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**The anti-parasitic drug miltefosine suppresses human eosinophil activation
and ameliorates murine allergic inflammation in vivo**

Short title: Miltefosine for the treatment of eosinophilic allergic diseases

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Abstract

Background and purpose: Miltefosine is an alkylphosphocholine drug with proven effectiveness against various types of parasites and cancer cells. Miltefosine is not only able to induce direct parasite killing but also modulates host immunity, for example by reducing the severity of allergies in patients. To date, there are no reports on the effect of miltefosine on eosinophils, central effector cells involved in allergic inflammation.

Experimental approach: We tested the effect of miltefosine on the activation of human eosinophils and their effector responses *in vitro* and in mouse models of eosinophilic migration and ovalbumin-induced allergic lung inflammation.

Key results: The addition of miltefosine suppressed several eosinophilic effector reactions such as CD11b up-regulation, degranulation, chemotaxis and downstream signaling. Miltefosine significantly reduced the infiltration of immune cells into the respiratory tract of mice in an allergic cell recruitment model. Finally, in a model of allergic inflammation, treatment with miltefosine resulted in an improvement of lung function parameters.

Conclusion and implications: Our observations suggest a strong modulatory activity of miltefosine in the regulation of eosinophilic inflammation *in vitro* and *in vivo*. Our data underline the potential efficacy of miltefosine in the treatment of allergic diseases and other eosinophil associated disorders and may raise important questions regarding the immunomodulatory effect of miltefosine in patients treated for Leishmania infections.

Key words: allergic inflammation, eosinophils, miltefosine,

Abbreviations: BAL (bronchoalveolar lavage), C5a (complement component 5a), fMLP (N-Formylmethionine-leucyl-phenylalanine), FSC (forward scatter), LPC (lysophosphatidylcholine), PI (propidium iodide), PI3K (phosphatidylinositol 3-kinase), PMNL (polymorphonuclear leukocytes)

Bullet point summary

What is already known

- Miltefosine is an orphan drug marketed for the treatment of leishmaniasis
- Miltefosine reduces the severity of allergies in patients

What this study adds

- Miltefosine inhibits activation of human eosinophils and suppresses human eosinophil effector responses
- Miltefosine inhibits the infiltration of immune cells in the airways and improves lung function of animals

Clinical significance

- Miltefosine may serve as a potential candidate for the treatment of eosinophil-related diseases
- Miltefosine treatment may influence eosinophil-host responses in leishmania infected patients

1. Introduction

To date miltefosine (Impavido®) is the only oral drug approved for the treatment of leishmaniasis with limited mild or moderate side effects (Pijpers et al., 2019). The development of miltefosine is a success story of public - private partnership and a breakthrough in medicine affordability and patient drug adherence, landing it on the WHO's List of Essential Medicines (Berger et al., 2017; Sunyoto et al., 2018). Miltefosine disrupts membrane structures and affects phosphatidylcholine synthesis in susceptible promastigote cells (Rakotomanga et al., 2007; Pinto-Martinez et al., 2018). Due to its detergent-like properties miltefosine is thought to interact with the mucosa of the gastrointestinal tract during oral use and cause its most commonly listed side effects – nausea, vomiting and diarrhoea (Bhattacharya et al., 2007). During prolonged treatment the severity of the side effects was reported to decrease over time (8.2% during week 1 to 3.2% during week 4) (Bhattacharya et al., 2007).

Miltefosine exerts immunomodulatory effects on human cancer cells by inhibiting the phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathway (Ruiter et al., 2003), induces interleukin (IL)-12-dependent Th1 responses (Wadhone et al., 2009) and shows anti-inflammatory effects in endothelial cells, suppressing vascular inflammation (Fang et al., 2019). However, the immunomodulatory effects of miltefosine on primary human cells have so far only been described for T cells (Bäumer et al., 2010) and mast cells (Weller et al., 2009).

In plasma, miltefosine is mainly bound to albumin (96-98%) (Kip et al., 2018) and accumulates predominantly in cholesterol-rich microdomains of the cell membranes (lipid rafts) (Malta de Sá et al., 2015). Miltefosine increases membrane fluidity (Moreira et al., 2014) and modulates lipid raft dependent signalling (Weller et al., 2009) and

could therefore be an attractive drug candidate for the treatment of diseases characterized by abundant lipid-raft activation, such as allergic diseases (Dölle et al., 2010). Miltefosine attenuates allergic inflammation in T-cell dependent mouse models of dermal inflammation, (Bäumer et al., 2010) improves local dermatitis in patients with atopic dermatitis (Dölle et al., 2010), inhibits activation and degranulation of mast cells and significantly reduces allergic disease manifestation in patients (Magerl et al., 2013; Maurer et al., 2013; Rubíková et al., 2018).

Surprisingly, there are no reports on the effects of miltefosine on eosinophils, a key cell type involved in the initiation and propagation of immune responses in allergic diseases (Stone et al., 2010). Here we studied in detail whether miltefosine exerts immunomodulatory effects on eosinophils *in vitro* and in mouse models of allergic lung inflammation.

2. Materials and methods

Materials

Unless otherwise indicated all purchased reagents were from Sigma (Vienna, Austria). Eotaxin-2 (CCL24) used for *in vivo* chemotaxis and recombinant human C5a were acquired from R&D systems (Minneapolis, MN, USA). Eotaxin-1 (CCL11) and eotaxin-2 (CCL24) used *in vitro* assays were obtained from Immunotools (Friesoythe, Germany). Antibody against phospho-Akt (Ser 473) (Cat#9271, RRID:AB_329825) was obtained from Cell Signaling Technology (Danvers, Massachusetts, USA), while secondary goat anti-rabbit Alexa fluoro-488 IgG antibody (Cat# A-11008, RRID:AB_143165) was from Life Technologies (ThermoFisher scientific, Waltham, MA, USA). Annexin V, Propidium Iodide (Cat# 556547), CD63-FITC (Cat# 561924, RRID:AB_10894192), Siglec-F-PE (Cat# 552126, RRID:AB_394341), CD3-PE Cy5

(Cat# 553065, RRID:AB_394598), CD11b-PE-Cy7 (Cat# 552850, RRID:AB_394491) and CD11c-BV421 (Cat# 562782, RRID:AB_2737789) were from BD Bioscience (Vienna, Austria). TruStain fcX CD16/32 (Cat# 101320, RRID:AB_1574975), Ly-6C-FITC (Cat# 128005, RRID:AB_1186134), Ly-6G-APC (Cat# 127613, RRID:AB_1877163), CCR3-BV421 (Cat#144517, RRID:AB_2565743) and I-A/I-E-V510 (Cat# 107635, RRID:AB_2561397) were from Biolegend (San Diego, CA, USA). Aluminum hydroxide gel used as an adjuvant was acquired from InvivoGen (Toulouse, France). CD11b-FITC mouse anti-human antibody (Cat# IMO53OU) used for measuring CD11b upregulation was obtained from Beckman Coulter (Krefeld, Germany). Miltefosine used in *in vivo* assays was purchased from Cayman Chemical (Ann Arbor, MI, USA). All functional assays of eosinophils were performed in assay buffer (PBS with Ca²⁺ and Mg²⁺, HEPES 10 mmol/L, glucose 10 mmol/L, bovine serum albumin 0.1%, pH 7.4).

Fixative solution was prepared by adding 9 ml of distilled water and 30 ml of FACS Sheath fluid (BD Biosciences) to 1 ml of CellFix (BD, Vienna, Austria) as described previously (Knuplez et al., 2020a).

