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Miltefosine suppresses inflammation in a mouse model of inflammatory bowel disease

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Abstract

Background: The repertoire of immunomodulators that can be used for the treatment of inflammatory bowel disease (IBD) is limited. The use of these drugs is further restricted by the occurrence of side effects in a proportion of patients. Miltefosine (hexadecylphosphocholine) is a lipid drug developed in the eighties for treatment of cancer but is nowadays best known for its application in the oral treatment of Leishmaniasis. Although the exact mechanism of action of miltefosine has yet to be elucidated the drug has previously been shown to inhibit phospholipases and protein kinase C, both key components of pro-proliferative signal transduction in T cells.

Methods: Stimulated PBL were treated with miltefosine and proliferation was measured. We use the CD45RBhigh T cell transfer colitis model to investigate the effect of miltefosine treatment on intestinal inflammation. Effects on the severity of colitis were studied by histochemical- and immunohistochemical staining and cytokine levels were determined using a cytokine bead array.

Results: Miltefosine inhibited T cell proliferation *in vitro*. In the transfer model miltefosine significantly ameliorated the severity of colitis as measured by clinical, (immuno)histochemical and biochemical parameters.

Conclusions: Miltefosine inhibits T cell proliferation and effectively reduces inflammation in the T cell transfer model. The drug may therefore be a candidate immunomodulator for IBD.

Introduction

A limited number of drugs is available for the treatment of inflammatory bowel disease. In Crohn's disease for example, glucocorticoids can be used for remission induction but are generally not advised for long term treatment because of their limited value for remission maintenance and important side effects. Thiopurines and methotrexate are widely used but unfortunately only work in a proportion of patients and are sometimes poorly tolerated. If tolerated, the drugs can show important side effects such as bone marrow suppression or pancreatitis. Even if patients are started on anti-TNF antibodies immunomodulators are often used as co-treatment to increase the efficacy of the anti-TNF and prevent the development of anti-TNF neutralizing antibodies. Therefore there is a clear need for the identification of novel candidate immunomodulators for remission induction or maintenance in patients with IBD.

Miltefosine is an ether lipid drug originally designed in the eighties for the treatment of cancer. Systemic treatment with the drug has been tested in phase I[20] and phase II clinical trials in advanced colorectal cancer,[13] soft tissue sarcoma,[19] squamous cell head and neck cancer.[18] In oncology miltefosine only made it a phase III trial that showed effectiveness as a topical palliative treatment for cutaneous metastases of breast cancer.[11] In the mean time however it was found that miltefosine showed activity against *Leishmania* in animal models[10] and clinical trials showed spectacular therapeutic efficacy in patients with visceral leishmaniasis.[8,15] Miltefosine is well absorbed, has a half life of 7 days[5] and is generally well tolerated at a dose of 100-150 mg/day that is used for the treatment of Leishmaniasis. The most important side effect of doses up to 150 mg/day are mild-to-moderate nausea and vomiting which occur mostly in the first two weeks of treatment and are often transient.[8,15] Furthermore, mild and reversible elevations in the aminotransferases and creatinine have been described.[8,15]

The mechanism of action of miltefosine is incompletely understood. Miltefosine is chemically related to membrane phospholipids and generally

believed to act by modulating signaling events at cellular membranes.[2] It has been reported that miltefosine inhibits phospholipase C, phospholipase D and protein kinase C (PKC).[2] Since activation of T cells critically depends on activation of phospholipase C and PKC we hypothesized that treatment with miltefosine might inhibit T cell activation. Indeed, it has previously been reported that miltefosine inhibits T cell proliferation in mixed lymphocyte reactions and showed inhibitory activity in an ovalbumin induced mouse model of delayed type hypersensitivity in mice[3] and in patients with atopic dermatitis.[4]

Here we find that miltefosine inhibits both phytohemagglutanin and staphylococcal enterotoxin B mediated T cell proliferation. Our experiments show that treatment of mice with miltefosine ameliorates severity of a T cell dependent murine model of IBD.

Material and Methods

Reagents and Antibodies

Anti-mouse CD3 (A0452), biotin labeled goat anti-rat and streptavidin-HRP complex were purchase from DAKO (Heverlee, Belgium). An poly HRP labeled anti–rabbit antibody (DPVR110HRP) was purchased from Immunologic (Duiven, The Netherlands). FITC rat anti-mouse Ly-6G (553127), FITC Rat anti-Mouse CD45RB (553099) and PE-Cy™5 rat anti-mouse CD4 (553050) were purchased from BD Pharmingen (Breda, The Netherlands). F4/80 Rat anti-Mouse, clone BM8 (T2006) was purchased from BMA Biomedicals (Augst, Switzerland). Rabbit F(ab')2 Anti-Rat IgG (6130-01) was purchased from Southern Biotech (Birmingham, Alabama, USA). Miltefosine was purchased from Cayman Chemical (Huissen, The Netherlands). Sheep anti-rat IgG Dynabeads were purchased from Invitrogen (Oslo, Norway)

