



Recognition and killing of *Brugia malayi* microfilariae by human immune cells is dependent on the parasite sample and is not altered by ivermectin treatment



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ABSTRACT

Mass administration of macrocyclic lactones targets the transmission of the causative agents of lymphatic filariasis to their insect vectors by rapidly clearing microfilariae (Mf) from the circulation. It has been proposed that the anti-filarial action of these drugs may be mediated through the host immune system. We recently developed an *in vitro* assay for monitoring the attachment to and killing of *B. malayi* Mf by human neutrophils (PMNs) and monocytes (PBMCs), however, the levels of both cell to worm attachment and leukocyte mediated Mf killing varied greatly between individual experiments. To determine whether differences in an individual's immune cells or the Mf themselves might account for the variability in survival, PMNs and PBMCs were isolated from 12 donors every week for 4 weeks and the cells used for survival assays with a different batch of Mf, thereby keeping donors constant but varying the Mf sample. Results from these experiments indicate that, overall, killing is Mf-rather than donor-dependent. To assess whether ivermectin (IVM) or diethylcarbamazine (DEC) increase killing, Mf were incubated either alone or with immune cells in the presence of IVM or DEC. Neither drug induced a significant difference in the survival of Mf whether cultured with or without cells, with the exception of DEC at 2 h post incubation. In addition, human PBMCs and PMNs were incubated with IVM or DEC for 1 h or 16 h prior to RNA extraction and Illumina sequencing. Although donor-to-donor variation may mask subtle differences in gene expression, principle component analysis of the RNASeq data indicates that there is no significant change in the expression of any genes from the treated cells versus controls. Together these data suggest that IVM and DEC have little direct effect on immune cells involved in the rapid clearance of Mf from the circulation.

1. Introduction

Filarial parasitic nematodes are responsible for several important neglected diseases of humans, including onchocerciasis (river blindness) and lymphatic filariasis. The parasites that cause lymphatic filariasis (LF), mostly *Wuchereria bancrofti* (responsible for 90% of cases) and *Brugia malayi* (responsible for ~10%) infect 120 million people worldwide, with 1.3 billion people at risk of infection. Control and elimination programs for LF rely on mass drug administration (MDA) of the anthelmintics ivermectin (IVM), diethylcarbamazine (DEC) and albendazole (ALB) to kill microfilariae and prevent transmission – the

drugs have limited effects on the adult parasites (Ottesen, 2006). Albendazole inhibits microtubule formation by binding to nematode β -tubulin (Lubega and Prichard, 1990; Robinson et al., 2004; Prichard, 2007). However, despite their widespread use in MDA, we know little about how IVM and DEC work against filarial species. It has been very clearly established, via clinical trials and community-based interventions, that treatment of infected people with DEC or IVM leads to a rapid (less than 24 h) and, in the case of IVM, sustained (up to 12 months) reduction in circulating Mf (Hawking and Laurie, 1949; Ottesen, 2006). This outcome is ideal for use in MDA programs whose aim is to reduce or eliminate transmission of the disease, but the

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mechanisms by which these rapid reductions are achieved are unknown. The paralytic effect of IVM is not directly responsible for Mf removal, as the concentrations of the drug that are required to paralyze filarial Mf *in vitro* are orders of magnitude higher than those which are effective *in vivo* (Tompkins et al., 2010; Storey et al., 2014). Similarly, the potent and rapid effect of DEC treatment on the numbers of circulating filarial Mf *in vivo* is not reproduced *in vitro* (Vickery et al., 1985; Maizels and Denham, 1992). This has led to considerable speculation that the anthelmintic effects of these drugs is mediated, at least in part, via the host immune system (Maizels and Denham, 1992; Kwarteng et al., 2016; Wolstenholme et al., 2016). Some time ago, it was shown that the innate immune system and leukocytes, but not T cells or complement, were involved in DEC's mode of action. The drug interferes with arachidonic acid metabolism, blocking both cyclooxygenase (COX) and lipoxygenase, and therefore has anti-inflammatory properties (Maizels and Denham, 1992). Increased concentrations of prostaglandins have been reported in the plasma of microfilaraemic patients and these were significantly reduced 12 h after treatment with DEC (Sankari et al., 2013). In mice, the activity of DEC and the reduction in COX expression in peritoneal exudate cells that it causes, is dependent on inducible nitric oxide synthase (iNOS), as the drug is ineffective in animals deficient in this enzyme (McGarry et al., 2005). The rapid removal of Mf from the peripheral circulation may be due to their sequestration in the central vasculature, where they may be subsequently killed by granulocytes and platelets (Cesbron et al., 1987); DEC increases the adherence of *B. pahangi* Mf to granulocytes (Johnson et al., 1988).