Mice

All animal experiments were performed in the animal facilities of the Medical University of Graz. The experimental procedure used in this study was approved by the Austrian Federal Ministry of Science, Research and Economy (protocol number: BMWFW-66.010/0207-WF/V/3b/2017); it conforms to Directive 2010/63/EU, and was performed in accordance with national and international guidelines. BALB/c mice (RRID:IMSR_CRL:028) were either bred in house or obtained from Charles River. Δdbl GATA-1 and interleukin-5 transgenic (IL-5Tg) mice (both BALB/c background)

were initially obtained from Dr. Helene Rosenberg (NIH, Bethesda, MD, USA) and bred in our facilities. IL-5Tg mice were originally generated by Lindsay A. Dent. A 10 kb genomic mouse Il5 sequences under the control of the dominant control region (DCR) from human CD2 was used for the transgene. Δ dbl GATA mice were originally generated by C. Yu. A high-affinity, palindromic "double" GATA protein binding site in the Gata1 promoter presumed to mediate positive Gata1 autoregulation was replaced by a floxed Pgk-neo cassette; transient Cre recombinase expression in ES cells left a single loxP site flanked by two Not1 sites. The 21-bp deleted segment comprised nucleotides -691 through -671 upstream of the last nucleotide of the first haematopoietically expressed exon of Gata1. Mice were housed in plastic sawdust floor cages at constant temperature (22°C) and a 12:12 h light–dark cycle with free access to standard laboratory chow and water. 8 - 12-week-old male and female mice were included in experiments, where there were randomly divided in three groups (negative control – vehicle; positive control – ovalbumin or eotaxin stimulated and miltefosine pretreated and ovalbumin or eotaxin stimulated group). Experiments, where BAL fluid was collected could not be performed blinded, due to investigator treating the mice prior to fluid collection. Lung function testing was performed blinded, since investigator 1 treated the mice and investigator 2 independently performed lung function testing on mice in a random order. For all animal experiments, at least five mice were included in each group, and at least two repeat experiments were carried out. Experiments were designed to make sample sizes relatively equal and randomized among comparison groups. Sample sizes were determined according to previous studies with similar analyses (Theiler et al., 2019; Knuplez et al., 2020a).

Blood sampling and eosinophil isolation

Blood sampling from healthy volunteers was approved by the Institutional Review Board of the Medical University of Graz (17-291 ex 05/06). All participants signed a written informed consent.

PMNL (polymorphonuclear leukocytes) preparations were purified from citrated whole blood as previously described (Curcic et al., 2015). Firstly platelet-rich plasma was removed by centrifugation. Next, red blood cells and platelets were removed by dextran sedimentation and PMNL preparations were obtained by density gradient separation. Eosinophils were isolated from PMNL by negative magnetic selection using a cocktail of biotin-conjugated antibodies against CD2, CD14, CD16, CD19, CD56, CD123, and CD235a (Glycophorin A) as well as anti-Biotin Microbeads from Miltenyi Biotec (Bergisch Gladbach, Germany). Eosinophil purity was determined by morphological analysis of Kimura-stained cells and was typically greater than 97%.

Eosinophil shape change

Eosinophil shape change was determined as described previously (Luschnig-Schratl et al., 2011). Approximately 5×10^4 eosinophils per sample were suspended in assay buffer with Ca^{2+} and Mg^{2+} , preincubated with miltefosine in different concentrations (15 min, RT) and then stimulated in water bath (4 min, 37 °C) with eotaxin-1 (CCL11). Afterwards cells were transferred to ice and ice-cold fixative solution was added to terminate the reaction and maintain the change in cell shape until analysis. The samples were analyzed on a FACS Canto II flow cytometer (Becton-Dickinson, Mountain View, CA), where shape change was determined as the increase in the forward scatter property of the cell and was normalized to unstimulated vehicle control.

CD11b upregulation

CD11b upregulation on eosinophils was determined as described in detail elsewhere (Knuplez et al., 2020a). Briefly, eosinophils were stained with anti-CD11b–FITC, pretreated either with vehicle or miltefosine (20 μ M) and stimulated with eotaxin-2 (CCL24) as indicated in the figure legend. Additionally, CD11b upregulation assay was performed in the PMNL fraction (see supplementary information), where cells were pre-stained with anti-CD16-PE to distinguish between eosinophil and neutrophil PMNL fractions. CD11b upregulation on neutrophils (CD16+ cells) was induced with N-Formylmethionyl-leucyl-phenylalanine (fMLP). Eosinophil or neutrophil CD11b expression was determined by flow cytometry as the geometric mean of the fluorescence in the fluorescein isothiocyanate (FITC) channel and expressed as percent of unstimulated vehicle response.

CD63 expression

Eosinophils were stained with anti-CD63-FITC (1:100) and preincubated with vehicle or miltefosine (20 μ M) (15 min, RT). Subsequently cells were primed with cytochalasin B (5 μ g/ml) and stimulated with C5a in two different concentrations (Schratl et al., 2006). Degranulation was analyzed by flow cytometry and expressed as fold increase in fluorescence over indicated vehicle mean.

Preparation of bone marrow derived eosinophils (BMDEs)

Mouse eosinophils were derived from bone marrow of BALB/c mice as described before (Dyer et al., 2008; Kienzl et al., 2020; Knuplez et al., 2020b). Briefly, following the lysis of erythrocytes in bone marrow the cells were cultured in RPMI + 20% HyClone FBS (GE Healthcare; # 10309433), 1% P/S, 25 mM HEPES (Thermo Fisher; # 15630–080), 1 x non-essential amino acids (Thermo Fisher; # 11140–035), 1 mM

sodium pyruvate (Thermo Fisher; # 11360–039) and 50 μ M beta-mercaptoethanol (Sigma-Aldrich; M3148) supplemented with 100 ng/ml stem cell factor (PreproTech; # 250–03) and 100 ng/ml FLT3L (PreproTech; # 250–31 L). On day four, medium was changed to media supplemented with 10 ng/ml IL-5 (Bio-Techne; # 405-MI-005) only, to differentiate progenitors into eosinophils. On day 14 purity and viability of BMDEs was assessed by flow cytometry staining for mouse eosinophil markers CCR3 (CCR3-BV421) and Siglec-F (Siglec-F-PE), and PI respectively. Cytospins of BMDEs were prepared, stained with a Hemacolor Rapid staining of blood smear and imaged on an Olympus BX41 microscope (Olympus, Vienna, Austria). Day 14 BMDEs were used for further *in vitro* analyses.

Calcium flux

Isolated human or differentiated mouse eosinophils were loaded with 2 μ M of Fluo-3 AM in the presence of 0.02 % pluronic F-127 for 1 hour at room temperature in the dark. Individual samples were treated as indicated in the figure legend. Changes in $[Ca^{2+}]_i$ were detected as fluorescence in the FL1 channel by a FACSCalibur flow cytometer (BD, Mountain View, CA, USA), as described previously (Heinemann et al., 2003; Knuplez et al., 2020a)

In vitro chemotaxis

Purified eosinophils were pretreated either with vehicle or miltefosine in different concentrations (15 min, RT) and were allowed to migrate to 10 nM eotaxin-2 (CCL24) in a HTS Transwell 96 well plate with a 5 μ m pore size polycarbonate membrane (1 h, 37 °C). Eosinophils that have migrated to the lower compartment were enumerated for 1 min by flow cytometric counting on a FACS Canto II (BD Mountain View, CA, USA). (Knuplez et al., 2020a)

Flow cytometric analysis of intracellular kinase phosphorylation

Isolated eosinophils were pretreated either with vehicle or miltefosine 20 (μ M) (15 min, RT). Following the pretreatment, cells were incubated with 10 nM eotaxin-1 (CCL11) (3 min, 37 °C). Subsequently cells were fixed, permeabilized and stained as described previously (Knuplez et al., 2020a). Phosphorylation of Akt residues in fixed eosinophils were quantified as the increase of fluorescence in the FITC fluorescence channel from unstimulated control.

