,B). It is plausible that in vivo a lower dosage is required to inhibit the proliferation of CD4\(^+\) T cells in vivo. Percentage of LP CD4\(^+\) CD44\(^{low}\) CD62L\(^{hi}\) naïve T cells from mice treated with HMPL-004 or MC. Means and individual data points are shown (n = 5/group). One representative experiment out of three independent experiments with similar results is shown. Statistical significance was determined by Student’s t-test. ***P < 0.005.

FIGURE 7. HMPL-004 inhibits the differentiation and/or expansion of LP effector T cells in vivo. Percentage of LP CD4\(^+\) CD44\(^{low}\) CD62L\(^{hi}\) naïve T cells from mice treated with HMPL-004 or MC. Means and individual data points are shown (n = 5/group). One representative experiment out of three independent experiments with similar results is shown. Statistical significance was determined by Student’s t-test. ***P < 0.005.

FIGURE 8. HMPL-004 inhibits the proliferation of CD4\(^+\) T cells in vitro. CD4\(^+\) CD25\(^-\) T cells were isolated from the spleen of mice and labeled with CFSE. T cells were stimulated with anti-CD3 and anti-CD28 in the presence of HMPL-004 (1–10 \(\mu\)g/mL) or MC for 4 days. (A) The proliferation of CD4\(^+\) T cells in the presence of HMPL-004 was analyzed using flow cytometry and was compared to MC-treated cells. (B) Secretion of IFN-\(\gamma\) was analyzed by ELISA. One representative experiment out of three independent experiments with similar results is shown.
and differentiation of naïve T cells into effector T cells than to inhibit committed Th1 or Th17 cells. Clinical trials in patients with mild to moderate active UC have shown a clear dose–response curve for HMPL-004. The higher dose of 1800 mg/day lead to a clinical response in 73% of the patients compared to 55% for 1200 mg/day and 44% in the placebo group. However, we do not know how the dosages used in the clinical trials compare to the ones that we used in mice.

Our findings that HMPL-004 inhibits the proliferation of CD4+ T cells and the differentiation into Th1 and Th17 cells are consistent with previous publications demonstrating that androgapholides, the main bioactive components of A. paniculata, inhibit IFN-γ production of murine T cells in vitro and in an in vivo model of multiple sclerosis.

In addition, we confirmed our in vivo finding by in vitro T-cell proliferation/differentiation experiments. HMPL-004 inhibits
the TCR-induced proliferation of CD4⁺ T cells in a dose-dependent manner. Furthermore, HMPL-004 also dose-dependently inhibited the differentiation of naïve CD4⁺ T cells into Th1 and Th17 effector cells. A. paniculata extracts have been shown to induce cell cycle arrest and apoptosis in various types of tumor cells as well as in fibroblast-like cells derived from patients with rheumatoid arthritis. Our data demonstrating inhibition of CD4⁺ T-cell proliferation by HMPL-004 are consistent with these findings; however, we did not observe cytotoxic effects of HMPL-004 on CD4⁺ T cells. Interestingly, we observed different dose–responses for HMPL-004 in different T-cell subsets. While 10 µg/mL HMPL-004 almost completely inhibited the
proliferation of Th0 cells, it has no effect on the proliferation of Th17 cells and partially inhibited Th1 proliferation (Fig. 9A). This effect is corroborated by ELISA data (Fig. 9B). These data suggest that different T-cell populations have a different sensitivity to the effects of HMPL-004. Additionally, we also observed that the induction of IFN-γ is much more sensitive than proliferation of CD4+ T cells (as measured by CFSE dilution) with a clear dose–response for HMPL-004 (Fig. 8B). We observed an ≈ 70% inhibition of IFN-γ with 1 μg/mL HMPL-004, while at the same concentration proliferation is not inhibited. These data suggest that HMPL-004 affects different cellular processes at a different concentration range (proliferation vs. cytokine secretion).

During colitis, several different immune cells have been implicated in the development of chronic colitis in mice and human, including macrophages, DC, T cells, innate lymphoid cells (ILC), and epithelial cells. Surprisingly, HMPL-004 had no inhibitory effect on the development of chronic DSS colitis, a tissue injury model that is characterized by initial macrophage-driven inflammation that develops into a Th1/Th17-driven immune response after repeated administration of DSS. These data suggest that the mechanism of action of HMPL-004 is not mediated via the inhibition of macrophage or DC function, at least in this model of colitis. Alternatively, the dosage of HMPL-004 used in the treatment experiments might have not been high enough to observe an inhibitory
effect. The more mature T cells present in the LP of DSS-treated mice might be less inhibitable than the naïve T cells present in the T-cell transfer model, as discussed above. Previously published data demonstrated an inhibition of DC function (i.e., DC maturation, presentation of antigens to T cells) by andrographolides leading to impaired T-cell proliferation and activation in an in vivo model of EAE. This is in contrast to our findings of a direct effect of HMPL-004 on T-cell activation, proliferation, and/or differentiation during colitis. A possible explanation of this discrepancy lies in dependency of antigen presentation (myelin oligodendrocyte glycoprotein peptide) by DC in the EAE model, while the T-cell transfer model of colitis depends on the early expansion of T cells in lymphopenic mice and their differentiation into effector T cells.

Although extracts from A. paniculata have been used in several models of inflammatory diseases, this is to our knowledge the first time A. paniculata extracts have been shown to be effective in an experimental model of chronic colitis. Andrographolides potently inhibit mortality in endotoxin-induced septic shock and prevent allergic lung inflammation by potently inhibiting the activation of the transcription factor NF-κB. Consistent with this publication, we observed that the expression of several NF-κB responsive genes was significantly reduced in the intestine of HMPL-004-treated mice (TNF-α, IL-1β). Furthermore, HMPL-004 treatment also led to reduced systemic inflammation as determined by reduced IL-6 and TNF-α serum concentrations; both cytokines are secreted in response to NF-κB activation.

In conclusion, our data demonstrate that HMPL-004 efficiently inhibits the development of chronic colitis in vivo by inhibiting the proliferation/differentiation of naïve T cells. Our data suggest that HMPL-004 might be an attractive, alternative therapeutic for the treatment of T-cell-driven pathologies including CD and UC.

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