Recently, we have described *in vitro* assays for the recognition of Mf by host immune cells, both neutrophils and peripheral blood mononuclear cells (PBMCs), using *Dirofilaria immitis*, the causative agent of heartworm disease in dogs, and *B. malayi* (Vatta et al., 2014; McCoy et al., 2017). In the case of *D. immitis*, canine neutrophil and PBMC attachment to Mf was increased by incubation with pharmacologically relevant concentrations of IVM, leading us to speculate that this might represent one possible mechanism of action for the drug against this parasite. In the case of *B. malayi*, we observed that human neutrophils and PBMCs could also attach to Mf, and cause parasite death. However, this finding was inconsistent and the amount of parasite killing that we observed varied greatly between experiments; this could be explained either by variations between the individual human volunteer donors that we used or by batch-to-batch variations in the *B. malayi* Mf. We also did not test the ability of DEC or IVM to stimulate this killing. In this study, we address those two questions by comparing the ability of leukocytes isolated from the same donors to kill independent batches of Mf isolated over four consecutive weeks, and by studying the effects of adding DEC and IVM on parasite killing and on gene expression in the human cells.

2. Materials and methods

2.1. Ethics statement

For studies using individual donors, experiments and informed consent procedures were approved by the Institutional Review Boards of the University of Georgia (permit number 2012–10769). Human subjects recruited under the guidelines of IRB-approved protocols were provided written informed consent. For the multiple donor study, human blood material was obtained from the Clinical and Translational Research Unit, University of Georgia; experiments and informed consent procedures were approved by the Institutional Review Boards of the University of Georgia (IRB ID: STUDY000044750).

2.2. Preparation of *B. malayi* Mf

Live *B. malayi* Mf isolated from the peritoneal cavity of infected Mongolian gerbils were provided by the Filarial Research Reagent

Resource Center (FR3: Athens, GA, USA) (Michalski et al., 2011). Mf were washed three times in phosphate buffered saline (PBS; centrifuged at $1500 \times g$ for 8 min) and re-suspended in RPMI-1640 (Gibco, Life Technologies, Grand Island, NY, USA). All RPMI-1640 used in this study was supplemented with 100 U/ml penicillin-streptomycin (Life Technologies, Grand Island, NY, USA) and 0.1 mg/ml gentamicin (Sigma, St. Louis, MO, USA). Re-suspended Mf samples were then filtered through a 5 μm Isopore™ membrane (Merck Millipore Ltd., Carrigtwohill, Cork, Ireland) to capture the Mf and exclude contaminating small particles. Membranes were soaked in RPMI-1640 at 37 °C and 5% CO₂ for 20–30 min to facilitate the migration of viable Mf from the membrane. Viable Mf were incubated overnight in RPMI-1640 at 37 °C and 5% CO₂. Mf samples were washed for a second time by 5 μm Isopore™ membrane filtration just before use. In some experiments the PBS washing steps were omitted.