Apoptosis

Eosinophil survival after preincubation with vehicle, positive control formaldehyde (3.8 %), or miltefosine (5-40 μ M) for different time points at 37 °C was assessed by annexin V/propidium iodide staining, as described previously (Heinemann et al., 2005; Knuplez et al., 2020a)

***In vivo* chemotaxis**

In vivo eosinophil migration was induced by intranasal application of 4 μ g eotaxin-2 CCL24 in eight-week-old male and female heterozygous interleukin- 5 transgenic (IL-5Tg) mice (BALB/c background). The mice and their littermate controls received oral gavages of miltefosine (20 mg/kg in 0.9 % NaCl) or vehicle for three consecutive days before CCL24 application. Bronchoalveolar lavage (BAL) fluid was collected 4 hours after experiment had started. Migration of eosinophils was evaluated by flow cytometric counting of highly granular (high side scatter) CD11c⁻/Siglec F⁺ cells, as described previously (Knuplez et al., 2020a). The gating strategy for evaluation of other immune cells was previously published (Knuplez et al., 2020a) and was as following: alveolar macrophages (Siglec F⁺/CD11c⁺), neutrophils (Siglec F⁻/CD11b⁺/Ly6G⁺), B cells (CD11c⁻/CD11b⁻/MHCII⁺), T cells (CD11c⁻/CD11b⁻/CD3⁺),

dendritic cells (Siglec F⁺/Ly6G⁻/Ly6C⁻/MHCII⁺) and inflammatory monocytes (Siglec F⁺/Ly6G⁻/MHCII⁻/LY6C⁺⁺).

Mouse model of allergic lung inflammation

Eight-week-old male and female BALB/c or eosinophil-deficient (Δ dbl GATA-1) mice were immunized by intraperitoneal injections of 20 μ g of OVA adsorbed to Al(OH)₃ on days 0 and 7. Mice were challenged by an aerosol of OVA (1 mg/ml in 0.9 % NaCl) on days 14 and 16. During the last 10 days of the model mice received daily oral gavages of miltefosine (20 mg/kg in 0.9 % NaCl) or vehicle. On day 17, either airway hyperresponsiveness to methacholine was recorded with the FlexiVent system (Scireq, Montreal, QC, Canada) or BAL fluid was taken and analyzed by flow cytometry. BAL fluid supernatants were collected and stored at -70 °C for further cytokine assessment. All animal subjects were randomized prior to inclusion in the experiments.

Cytokine measurements in BAL fluid

Cytokine concentrations in stored BAL fluid supernatants from BALB/c and Δ dbl GATA-1 mice subjected to OVA/Alum were evaluated using the custom ProcartaPlex™ immunoassay (eBioscience, San Diego, CA, United States) according to the manufacturer's specifications. Fluorescent signals were quantified with the Bio-Plex 200 multiplex suspension array system equipped with Luminex® xMAP® technology combined with the Bio-Plex 5.0 software (BioRad, Hercules, CA, United States). All cytokine concentrations were evaluated in duplicates.

Corticosterone measurement in plasma

Corticosterone levels were assessed in plasma of BALB/c mice treated with oral gavages of miltefosine (20 mg/kg) once daily for 3 days. A blood sample was collected

via cheek bleed 5 hours after first miltefosine application on day 1, as well as 4 hours after last treatment on day 3. Corticosterone levels were determined with a specific enzyme immunoassay kit (Assay Designs, Ann Arbor, MI, United States) with a sensitivity of 0.027 ng/ml as previously described (Farzi et al., 2015) and according to the manufacturer's specifications.

Statistical analysis

The manuscript complies with BJP's recommendations and requirements on experimental design and analysis (Curtis et al., 2018). Statistical analysis was preformed using the GraphPad Prism™ 6 software (GraphPad Software, Inc, CA, USA). Data were normalised to baseline (1 or 100%) of the means of negative control in experiments performed with eosinophils isolated from human donors to reduce interindividual source of variation.

Statistical analysis was only performed for groups where $n \geq 5$. Additional preliminary data ($n=3$) on p-Akt phosphorylation in eosinophils were included in the manuscript to suggest a mechanism previously shown for other cell types (Ruiter et al., 2003; Chugh et al., 2008). The group size given for each experiment is the number of independent values (individual human eosinophil donors or mice). Statistical analysis was performed using these independent values.

Data was tested for normality using D'Agostino & Pearson omnibus normality test. If normality was assumed, comparisons among multiple groups were performed with one-way ANOVA or two-way ANOVA. For these analyses, post hoc pairwise comparisons were performed using Bonferroni's multiple comparison test (or Dunnett's multiple comparison test, when comparing samples to the control group), only if a main effect for at least one factor or the interaction between two factors showed statistical

significance and if there was no significant variance in homogeneity. Cytokine levels were compared using Mann-Whitney U test. Significance level for the analyses was set to $\alpha=0.05$ and significant differences are indicated with the corresponding p-value

* $p \leq 0.05$.

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019).

3. Results

3.1 Miltefosine suppresses eosinophil activation *in vitro*

First, we tested the viability of eosinophils after pretreatment with different concentrations of miltefosine. Importantly, miltefosine (up to 20 μ M, in the presence of 1 mg/ml bovine serum albumin) showed no toxic effects on eosinophils (**Supplemental Figure S1**).

During the state of allergic inflammation, elevated concentrations of cytokines and chemoattractants in the blood activate eosinophils, which leads to a rearrangement of their actin filaments (the so-called "shape change") (Willettts et al., 2014) and results in an upregulation of the adhesion molecules integrins (e.g. CD11b/CD18; Mac-1) on the cell surface (Jia et al., 1999). When human eosinophils were pretreated with miltefosine, we could observe a statistically significant inhibition of their shape change (by approx. 50%) induced by CCL11 stimulation (**Figure 1 A-B**) when using the

highest concentration of miltefosine (20 μ M). Miltefosine addition did not alter eosinophil shape change in the absence of external stimuli (**Supplemental Figure S2**). When isolated eosinophils were pretreated with 20 μ M miltefosine, upregulation of CD11b was reduced by about 50 % (**Figure 1 C-D**). Similarly, miltefosine suppressed CD11b expression of CCL11 activated eosinophils (CD16⁻ cells) in the PMNL fraction (**Supplemental Figure S3 A**). Notably, miltefosine did not alter CD11b expression of fMLP stimulated neutrophils (CD16⁺ cells) (**Supplemental Figure S3 B**).

To determine whether miltefosine has an effect on the chemotaxis of human eosinophils, we performed *in vitro* chemotaxis assays using eotaxin-2 (CCL24) as chemoattractant. Miltefosine significantly inhibited eosinophilic chemotaxis in a dose-dependent manner (**Figure 2 A-B**).

We next assessed whether miltefosine affects degranulation-associated processes in eosinophils. For that purpose, eosinophils were pretreated with miltefosine and subsequently stimulated with recombinant C5a, a potent inducer of degranulation. Miltefosine effectively suppressed C5a (0.5 nM) induced CD63 expression, a marker of eosinophilic degranulation (Carmo et al., 2016). (**Figure 2 C-D**). CD63 expression induced with very high concentrations of C5a (100 nM) was not affected by miltefosine (**Figure 2 E-F**).

Previous studies have shown that miltefosine inhibits (PI3K)/Akt kinase signaling with an IC₅₀ in the range of 5 to 35 μ M, depending on the cell line tested. (Rybczynska et al., 2001; Kaleağasıoğlu et al., 2018). Our preliminary results show a tendency of miltefosine (20 μ M) inhibiting Akt phosphorylation (**Figure 3 A-B**). Moreover, we could demonstrate that intracellular calcium flux in CCL11-stimulated eosinophils was

inhibited by about 50% (**Figure 3 C-D**) after only 1 minute of miltefosine addition (20 μ M).

3.2 Miltefosine ameliorates ovalbumin induced lung inflammation

Next, we investigated whether the *in vitro* results obtained with isolated human eosinophils are also relevant *in vivo*. We first performed Ca^{2+} flux assays using mouse bone marrow derived eosinophils to test whether mouse eosinophils behave similar to human isolated eosinophils (**Figure 4 A-B**). For that purpose, eosinophils were differentiated from bone marrow cells of BALB/c mice following an established protocol (Kienzl et al., 2020), which yields a pure population of cultured eosinophils as determined by a single population positive for mouse eosinophil markers CCR3 and Siglec-F (**Supplemental Figure S4 A**). Microscopic analysis of cytopins of BMDEs shows a uniform population of cells exhibiting typical eosinophil staining and granule morphology (**Supplemental Figure S4 B**). These mature bone marrow eosinophils were used to perform Ca^{2+} flux assays under similar experimental conditions as isolated human eosinophils (**Figure 4 A-B**). Our data shows the level (approximately 50%) and kinetic of Ca^{2+} flux inhibition in mouse eosinophils resembling that of human eosinophils pretreated with miltefosine and stimulated with CCL24 (**Figure 4 A-B**).