Cells

Human peripheral blood lymphocytes (PBLs) were isolated from whole blood of healthy volunteers by Ficoll-Isopaque density gradient centrifugation. After washing monocytes were separated from lymphocytes by percoll density gradient centrifugation. The lymphocytes were cultured in IMDM (Gibco, Verviers, Belgium) supplemented with 10% heat inactivated fetal calf serum. For proliferation experiments cells were stimulated for 24 hours. Lymphocytes were stimulated with PHA (10µg/ml) or SEB (100 ng/ml). Proliferation was measured using a ^{3}H-thymidine incorporation assay. Mouse splenocytes were isolated from spleen from C57BL/6 mice. Spleens were homogenized and passed through a 70 □m strainer.

Mouse experimental colitis CD45RB^{High} transfer model

C.B-17 SCID mice and wild type BALB/c mice were ordered from Harlan (Boxmeer, The Netherlands). Transfer of CD45RB^{High} CD4^{+} cells was performed as previously described by Read and Powrie.[14] In short; for

every three SCID mice $CD45RB^{High}$ cells were isolated from the spleen of a single wild type BALB/c mouse. A single cell suspension was created by forcing the spleens through a cell strainer. Erythrocytes were removed by adding erythrocyte lysis-buffer followed by negative depletion of macrophages, B cells and $CD8^+$ cells using magnetic beads. The remaining $CD4^+$ cell enriched cell suspension was labelled and $CD45RB^{High}$ and $CD45RB^{Low}$ cells were isolated on a FACS sorter. Colitis was induced by injecting $4 \cdot 10^5$ $CD45RB^{High}$ cells intraperitoneally. Control mice received a combination of $4 \cdot 10^5$ $CD45RB^{High}$ cells and $2 \cdot 10^5$ $CD45RB^{Low}$ cells. The inflammation developed over a period of 8 to 10 weeks. Two groups of ten SCID mice each were injected with $CD4^+CD45RB^{High}$ cells and one group of ten mice was injected with a combination of $CD4^+CD45^{High}$ and $CD4^+CD45RB^{Low}$ cells. Because the first two groups have a reconstituted T cell repertoire lacking regulatory T cells, they will develop colitis. The last group does have regulatory T cells (contained in the $CD4^+CD45RB^{Low}$ compartment), will not develop colitis and acts as a control group.

Miltefosine was dissolved in 10% DMSO in PBS. Each mouse received 50 mg/kg miltefosine twice weekly in 200 µl solution by oral gavage or in the case of the positive control group 200 µl 10% DMSO in PBS.The mice were euthanized by CO_2 inhalation followed by removal of the spleen and colon.

Body weights were recorded three times a week, and wasting disease progression was calculated by percentage of weight loss from initial body weight. Animals were withdrawn from the study when the weight loss was more than 15% compared with the starting weight. Both the colon and the spleen were removed and weighed. Two independent investigators blinded for treatment allocation scored the colons for stool consistency, visible fecal blood, and macroscopic inflammation using a scale of 0–3 per item with a maximum score of 9. Tissue weights were recorded and used as an index of disease related intestinal wall thickening. Colons were subsequently divided longitudinally into two parts: one part was immediately frozen in liquid nitrogen for protein extraction and cytokine determination, while the second part was stored in formalin and embedded in paraffin for (immuno)histological evaluation.

Histological Analysis

H&E sections were blindly scored by an independent experienced GI pathologist (SLM). The histology damage score was calculated as the total score of points that were scored on 6 criteria. (A) Leukocyte infiltration: 0 = normal, 1 = increase in mucosa, 2 = increase in mucosa + submucosa, 3 = extending into tunica muscularis. (B) Loss of goblet cells: 0 = none, 1 = < 10% depletion, 2 = 10-50%, 3 = >50%. (C) Crypt loss: 0 = none, 1 = < 10% loss, 2 = 10-50%, 3 = >50%. (D) Epithelial hyperplasia: 0 = normal, 1 = slight, 2 = 2-3x increased crypt length, 3 = >3x increase. (E) Ulceration: 0 = none, 4 = present. (F) Crypt abcesses: 0 = none, 4 = present. The histological damage score ranged from 0 points to a maximum of 20 points.