2.3. Isolation of human neutrophils, PBMCs and autologous serum

Leukocytes were isolated from freshly donated peripheral blood drawn from healthy U.S. residents at the Health Center of the University of Georgia (single individuals) or the Clinical and Translational Research Unit, University of Georgia (multiple donor study). 30 ml of blood was collected into heparinized tubes (single donor studies) or 18 ml in EDTA containing tubes (for the multiple donor study). For the multiple donor study, samples were randomized prior to cell isolation. Neutrophils were isolated using the EasySep™ Direct Human Neutrophil Isolation Kit (STEMCELL Technologies, Vancouver, BC, Canada) according to manufacturer's instructions. PBMCs were isolated using SepMate™-50 Tubes (STEMCELL Technologies, Vancouver, BC, Canada) according to manufacturer's instructions. To remove contaminating platelets, the optional extended wash step ($120 \times g$ for 10 min) of the SepMate™ protocol was included. Isolated PMNs and PBMCs were washed in PBS (centrifuged at $300 \times g$ for 5 min), re-suspended in a 1:1 mixture of RPMI-1640-10 mM HEPES buffer and autologous serum, stored at room temperature and used within 6 h post-isolation. Cell counts were performed on a 1:10 dilution of each preparation of cells containing 0.4% trypan blue (Gibco, Life Technologies, Grand Island, NY, USA) using a BioRad TC10™ automated cell counter (BioRad, Hercules, CA, USA). To estimate the purity of isolated cell populations, cell slides were prepared using the Cytospin®3 Cell Preparation System (Shandon Scientific Limited, Astmoor, Runcorn, Cheshire, England) and stained with Modified Wright's stain (Hema 3® Stat pack, Fisher Scientific, Kalamazoo, MI). Serum was prepared from 5 to 10 ml of autologous blood collected as above but allowed to clot in the absence of heparin or EDTA (incubated for ~2 h at room temperature). The liquid fraction of the blood sample was removed to a fresh tube and centrifuged at $10,000 \times g$ for 5 min. The resulting supernatant was filter sterilized and used as described below.

2.4. Mf survival assays and drug treatments

Assays were set up in Corning® 96 Well TC-treated microplates (Millipore-Sigma, Burlington, MA, USA). Four components were added to each well in 50 μl volumes, giving a total volume of 200 μl : ~100 *B. malayi*, autologous serum and either no cells, ~150,000 PMNs, or 150,000 PMNs and ~150,000 PBMCs. To create the respective controls, 50 μl of RPMI-1640 was substituted for the relevant component. The tissue culture plates were incubated at 37 °C and 5% CO₂. Viable Mf were counted on a Nikon™ TS2 microscope (Nikon Instruments Inc., Melville, NY, USA) at 1, 24 and 120 h post-experimental set up for most assays. Mf were considered to be 'viable' if there was any detectable movement observed within approximately 10–20 s. In experiments testing effects of MLs on survival, IVM was included at a final concentration of 1 μM and DEC at 100 μM and was present from initial setup. Although IVM treatment does affect movement at high concentrations, minimal effects on motility are observed at 1 μM

(Tompkins et al., 2010; Storey et al., 2014). For pre-treatment of cells with drug, the assay was set up with all components except Mf and incubated for either 1 h or 24 h prior to addition of the worms and counted at 72 or 48 h after addition of Mf. The number of motile Mf at each time point was normalized relative to the number of moving Mf scored 1 h post-set up (= 100%), and expressed as a relative percentage or reported as the number of Mf present. Each biological replicate represents the mean of three technical replicates. Images of cells and Mf were obtained using an HD Camera 1080p output (1920 × 1080 at 60 fps) HDMI with ISCapture Software (Southern Microscope Inc., Haw River, NC, USA).