Next, we performed an *in vivo* eosinophilic migration test using IL-5Tg mice. This strain of mice is characterized by eosinophilia due to increased production of IL-5. Together, intranasal eotaxin application in IL-5 primed eosinophils results in abundant and eosinophil accumulation in the BAL fluid and lungs of animals (Ochkur et al., 2007). We treated IL-5Tg mice for three consecutive days perorally with miltefosine (20 mg/kg) (**Figure 4 C**). We used a dosing regimen comparable to other studies in mice testing miltefosine (Bäumer et al., 2010; Dorlo et al., 2012). Remarkably, miltefosine

significantly suppressed the migration of eosinophils to intranasal CCL24 into the BAL of animals (**Figure 4 D**). A trend towards reduced infiltration of immune cells was observed for all detected cell types (**Figure 4 D**). Analysis of the blood immune cell composition revealed an increase in the percentage of neutrophils in blood of IL-5Tg mice treated with miltefosine (**Supplemental Figure S5 A**), however when BALB/c mice were treated with miltefosine no increase in neutrophils was observed (**Supplemental Figure S5 B**). By testing plasma of BALB/c mice for their corticosterone levels, we observed no significant differences at both of the two tested time points (**Supplemental Figure S6 A-B**).

We next tested the efficacy of miltefosine in an acute model of allergic lung inflammation. Ovalbumin was used as a model allergen to reproduce key features of clinical asthma, such as airway hyperresponsiveness to methacholine (Kumar et al., 2008). The treatment protocol of the model is shown in **Figure 5 A**. We observed that daily peroral treatment with miltefosine markedly reduced the number of several infiltrating immune cells into airways of ovalbumin-challenged wild-type mice. Flow cytometric analysis of the composition of immune cells showed that the number of eosinophils as well as infiltrating T cells, B cells and dendritic cells was reduced by 50 % upon miltefosine treatment (**Figure 5 B**). Of note, mice treated with miltefosine showed significantly improved lung resistance and a trend towards improved lung compliance (**Figure 5 C**). In order to test, whether a decrease in eosinophil numbers was responsible for the reduction of other immune cells eosinophil-deficient (Δ dbl GATA-1) mice were exposed to the same ovalbumin-induced allergic model. In this mouse strain, treatment with miltefosine only had an impact on the number of dendritic cells (**Figure 6**), while other subgroups of immune cells were not affected.

Supernatants of BAL fluid of ovalbumin stimulated (vehicle) and miltefosine treated and ovalbumin stimulated BALB/c and Δ dbl GATA-1 mice were further analysed for their cytokine content (**Supplemental Figure S7 -S8**). We could observe significantly reduced levels of immunomodulatory cytokine IFN- γ in the BAL fluid of miltefosine treated BALB/c mice (**Supplemental Figure S7 A**), while no such inhibition was observed in Δ dbl GATA-1 mice (**Supplemental Figure S8 A**). Cytokine content of CCL11, TNF- α , IL-2 and IL-5 in BAL fluid was not significantly altered by miltefosine treatment in either BALB/c or Δ dbl GATA-1 mice (**Supplemental Figure S 7-8**).

4. Discussion

In the present study we show for the first time that the FDA-approved drug miltefosine inhibits the activation of human eosinophils. A short pre-treatment with miltefosine suppressed human eosinophilic effector responses after stimulation with various agonists *in vitro*. We were able to transfer our *in vitro* findings to preclinically relevant endpoints in an *in vivo* model of eosinophilic migration and allergic inflammation. Most importantly, in a model of ovalbumin-induced allergic lung inflammation, peroral administration of miltefosine significantly reduced the infiltration of immune cells into the lung while improving lung function parameters.

The effects of miltefosine have previously been studied on some other immune cells. Notably, miltefosine was found to inhibit degranulation and antigen-induced chemotaxis of mast cells by modulating lipid rafts and by inhibiting cytosolic protein kinase C (Rubíková et al., 2018). In contrast to our findings with eosinophils, calcium flux in mast cells was apparently not affected by miltefosine pretreatment, indicating cell type-specific differences. However, similar to mast cells, miltefosine led to an

inhibition of effector functions and mediator release in eosinophils. In macrophages, miltefosine was found to increase cholesterol release and phosphorylation of kinases associated with autophagy. Importantly, miltefosine decreased toll-like receptor 4 (TLR-4) recruitment to the cell surface of macrophages and dampened IL-1 β release following stimulation with lipopolysaccharide (Iacano et al., 2019). Given the fact that TLR-4 stimulation on eosinophils can help polarize macrophages towards pro- or anti-inflammatory phenotypes (Yoon et al., 2019), this finding further supports the evidence that miltefosine may influence the interplay and balance between various immune cell types during the state of inflammation.

It is noteworthy that in all our *in vitro* experiments non-toxic concentrations of miltefosine were used to distinguish our results from the non-specific cytolytic effects of the drug. In particular, since homeostatic functions such as tissue remodelling and plasma cell survival (Jacobsen et al., 2012) have recently been attributed to eosinophils, we were mainly interested in inhibiting eosinophil overactivation, to prevent their potential tissue-damaging effector functions. For our *in vivo* experiments, we used a dosage regimen, comparable to other studies in mice testing miltefosine (Bäumer et al., 2010).

Results from our *in vivo* experiments show significantly decreased numbers of infiltrating eosinophils in miltefosine treated animals compared to vehicle challenged controls. The data correspond to our *in vitro* experiments in which miltefosine inhibited the activation, migration and upregulation of adhesion molecules on eosinophils. Interestingly, we additionally discovered a trend towards decreased numbers of other infiltrating immune cells in miltefosine-treated animals, while the numbers of tissue resident alveolar macrophages remained the same across all treatment groups. In order to confirm, whether infiltration of other immune cells is directly affected by

miltefosine, we performed control experiments in eosinophil-deficient Δ dbl GATA-1 mice. We discovered that the decreased infiltration of most immune cells was at least partially due to the decreased eosinophil numbers. This is not unexpected, since activated eosinophils are known to attract and activate other immune cell types such as neutrophils (Yousefi et al., 1995) or B cells (Chu et al., 2011). Moreover, eosinophil-derived CCL17 and CCL22 have proven to be crucial in attracting effector T cells in localized allergic inflammation (Jacobsen et al., 2008). Interestingly, we observed a decrease in dendritic cell ($CD11c^+$ / $MHCII^+$ / $Siglec F^-$ / $Ly6G^-$ / $Ly6C^-$) numbers following miltefosine treatment compared to challenged controls both in wild-type and in eosinophil-deficient mice. It has been reported previously that combination therapy of paromomycin/miltefosine can influence TLR-9 on dendritic cells and therefore modulate Th1 host immune responses in leishmaniasis therapy (Das et al., 2014). As of yet, however, the direct effect of miltefosine on human dendritic cells remains unclear.

To assess whether the observed differences in immune cell count are a consequence of miltefosine directly inhibiting immune cell infiltration or rather indirectly altering the cytokine milieu in the lung, we additionally tested BAL supernatants from both BALB/c and Δ dbl GATA-1 mice for cytokine expression. Cytokine concentrations of CCL11, $TNF-\alpha$, IL-2 and IL-5 were not altered in miltefosine treated and ovalbumin stimulated mice of both genotypes. Interestingly however, we observed significantly reduced levels of the immunomodulatory cytokine IFN- γ in the miltefosine treated group of BALB/c mice. This data corroborates previous findings from Verhaar et al (Verhaar et al., 2013), where they observed similarly reduced levels of IFN- γ in miltefosine treated animals in a mouse model of inflammatory bowel disease. IFN- γ has on one hand long been considered to be beneficial in allergic inflammation as reviewed by Teixeira et al

(Teixeira et al., 2005) while on the other hand, recent studies recognize its pro-inflammatory functions. Our findings of reduced IFN- γ expression in BALB/c mice and not in Δ dbl GATA-1 mice are of particular interest, since it was discovered that eosinophil-derived IFN- γ induces airway hyperresponsiveness and lung inflammation even in the absence of lymphocytes (Kanda et al., 2009). Interestingly, IFN- γ was also found to upregulate several eosinophil effector functions (Ishihara et al., 1997; Takaku et al., 2011) and promote their survival (Fujisawa et al., 1994).