Immunohistochemistry

For immunohistochemical staining slides were deparaffinized, dehydrated and immersed in 1.5% H_2O_2 in phosphate-buffered saline (PBS) for 30 min. Different methods of antigen retrieval were used for the different antibodies. For the F4/80 staining slides were cooked for 10 min in 0.1 M sodium citrate (pH 6), for the CD3 staining they were cooked for 10 min in Tris/EDTA buffer (10 mM Tris, 1 mM EDTA (pH 9)). For the Ly6G staining antigen retrieval was performed by incubation of the slide in 0.025% pepsin in 0.1 M HCl at 37 °C for 15 min. Subsequently slide were blocked with Teng-T (10 mM Tris, 5 mM EDTA, 0.15 M NaCl, 0.25% gelatin, 0.05% [vol/vol] Tween-20, pH 8.0) for 30 min, followed by incubation overnight at 4 °C with the primary antibody in PBS with 0.1% Triton X-100 and 1% bovine serum albumin. For detection of F4/80 an avidin–biotin detection system was used. Secondary antibodies were diluted in PBS with 10% human serum and incubated for 1 h at room temperature (RT). Slides were then incubated for 1 h with horseradish peroxidase-conjugated avidin–biotin complex. For detection of CD3 and Ly6G the BrightVision detection system (Immunologic) was used. Peroxidase activity was detected with Sigma Fast 3,3-diaminobenzidine Tablets (Sigma, D4293). Sections were counterstained with hematoxylin, dehydrated and mounted with Pertex

(Histolab 00801). Macrophages, neutrophils and T cells were counted blindly in 10 intercrypt spaces per mouse.

Cytokine Measurements

Human PBL were seeded in a 96 well plate and treated and stimulated for 48 hours. IL-2 cytokine levels were determined by ELISA (Biolegend, San Diego, USA) following manufacturer's instructions.

Cytokine levels in mice were determined in colon mucosa. Colon homogenates were obtained using a Heidolph SilentCrusher M homogenizer at 4°C in 500 µl lysis buffer (Cell Signaling Technology, Beverly, CA) supplemented with protease inhibitors (Roche, Almere, The Netherlands). Samples were centrifuged at 24,000g for 10 minutes at 4°C and stored at -80°C until cytokine measurement. Protein content was determined using the BCA Protein Assay (Thermo Scientific Pierce, Etten-Leur, the Netherlands), and cytokine levels in homogenates were measured using the Cytometric Bead Array System (BD Biosciences, Breda, the Netherlands) following the manufacturer's instructions.

Statistical Analysis

Results are shown as means ± standard error of the mean (SEM) unless otherwise indicated. For statistical analysis, one way analysis of variance (ANOVA) was used followed by a Bonferroni post-test. Results were considered significant when $P < 0.05$.

Results

Miltefosine inhibits T cell proliferation

Previous studies have shown that miltefosine treatment inhibits T cell proliferation in a mixed lymphocyte reaction.[3] We used two stimuli that directly activate the T cell receptor in different ways to confirm this observation. The concentration miltefosine used is within physiologically relevant range.[5] Phytohemagglutinin (PHA) binds CD3 directly[17] and nonspecifically. This results in the potential activation of all T cells. Staphylococcal Enterotoxin B (SEB) is a superantigen that activates a large proportion of the T cell population by binding directly to the Vß chain of the T cell receptor, activating over 20% of the total T cell repertoire.[6] We chose SEB in addition to PHA as it has previously been described that SEB induces steroid resistance in T cells.[7] T cells stimulated with PHA were highly sensitive to dexamethasone whereas SEB induced a state of partial steroid resistance as described (Fig. 1A). In contrast, T cell proliferation induced with PHA and SEB was equally sensitive to miltefosine (Fig. 1B). We subsequently isolated mouse splenocytes from C57BL/6 mice and stimulated these with both PHA and SEB. The sensitivity of mouse splenocytes to both PHA and SEB is less than that of human cells (Fig. 1C) but as in the human cells PHA and SEB showed a similar sensitivity to miltefosine treatment. And finally we measured IL-2 cytokines levels in the medium of PHA or SEB stimulated human PBL treated with different doses of miltefosine (Fig. 1D). IL-2 secretion by both PHA and SEB stimulated PBL showed sensitivity to miltefosine treatment. Stimulation of human PBL with PHA results in very low levels of IL-2 secretion[9].

These results confirm the previous observation that miltefosine inhibits proliferation of T cells and suggests that miltefosine may suppress proliferation of T cells in conditions that cause steroid resistance.