2.5. RNA extraction

PMNs and PBMCs were extracted as described in Section 2.2. PMNs and PBMCs were incubated in RPMI containing 5% autologous serum and either 0.1% DMSO (control), 50 μM DEC or 100 nM IVM for either 1 h or 16 h respectively prior to RNA extraction using Trizol LS (Thermo-Fisher Scientific) following manufacturer's instructions. Briefly, 0.75 ml Trizol added per 0.25 ml sample (5–10 × 10⁶ cells). Cells were lysed by pipetting up and down. Homogenized samples were incubated for 5 min at room temperature. Chloroform (0.2 ml/1 ml Trizol) was added and the sample shaken for 15 s followed by a 3 min incubation at room temperature. Samples were centrifuged at 12,000 × g for 15 min at 4 °C. The aqueous phase is removed and the RNA precipitated by adding 0.5 ml isopropanol (including 15 μg GlycoBlue™ Coprecipitant, Invitrogen, Carlsbad, CA) to the aqueous phase and incubating at room temperature for 10 min. After centrifugation at 12,000 × g for 10 min, 4 °C the supernatant was removed and the pellet washed in 1 ml 75% ethanol per 1 ml Trizol. After a brief vortex, the sample was centrifuged at 7500 × g for 5 min (4 °C). Ethanol was removed and the pellet air dried for 5–10 min prior to resuspending in RNase-free water. Five biological replicates were generated for each treatment group.

2.6. RNASeq

Total RNA samples (1–100 μg, with a RIN score of at least 7.6) were submitted to the Georgia Genomics and Bioinformatics Core, University of Georgia for library preparation and sequencing. RNA-Seq libraries were synthesized using a TruSeq Stranded Total RNA LT Sample Prep Kit (Illumina, San Diego, CA). Paired-end sequencing was performed on two Illumina NexSeq 500 runs (150 bp, high output). Raw read data were demultiplexed and converted to fastq using bcl2fastq from Illumina. Bioinformatics analyses were carried out by the UGA Quantitative Biology Consulting Group as follows: Raw and trimmed read data were quality assessed using FastQC (ver. 0.11.4) software (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and residual adapter/index removal and quality trimming was done with Trimmomatic software (Bolger et al., 2014). A minimum read length cutoff of 50 bases was employed and average base and read qualities were ≥ QC 32. Average loss of data after trimming was typically 6–7%. The HG38 human genome (GCF_000001405.31_GRCh38.p5) and associated GFF files were obtained from NCBI and trimmed reads were mapped using Tophat2, ver. 2.0.14, run at default settings (Kim et al., 2013). Mapping resulted in a typical concordant pair alignment of > 94%. BAM files were name sorted with SAMtools (Li et al., 2009) and HTSeq (Anders et al., 2015) was used to extract raw count data for each sample. HTSeq output files containing gene-to-count information for each sample were concatenated to generate the count matrices used in subsequent expression analyses. The DESeq2 Bioconductor package (Love et al., 2014) was run in the R computing environment to normalize count data, estimate dispersion and test for differential expression using negative binomial generalized linear models for analysis of differentially expressed genes. A meta data table was generated containing relevant sample and replicate associations for each

experimental and control group and included a secondary (batch) analysis column to account for differences in sample collection times of replicates within each group. P-values were adjusted for multiple testing for determination of false discovery rate (FDR) using the Benjamini-Hochberg correction (Benjamini and Hochberg, 1995). Regularized logarithm (rlog) transformation of raw count matrices was used to generate principle component analysis (PCA) plots. All the data are available at NCBI BioProject ID PRJNA328502.

2.7. Data analysis

Comparisons of survival with and without immune cells over time or with and without drug were analyzed by 2-way ANOVA and Sidak's multiple comparisons test using Graphpad Prism[®], v5 (GraphPad Software, INC., San Diego, CA).