When we examined the composition of immune cells in mouse blood, miltefosine-treated and CCL24 stimulated IL-5Tg animals showed an increased neutrophil count, yet miltefosine treated BALB/c animals showed no altered neutrophil numbers at baseline. A previous study showed that patients treated with miltefosine exhibited increased levels of the neutrophilic chemokine IL-8 (Mukhopadhyay et al., 2011), therefore this finding remains to be confirmed in mice. Increased corticosterone levels in mice induced by miltefosine, could be another plausible explanation for both increased neutrophil numbers (Liles et al.) and decreased airway inflammation (Suqin et al., 2009). Furthermore, an inverse association between endogenous glucocorticoid and IFN- γ levels were observed in allergic lung inflammation (Suqin et al., 2009). Nonetheless, we observed no significant alterations in corticosterone levels in miltefosine treated mice.

Fang et al. additionally showed that miltefosine acts on endothelial cells by downregulating E-selectin, which is important for leukocyte adhesion and infiltration (Leung et al., 2007). Therefore, in our ovalbumin model of allergic inflammation we cannot neglect additional anti-inflammatory effects of miltefosine.

In another point, our work raises important questions regarding the immunomodulatory effect of miltefosine in patients treated for Leishmania infections. So far, little has been reported about the drug's effect on the host responses responsible for fighting the infection. However, some *in vitro* findings report a strong reversal of Th2 responses of leishmania-infected macrophages towards Th1-type following miltefosine treatment (Wadhone et al., 2009). Since eosinophils are one of the primary cells recruited to the sites of leishmania infection (de Oliveira Cardoso et al., 2010) and have been shown to help control parasite load (Watanabe et al., 2004) in mice, it might be of interest to further investigate this issue in patients treated with miltefosine. In line with the present study, we have previously shown that saturated lysophosphatidylcholines, which are structurally similar to miltefosine, inhibit eosinophil effector responses (Trieb et al., 2019; Knuplez et al., 2020a, 2020b).

A limitation of our work needs to be noted. Ovalbumin was used as a model allergen in our *in vivo* studies, albeit this model fails to completely reflect the aetiology of human asthma and its multi-step developmental process, including environmental factors associated with the disease. Further experiments with other physiological relevant antigens are needed to validate the relevance of our data in human disease setting.

In summary, we demonstrate the inhibitory effect of the orphan drug miltefosine on human eosinophils and its anti-inflammatory effect *in vivo* in a model of allergic inflammation. Our data highlight the potential efficacy of miltefosine or related molecules in the treatment of allergic diseases and other eosinophil-associated disorders.

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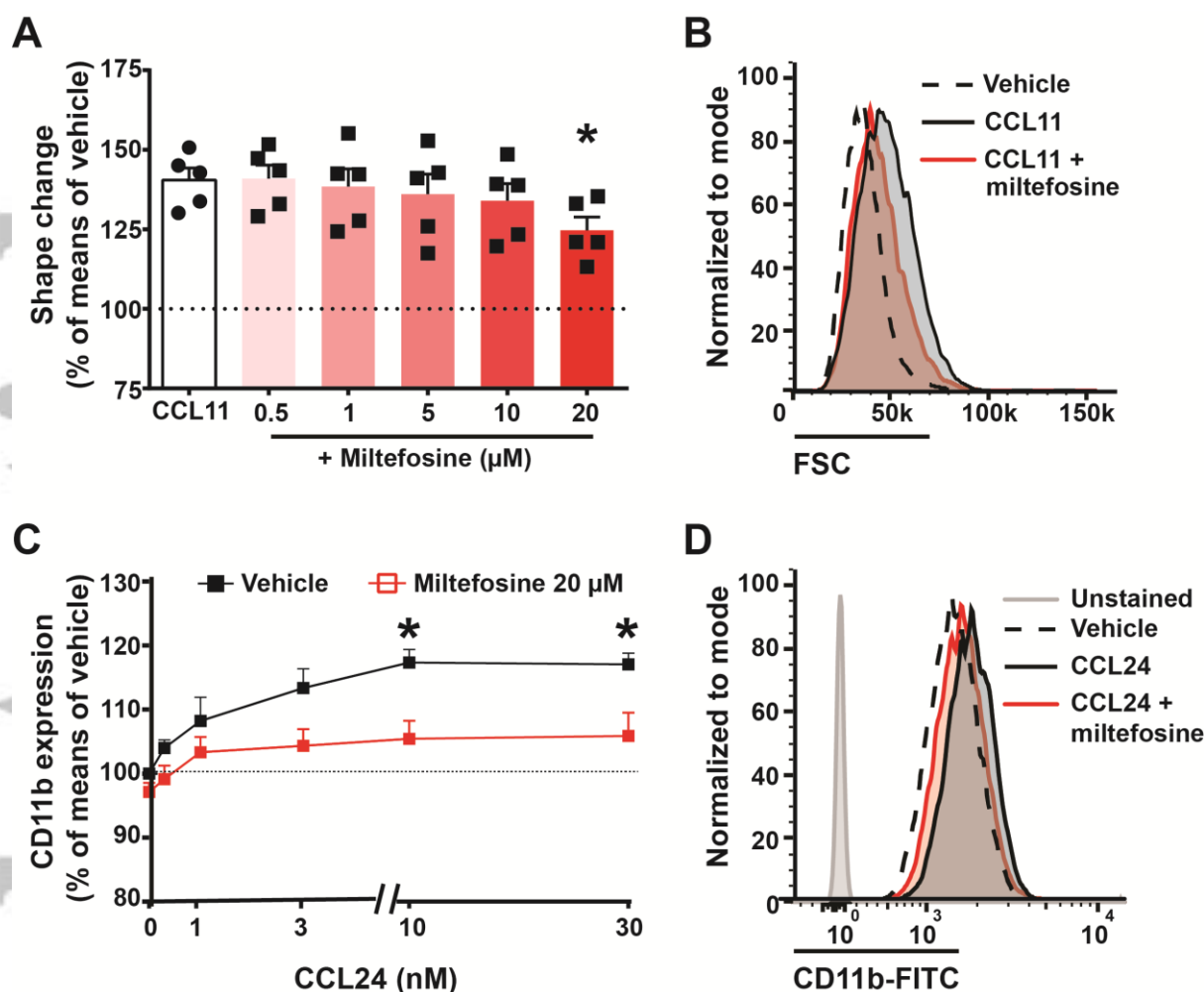


Figure 1. Miltefosine concentration-dependently inhibits eosinophil activation. (A-B) Eosinophils were pretreated with miltefosine in different concentrations (0.5-20 μM) (15 min, RT) and stimulated with 10 nM CCL11 (4 min, 37 °C). Cells were fixed and the change in cell size (FSC) was evaluated by flow cytometry. (A) Eosinophil shape change is expressed as percent of unstimulated vehicle response. Data are shown as mean + SEM from five individual experiments. *p<0.05, vs CCL11-stimulated vehicle (One-Way ANOVA with Dunnett's post hoc test). (B) Representative histogram of eosinophil FSC with miltefosine (20 μM) pretreatment and CCL11 stimulation (10 nM). (C-D) Eosinophils were stained with anti-CD11b and treated either with miltefosine (20 μM) or vehicle control (15 min, RT). Subsequently, cells were stimulated with CCL24 (4 min, 37 °C) and analyzed by flow cytometry. Eosinophil CD11b expression is expressed as percent of unstimulated vehicle response. Data are shown as mean + SEM from five individual experiments. *p<0.05, vs vehicle (Two-Way ANOVA with Bonferroni post hoc test.) (D) Representative histogram of CD11b upregulation with miltefosine (20 μM) pretreatment and CCL24 (10 nM) stimulation.