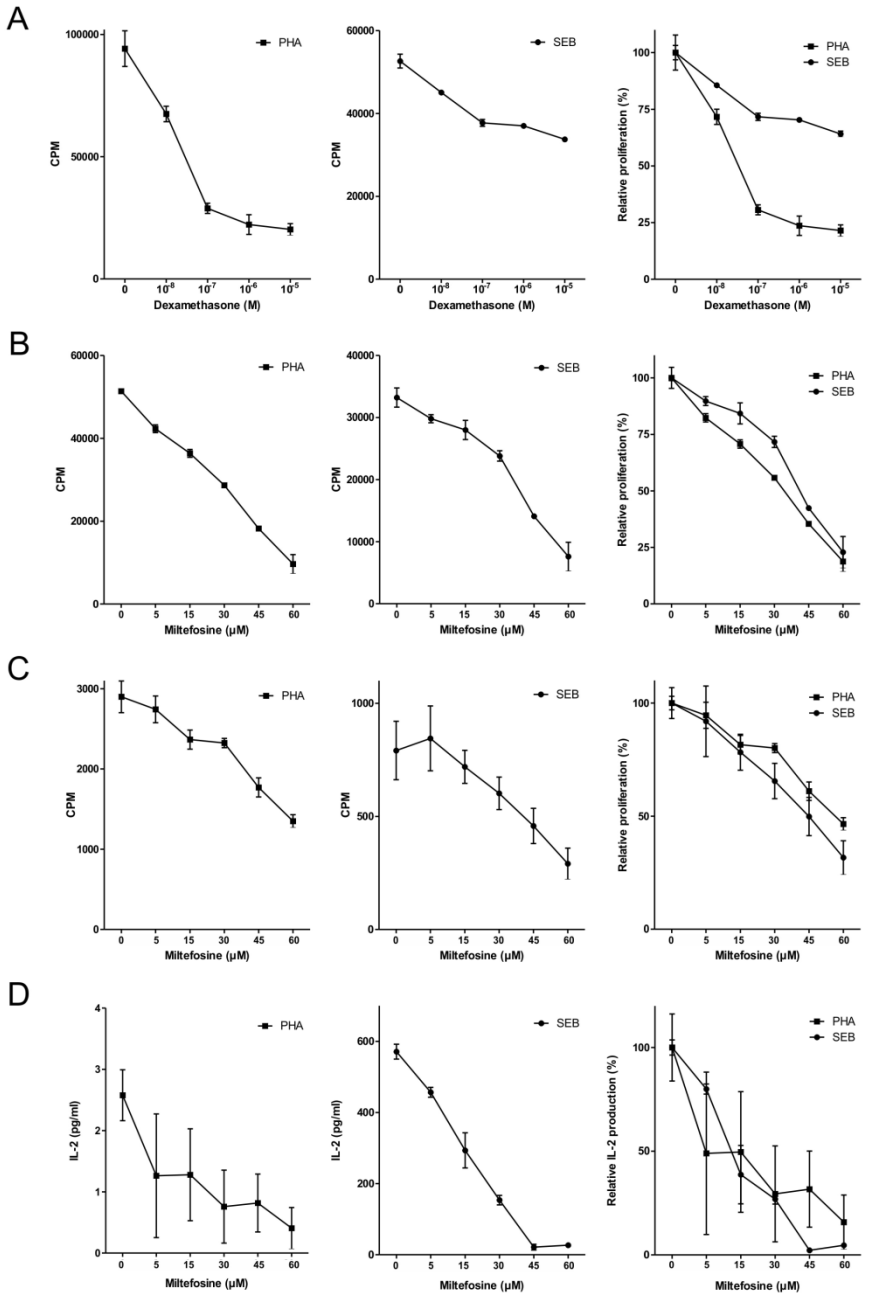

FIGURE 1. Miltefosine inhibits T cell proliferation. (A) Human PBL were stimulated with either PHA (10 μg/ml) or SEB (100 ng/ml) for 24 hours in the presence of

different doses of dexamethasone. Data shown are absolute and relative proliferation as measured by ^{3}H incorporation. PBL treated with SEB show partial steroid insensitivity as previously described. (B) Human PBL were stimulated with either PHA (10 µg/ml) or SEB (100 ng/ml) for 24 hours in the presence of different doses of miltefosine. Data shown are absolute and relative proliferation as measured by ^{3}H incorporation. PBL treated with SEB show no insensitivity to miltefosine. (C) Mouse splenocytes were stimulated with either PHA (10 µg/ml) or SEB (100 ng/ml) for 24 hours in the presence of different doses of miltefosine. Data shown are absolute and relative proliferation as measured by ^{3}H incorporation. Mouse splenocytes show sensitivity to miltefosine that is similar to humans. Each data point represents the mean and SEM of four independent samples. (D) Human PBL IL-2 cytokine levels as measured by ELISA. Human PBL were stimulated with either PHA (10 µg/ml) or SEB (100 ng/ml) for 48 hours in the presence of different doses of miltefosine. Both the production of IL-2 by PHA and by SEB stimulated PBL are sensitive to miltefosine. Each data point represents the mean and SEM of four independent samples.