3. Results

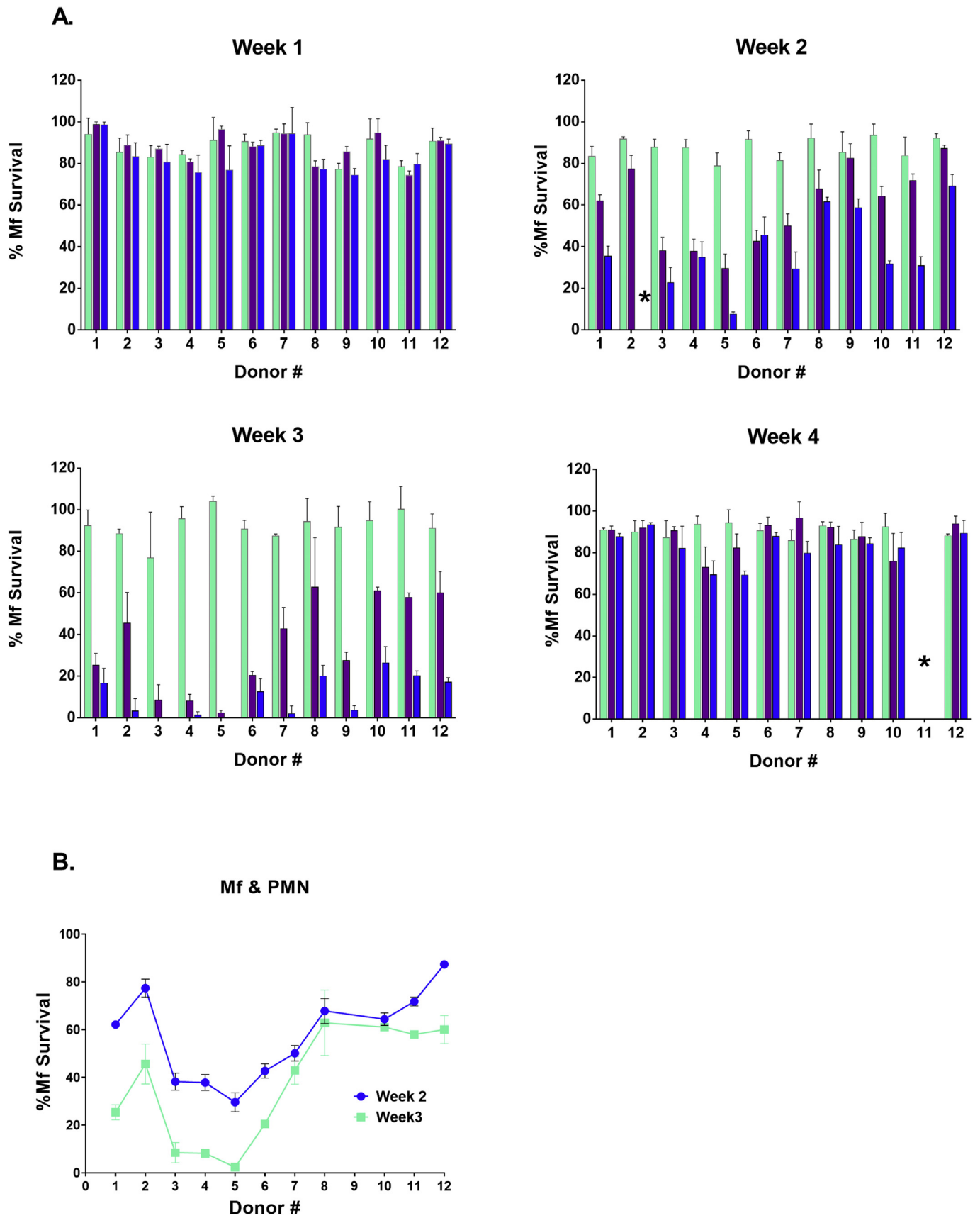
3.1. Killing of *B. malayi* Mf by human immune cells correlates with Mf cohort

Prior studies in our laboratory established an assay system designed to monitor the attachment and killing of *B. malayi* Mf by neutrophils (PMNs) and peripheral blood mononuclear cells (PBMCs) derived from human donors (McCoy et al., 2017). Results from those experiments fell mainly into two categories; 1) Mf survived co-culturing with immune cells to similar levels as controls without cells (“non-killing”) and 2) Mf survival was significantly inhibited compared to the no cell controls (“killing”). Each of these experiments was carried out using cells from a different donor. Since the human immune system varies greatly between individuals (Brodin and Davis, 2017), we originally hypothesized that the differences in Mf survival might be due to this variation. However, both the human cells and the parasites were isolated from different individuals every week. Therefore, to determine whether it was human donor or Mf batch responsible for this variation we conducted the following experiment.