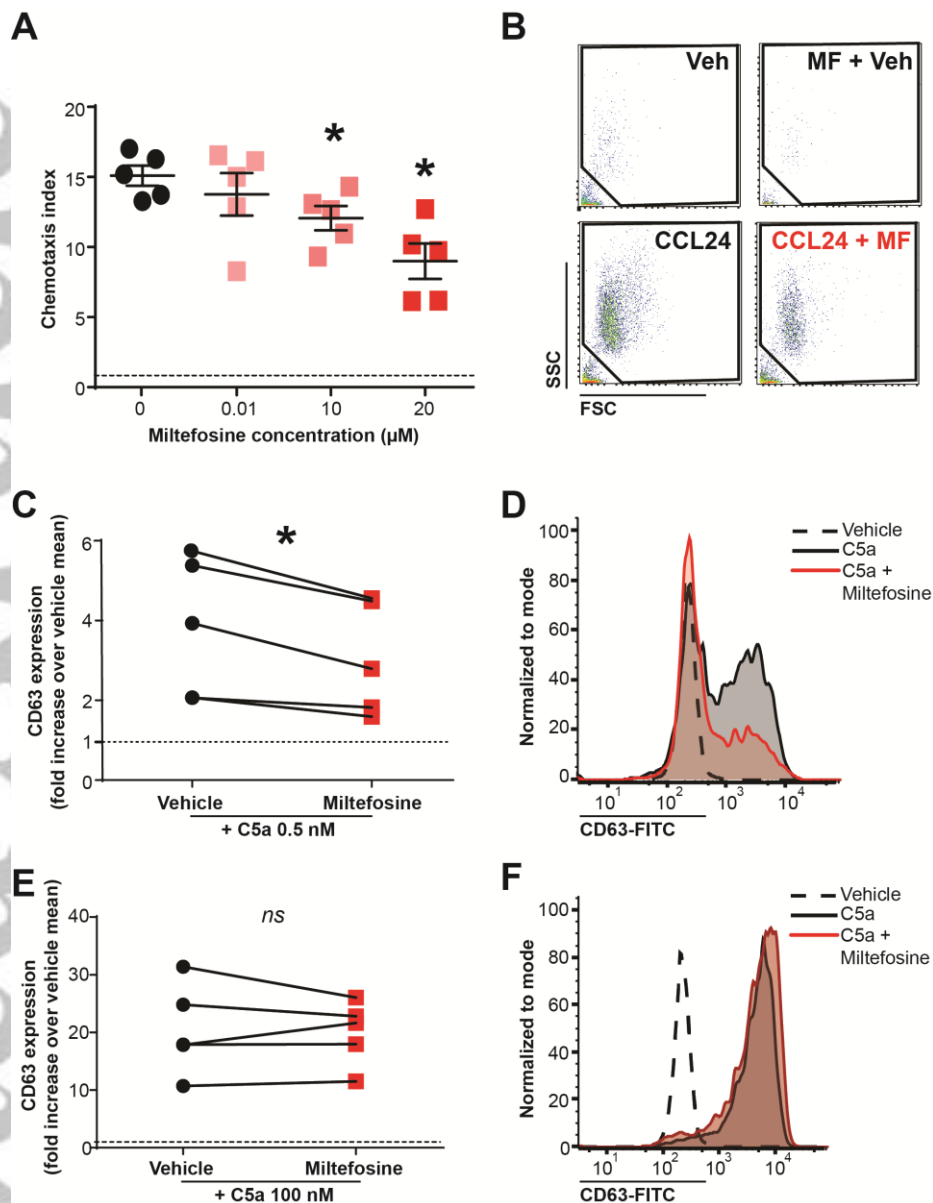


Figure 2. Miltefosine inhibits chemotaxis and CD63 expression of eosinophils.

(A-B) Purified human eosinophils were treated with miltefosine in different concentrations (15 min, RT) or vehicle and allowed to migrate towards CCL24 (10 nM) in transwell plates **(A)**. Migrated cells were enumerated by flow cytometry for 1 min. Results are presented as chemotactic index (migrated cell number/cell number migrated to unstimulated vehicle) and shown as mean $\pm$ SEM from five individual experiments. * $p < 0.05$ vs vehicle (One-Way ANOVA with Dunnett's post hoc test). **(B)** Representative scatter blots of cells migrating toward vehicle / miltefosine (20 μM) in the bottom compartment or cells pretreated with vehicle or miltefosine (20 μM) migrating towards CCL24. **(C-F)** Eosinophils were stained with anti-CD63-FITC followed by incubation with miltefosine (20 μM) or vehicle (15 min, RT). Subsequently, cells were primed with cytochalasin B and stimulated with C5a 0.5 nM **(C-D)** or 100 nM **(E-F)** for 20 min at 37 $^{\circ}\text{C}$. CD63 expression is expressed as fold increase of fluorescence intensity over unstimulated vehicle for individual donors. * $p < 0.05$, miltefosine vs vehicle treated (paired Student's t-test). Abbreviations represent: Veh, vehicle; MF + Veh, miltefosine + vehicle; CCL24 + MF, CCL24 + miltefosine.