Miltefosine suppresses inflammation in the CD45RB^{High} transfer model

To investigate if miltefosine might be a candidate immunomodulator for IBD we used the experimental transfer colitis model. In this model SCID mice are reconstituted with $CD4^{+}CD45RB^{High}$ cells. This population lacks the subset of T cells present in the $CD4CD45RB^{Low}$ population that are required to suppress the development of colitis.[1] We treated mice with a twice weekly dose of 50 mg/kg miltefosine by oral gavage. To allow the transferred T cells to home to the intestine and repopulate the mice, we started treatment with miltefosine at two weeks after adoptive transfer. Oral gavage with vehicle was used as a placebo treatment. In the course of two months placebo treated $CD45RB^{high}$ transplanted SCID mice developed severe inflammation of the gut. Two mice died before the end of the experiment. One mouse in the miltefosine treated group died due to a complication of the oral gavage. The other mouse was in the group of placebo mice and was taken out before the end of the experiment due to weight loss > 15%. At completion of the experiment at day 60, mice treated with miltefosine had lost significantly less weight than placebo treated mice (Fig. 2A) suggesting a reduction in the severity of disease. Indeed, inspection of the colon showed that the colon of placebo treated animals

was severely affected whereas colons of miltefosine treated animals showed remarkably less edema and contained normal stool pellets (Fig. 2B). Treatment with miltefosine significantly reduced the disease activity index to levels comparable to controls (Fig. 2C). Colon weight was substantially increased in CD45RB^{high} reconstituted placebo treated mice compared to control mice. Colon weight of miltefosine treated mice was substantially reduced compared to placebo treated animals and not significantly different from control mice that were reconstituted with both CD45RB^{high} and CD45RB^{low} cells (Fig. 2D). A similar observation was made for spleen weight which was reduced to levels controls by treatment with miltefosine (Fig. 2E).

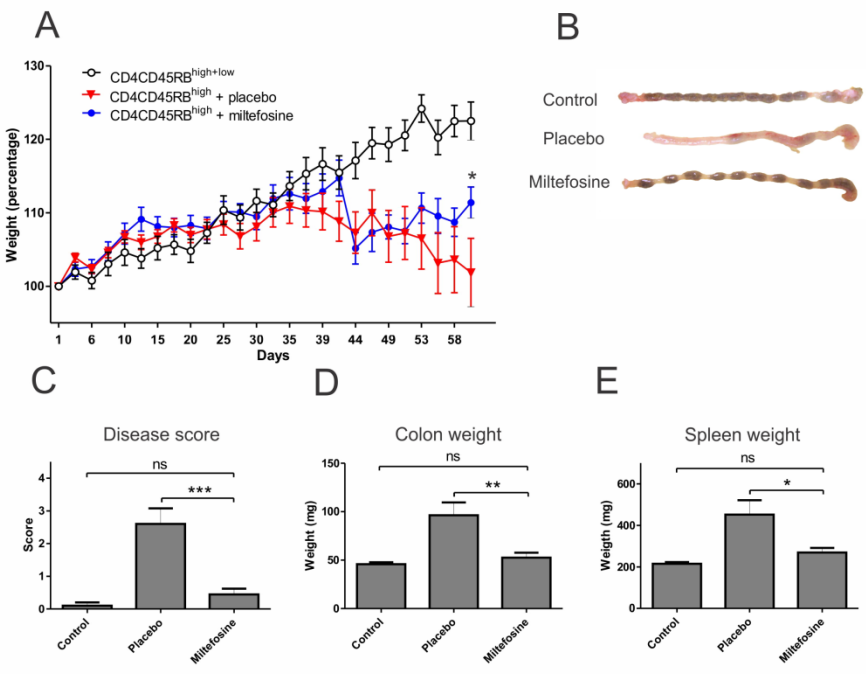

FIGURE 2. Miltefosine treatment reduces inflammation in CD4CD45RB^{High} transfer model. (A) Average weight of each group of mice as a percentage of the starting weight during the course of the experiment. The control group (black open circle) received both CD4CD45RB^{High} and CD4CD45RB^{Low} and did not develop

inflammation. Both the placebo treatment group (red inverted triangle) and the miltefosine treated (50 mg/kg twice weekly) group (blue closed circle) received CD4CD45RB^{High} only. (B) Representative examples of colons from mice in the indicated groups. The colon in the colitis group is clearly inflamed and void of stool while the colon of the treated group shows no signs of inflammation and is filled with solid pellets, comparable to the healthy control. (C) Disease score for different groups. (D) Colon weight. (E) Spleen weight. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$. Control = CD4CD45RB$^{high+low}$, placebo = CD4CD45RB^{high} + placebo, miltefosine = CD4CD45RB^{high} + miltefosine.

To confirm the gross morphological examination at the histopathological level, coded H&E stained slides where examined by a blinded experienced GI pathologist (SLM) and the degree of inflammation was scored. Mice in the placebo treated group showed clear infiltrates of leukocytes, loss of goblet cells, crypt damage, epithelial hyperplasia, ulcerations and crypt abcesses (Fig. 3A-C). In contrast, in the mucosa of mice that were treated with miltefosine, all aspects of inflammation were significantly reduced compared to placebo treated mice (Fig. 3A-C). None of the histopathological hallmarks of colitis in the miltefosine treated animals was significantly different from control animals without colitis (Fig. 3A).