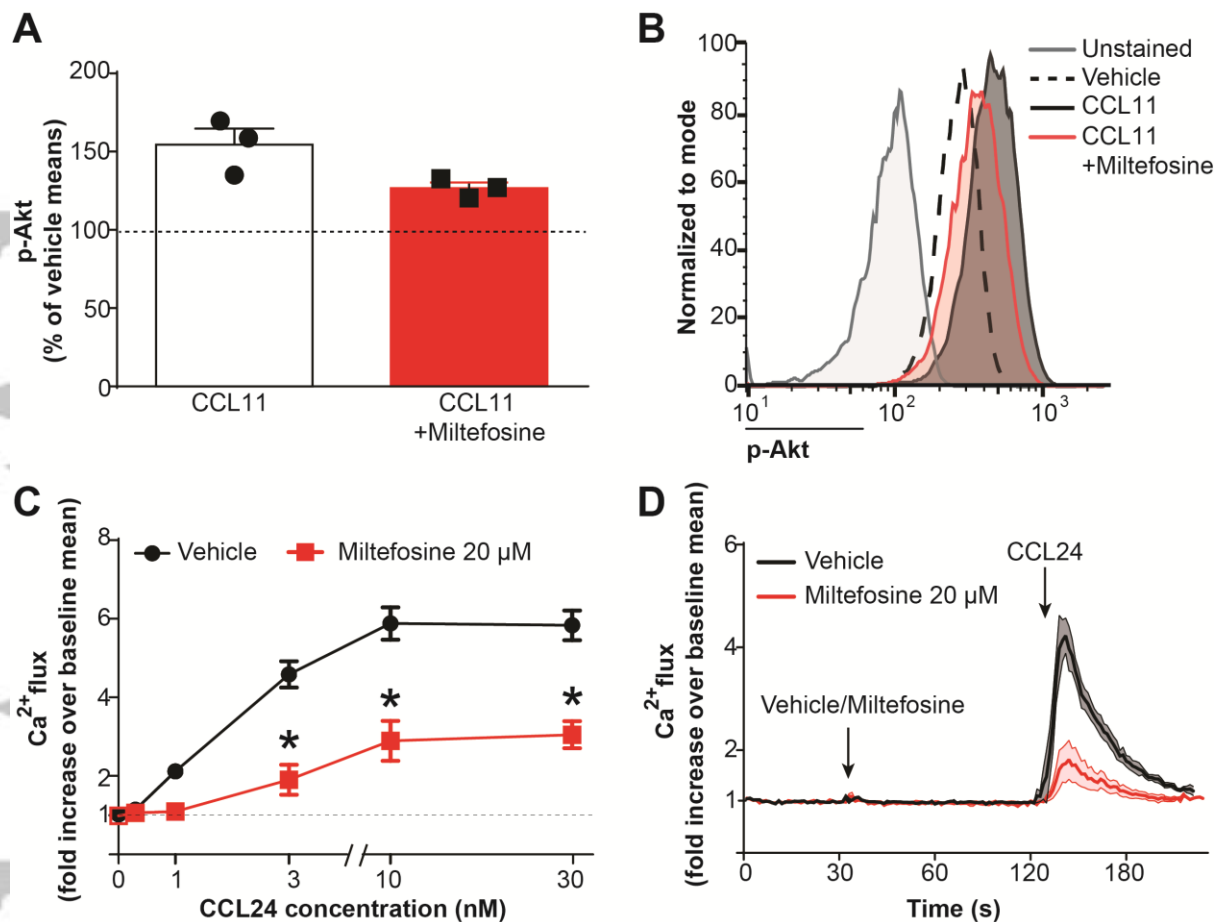


Figure 3. Miltefosine inhibits Akt phosphorylation and Ca^{2+} flux in human eosinophils. (A-B) Eosinophils were pretreated with miltefosine (20 μM) or vehicle (15 min, RT) followed by addition of CCL11 (3 min, 37 $^{\circ}\text{C}$). Subsequently cells were fixed, permeabilized and stained. (A) Phosphorylation of Akt residues was quantified as the increase of fluorescence in the FITC fluorescence channel and expressed as percent of unstimulated vehicle control. Data are shown as mean $\pm$ SEM from three individual experiments. (B) Representative histogram of p-Akt staining following miltefosine pretreatment. (C-D) Eosinophils were labeled with Fluo-3 AM and changes in $[\text{Ca}^{2+}]_i$ were detected by flow cytometry. Eosinophils were stimulated with increasing concentrations of CCL24 (0-30 nM) in the presence or absence of miltefosine (20 μM). (C) Results represent fold increase in $[\text{Ca}^{2+}]_i$ over unstimulated vehicle. Data are shown as mean $\pm$ SEM from five individual experiments * $p < 0.05$ miltefosine (20 μM) vs vehicle (Two-Way ANOVA with Bonferroni post-hoc test). (D) Time course of Ca^{2+} flux in eosinophils. Following baseline measurement (30 s), miltefosine (20 μM) or vehicle was added. After 1 min, CCL24 (10 nM) was added to induce Ca^{2+} flux. Data are shown as mean $\pm$ SEM from five individual experiments.

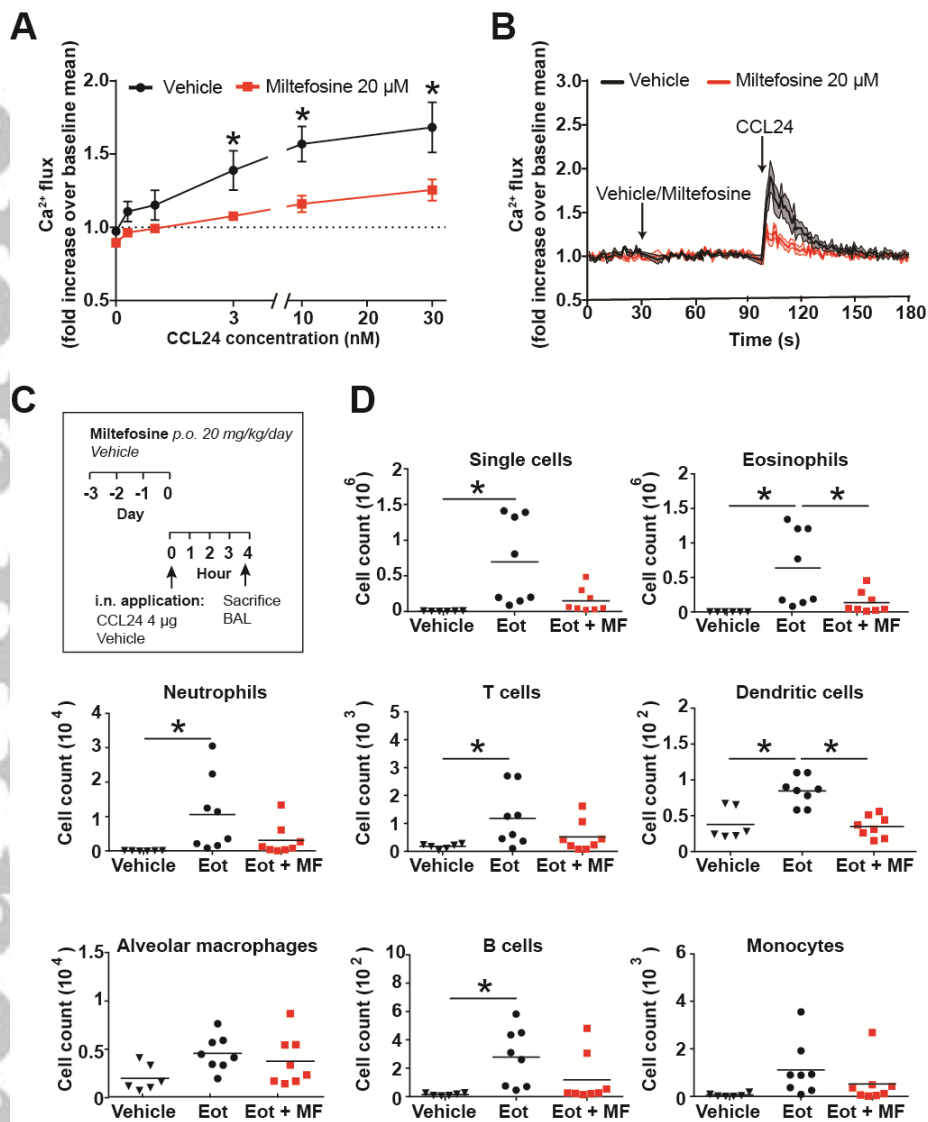


Figure 4. Miltefosine inhibits Ca²⁺ flux in mouse eosinophils and suppresses migration of eosinophils *in vivo*. (A-B) Bone marrow derived mouse eosinophils were labeled with Fluo-3 AM and changes in [Ca²⁺]_i were detected by flow cytometry. Eosinophils were stimulated with increasing concentrations of CCL24 (0-30 nM) in the presence or absence of miltefosine (20 μ M). (A) Results represent fold increase in [Ca²⁺]_i over unstimulated vehicle. Data are shown as mean $\pm$ SEM from five individual experiments *p<0.05 miltefosine (20 μ M) vs vehicle (Two-Way ANOVA with Bonferroni post-hoc test). (B) Time course of Ca²⁺ flux in eosinophils. Following baseline measurement (30 s), miltefosine (20 μ M) or vehicle was added. After 1 min, CCL24 (10 nM) was added to induce Ca²⁺ flux. Data are shown as mean $\pm$ SEM from five individual experiments. (C-D) Eight-week-old IL-5Tg mice received either miltefosine (20 mg/kg) or vehicle (0.9% NaCl) per os for three consecutive days, followed by intranasal application of CCL24 (4 μ g) or vehicle. After four hours, mice were sacrificed and BAL fluid was collected. (B) Immune cell composition in BAL fluid was analyzed by flow cytometry. Data are shown from 6-8 mice from three individual experiments. *p<0.05, vs Eot group, (One-Way ANOVA with Dunnett's post-hoc test). Abbreviations represent: Eot, eotaxin (CCL24); Eot + MF, eotaxin (CCL24) + miltefosine.

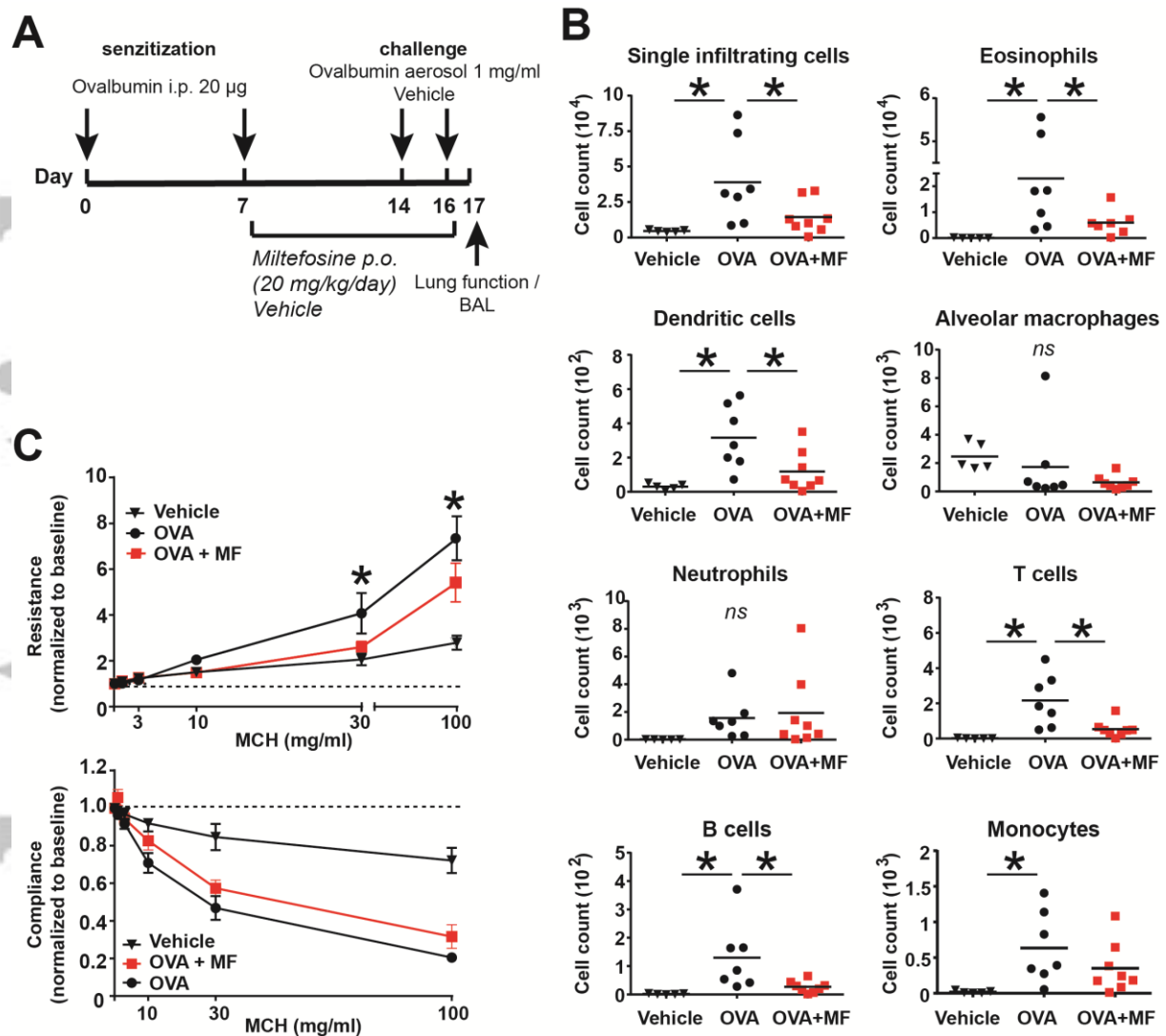


Figure 5. Miltefosine ameliorates ovalbumin-induced allergic lung inflammation. (A) Eight-week-old BALB/c mice were sensitized with ovalbumin intraperitoneal (i.p.) at day 0 and at day 7 and were subsequently treated with miltefosine (20 mg/kg) or vehicle per os daily from day 7 to day 17. Afterwards mice were challenged with an ovalbumin aerosol on day 14 and day 16 followed by lung function testing or sampling of BAL fluid. (B) Immune cell composition in the BAL fluid was analyzed by flow cytometry. Representative results from two individual experiments are shown. * $p < 0.05$, vs OVA group (One-Way ANOVA with Dunnett's post-hoc test). (C) Lung function of mice was assessed while applying increasing doses of methacholine (0-100 mg/ml). Data are shown as mean $\pm$ SEM from two individual experiments performed with 5-8 mice. * $p < 0.05$, OVA + MF vs OVA group, (Two-Way ANOVA with Dunnett's post-hoc test). Abbreviations represent: OVA, ovalbumin; OVA +MF ovalbumin + miltefosine, MCH, methacholine.

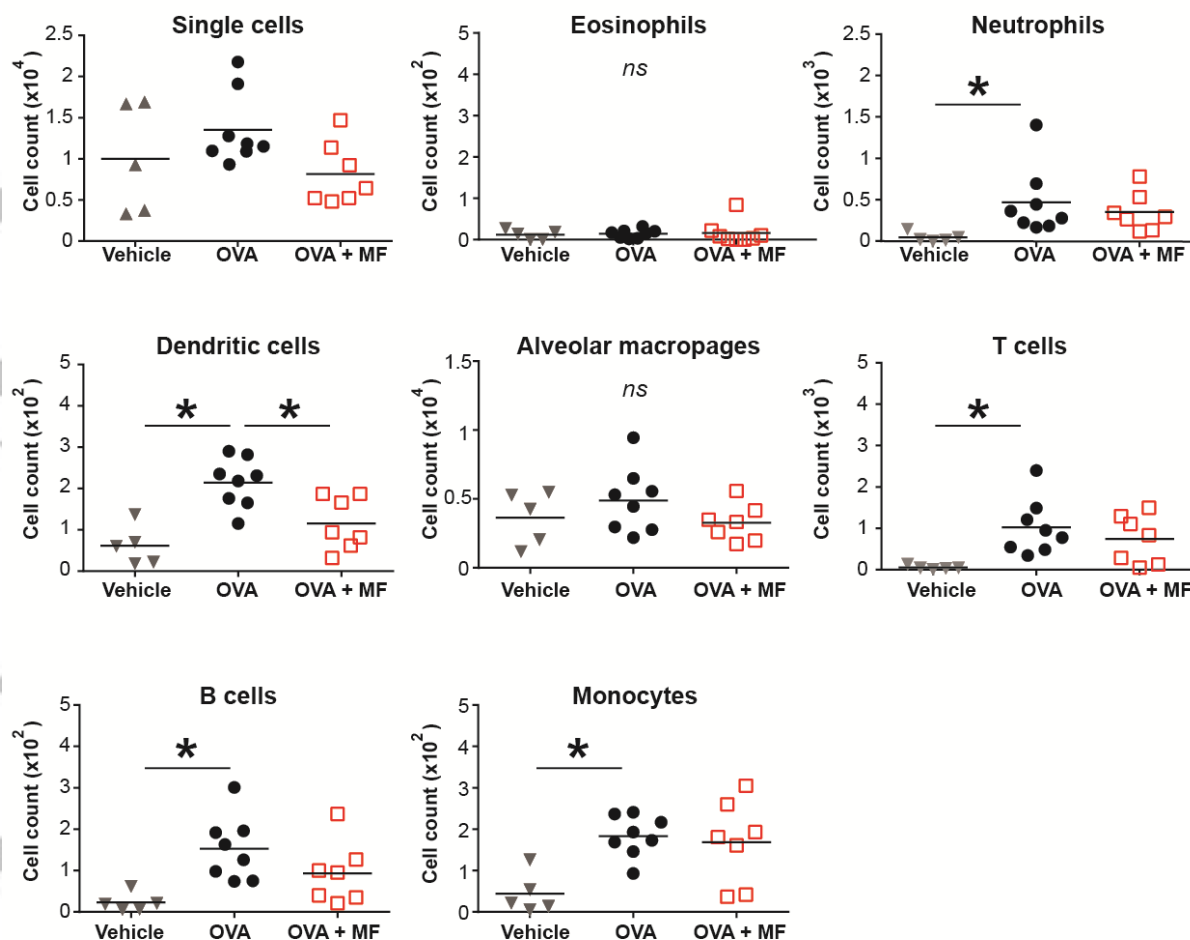


Figure 6. The effect of miltefosine treatment in eosinophil-deficient mice. Eight-week-old Δ dbl GATA-1 mice were sensitized with ovalbumin intraperitoneal (i.p.) at day 0 and at day 7 and subsequently treated with miltefosine (20 mg/kg) or vehicle per os daily from day 7 to day 17. Afterwards mice were challenged with an ovalbumin aerosol on day 14 and day 16 followed by sampling of BAL fluid. Immune cell composition in the BAL fluid was analyzed by flow cytometry. Results from two individual experiments with 5-7 mice are shown. * $p < 0.05$ vs OVA group (One-Way ANOVA with Dunnett's post-hoc test). Abbreviations represent: OVA, ovalbumin; OVA+MF ovalbumin + miltefosine.