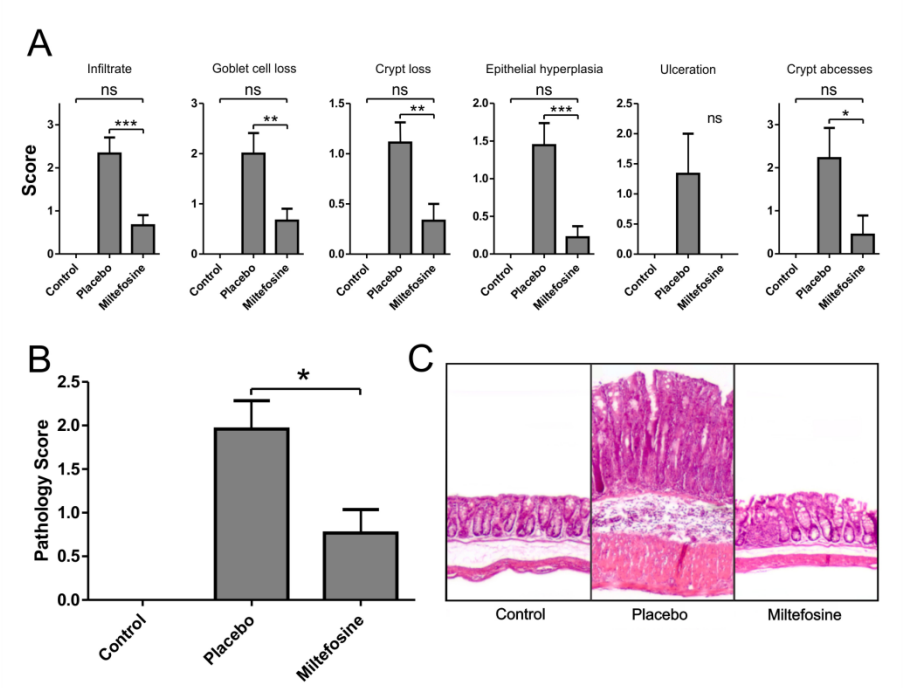

FIGURE 3. Miltefosine treatment improves the histopathological score of colitis. (A) Individual components of the histopathological colitis score. (B) Total score. (C) Representative images of H&E stained slides of colons from mice in the indicated groups. Control = $CD4CD45RB^{high+low}$, placebo = $CD4CD45RB^{high}$ + placebo, miltefosine = $CD4CD45RB^{high}$ + miltefosine. Original magnification in C: 100x. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

We subsequently analyzed the recruitment of individual leukocyte subsets to the mucosa (Fig. 4). We used F4/80 as a marker of macrophages, Ly6G for neutrophils and CD3 for T cells. The number of infiltrating cells of each type was counted per intercrypt space. Per mouse 9 intercrypt spaces were counted in 3 high power field images (3 adjacent inter crypt spaces per image). In placebo treated animals with colitis, the number of infiltrating macrophages, neutrophils and T cells was substantially increased compared to controls. Treatment with miltefosine significantly reduced influx of all three cell types. Both macrophage and neutrophil infiltration were not significantly different from unaffected control animals (Fig. 4A-C).

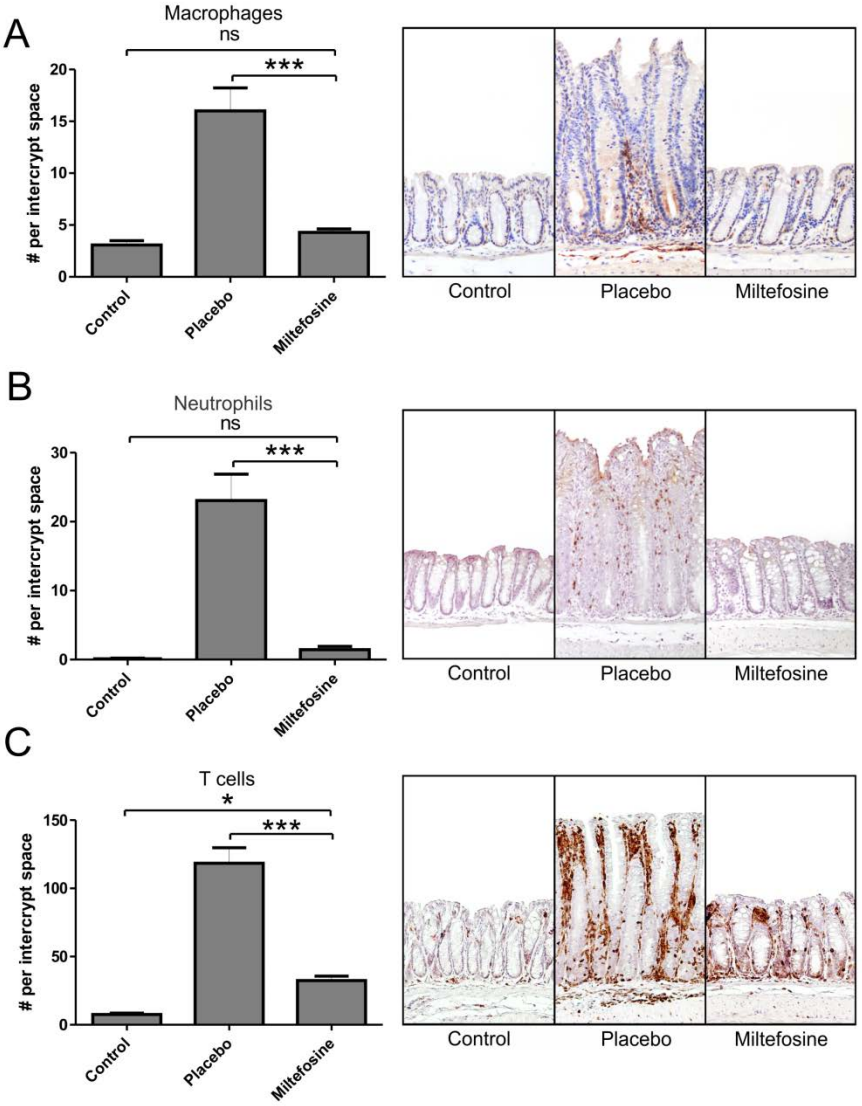

FIGURE 4. Reduced influx of different leukocyte subsets in miltefosine treated animals. (A) Quantification and representative images of the presence of macrophages as determined by immunohistochemistry for F4/80. (B) Quantification and representative images of the presence of neutrophils as determined by immunohistochemistry for Ly6G. (C) Quantification and representative images of the presence of T cells as determined by

immunohistochemistry for CD3. Control = $CD4CD45RB^{high+low}$, placebo = $CD4CD45RB^{high}$ + placebo, miltefosine = $CD4CD45RB^{high}$ + miltefosine. Original magnifications 100x. * = $P < 0.05$, *** = $P < 0.001$.

To investigate the immunomodulatory effect of miltefosine treatment on cytokine production, cytokine levels were determined in whole colon lysates (Fig. 5). In the colitis group the inflammation caused a significant increase in the levels of the pro-inflammatory cytokines IL-6, TNFα, INFγ and IL-17. The levels of the Th2 related cytokine IL-4 and of the anti-inflammatory cytokine IL-10 remained unchanged. In mice treated with miltefosine the increase of pro-inflammatory cytokines IL-6, TNFα, INFγ and IL-17 was significantly reduced.

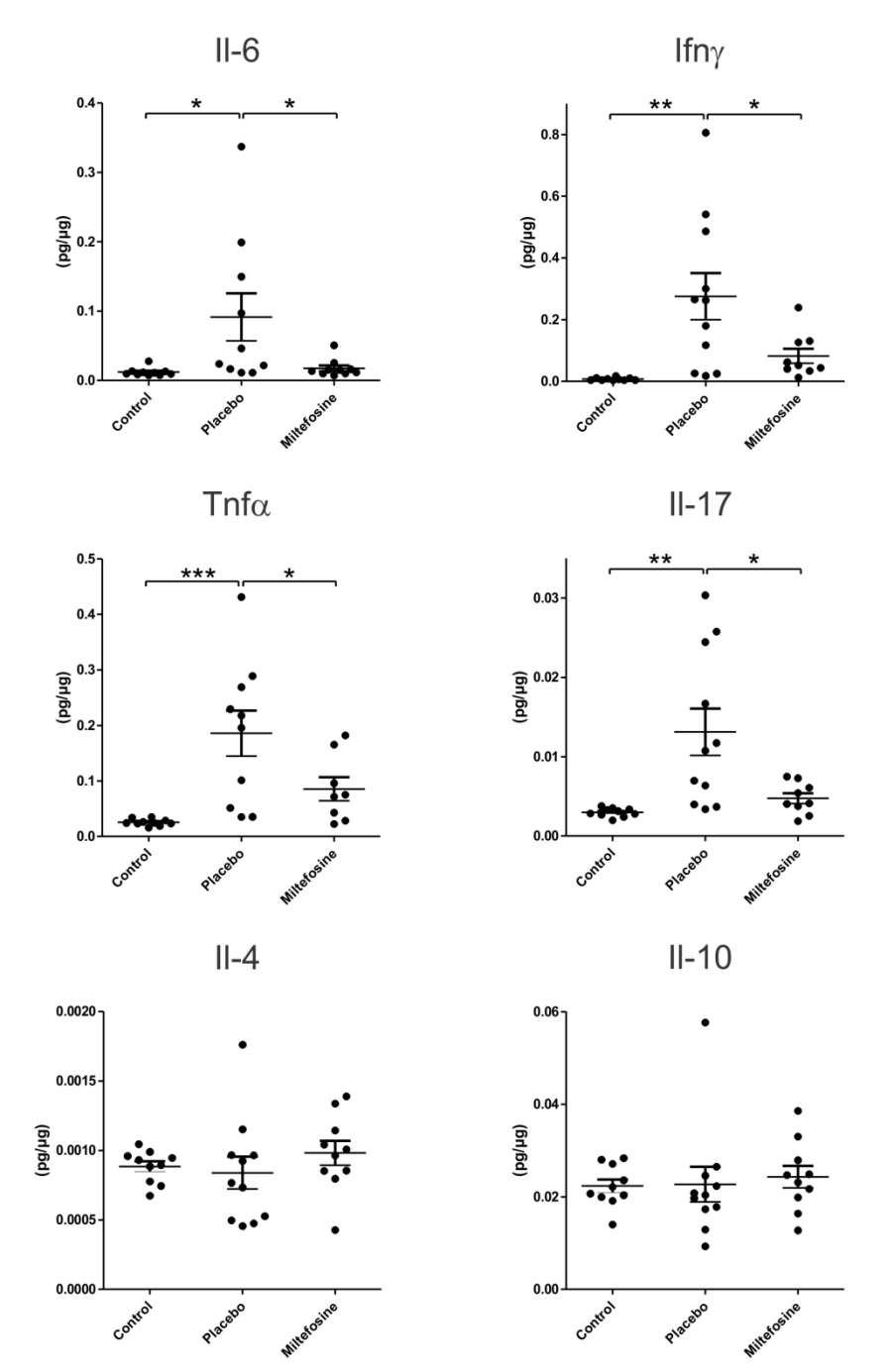
Il-6
Ifnγ
Tnfα
Il-17
Il-4
Il-10
(pg/µg)
Control
Placebo
Miltefosine
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FIGURE 5. Miltefosine treatment reduces the production of pro-inflammatory cytokines. Measurement of mucosal levels of different cytokines in colonic homogenates by cytokine bead array. Control = CD4CD45RB$^{high+low}$, placebo = CD4CD45RB^{high} + placebo, miltefosine = CD4CD45RB^{high} + miltefosine. ns = not significant, * = $P < 0.05$.

Discussion

Miltefosine was initially developed as an anti-cancer drug but has largely failed clinical development for oncology. After preclinical experiments in mice[10] it was found that miltefosine has a surprising efficacy for the treatment of Leishmaniasis.[8,15] Since it was previously found that miltefosine may suppress T cell activation,[3,4] we investigated miltefosine as a potential immunomodulator in a mouse model of colitis. We found that miltefosine efficiently inhibited PHA mediated T cell proliferation at physiologically attainable concentrations.[5] In addition, miltefosine fully inhibited SEB induced T cell proliferation which is known to be only partially sensitive to dexamethasone. To test the feasibility of miltefosine treatment as a way to suppress intestinal inflammation we used the experimental CD4CD45RB^{High} transfer colitis mouse model. We chose this model as it is characterized by an expression profile of proinflammatory molecules that is relatively similar to human IBD, especially compared to the chemical colitis models.[16] Treatment with miltefosine was started two weeks after the T cell transfer as in previous studies using this model the mice started losing weight after two weeks.[12] The mice were dosed 50 mg/kg twice per week as miltefosine has a long half life. Treatment with miltefosine resulted in a remarkable reduction of all aspects of the severity of colitis that we examined. Most of the hall marks of colitis that we measured in this model were no longer significantly different between CD45RBhigh transplanted colitic mice treated with miltefosine and control mice that received both CD45RB^{high} and CD45RB^{low} cells.

As miltefosine is already marketed for use in Leishmaniasis under the brand name Impavido (by Zentaris) it could be an attractive immunomodulator to examine for use in humans with IBD. Miltefosine has an excellent oral availability and the pharmacokinetics have been well described.[5] Clinical

trials in humans have shown an acceptable safety profile with reversible abnormalities in liver biochemistry and renal function.[8,15] The major side effects of miltefosine are nausea and vomiting which could be problematic in patients with IBD. However these side effects are often transient.[8,15] The safety and side effects of long term miltefosine use that would be required for maintenance therapy in patients with IBD have not been established.

In conclusion, we find that miltefosine inhibits T cell proliferation at physiologically relevant concentrations *in vitro*. Miltefosine strongly reduced severity of colitis in the murine transfer model of colitis. Our data suggest that miltefosine is an interesting candidate anti-inflammatory drug for patients with IBD.

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