Twelve healthy uninfected individuals were recruited. PMNs and PBMCs were isolated and survival assays conducted with a different batch of Mf over four consecutive weeks. In weeks 1 and 4, no significant difference was detected between Mf cultured for six days without cells and those incubated with either PMNs alone or PMNs and PBMCs from any of the 12 donors – these were “non-killing” weeks. In contrast, in weeks 2 and 3 a significant amount of killing occurred with both PMNs and PMNs and PBMCs from all of the 12 donors (Fig. 1A). In these weeks there was also statistically significant variation in the amount of killing between individual donor cells. Although the absolute amount of killing varied for each set of donor cells in these weeks, a comparison of all 12 individual results with either PMNs or PMNs and PBMCs revealed a striking similarity in the relative amounts of inter-individual killing from week to week (Fig. 1B). These results clearly indicate the major component responsible for the variability in killing we observed between experiments is the Mf rather than differences between cells or serum from individual human donors. However, when killing does occur, there were inter-donor differences in the ability to kill Mf and this supports at least a partial role for the individual's immune response in this variation.

3.2. Mf processing affects survival when co-incubated with PMNs and PBMCs

Our multi-donor experiments indicated that Mf variation is the major factor responsible for the variability in immune cell killing. After 5–6 days in co-culture with immune cells, the number of Mf surviving was determined by counting intact, motile worms. In these assays, we observed few if any intact dead worms at the final time point. Many of the starting cohort appeared to be coated in immune cells (Fig. 2A,



(caption on next page)

Fig. 1. Mf survival in co-cultures with PMNs and PBMCs is Mf-dependent. A) One batch of Mf was incubated either alone (light green bars), with PMNs (purple bars) or with PMNs and PBMCs (blue bars) isolated from 12 different donors. Mf survival was counted at 1, 3 and 6 days, 6 day results are shown. In weeks 1 and 4 Mf survived in the absence or presence of immune cells. In weeks 2 and 3, both PMNs and PMNs and PBMCs from all donors were capable of killing Mf. Error bars represent standard error of the mean. Asterisks (*) denote missing data due to lack of cells (Donor 2, week 2) or donor samples (Donor 11, week4). B) Percent survival from Mf plus PMNs in ‘killing’ weeks 2 and 3 were plotted to highlight the inter-individual variation. Error bars represent standard error of the mean. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

middle panel, Mf & PMNs) with only a portion of the worm clearly visible (arrowhead in Fig. 2A, bottom panel, Mf & PMNs & PBMCs). Aggregates containing no detectable Mf were also observed. Considering the number of visible Mf after 6 days of co-culture was much lower than the starting numbers, one explanation is that parasites were degraded and/or consumed by the immune cells. We sought to determine whether Mf needed to be viable for immune cell recognition and degradation by comparing the ability of cultured leukocytes to recognize and consume heat-killed Mf versus living parasites. Mf were processed as described in Materials and Methods prior to dividing the Mf into two aliquots. One half was killed by heat at 55 °C for 30 min.

Heat-killed (HK) Mf exhibit a characteristic rod-like appearance in contrast to the motile profile of the viable worms (Fig. 2A, top panel, Mf (left) versus heat-killed Mf (right)). Control or heat-treated Mf were incubated either without cells (Fig. 2A, top panels; Fig. 2B blue bars) or with the same preparation of PMNs alone (Fig. 2A, middle panels, Fig. 2B green bars), or PMNs and PBMCs (Fig. 2A, bottom panels, Fig. 2B purple bars). The number of detectable Mf were counted at 2, 24 and 120 h post-incubation. Control Mf were killed as early as 2 h post-incubation in the presence of PMNs and PBMCs and at 120 h less than 10% remained (Fig. 2B). In contrast, the number of heat-killed Mf remained unchanged either with or without cells (Fig. 2B).

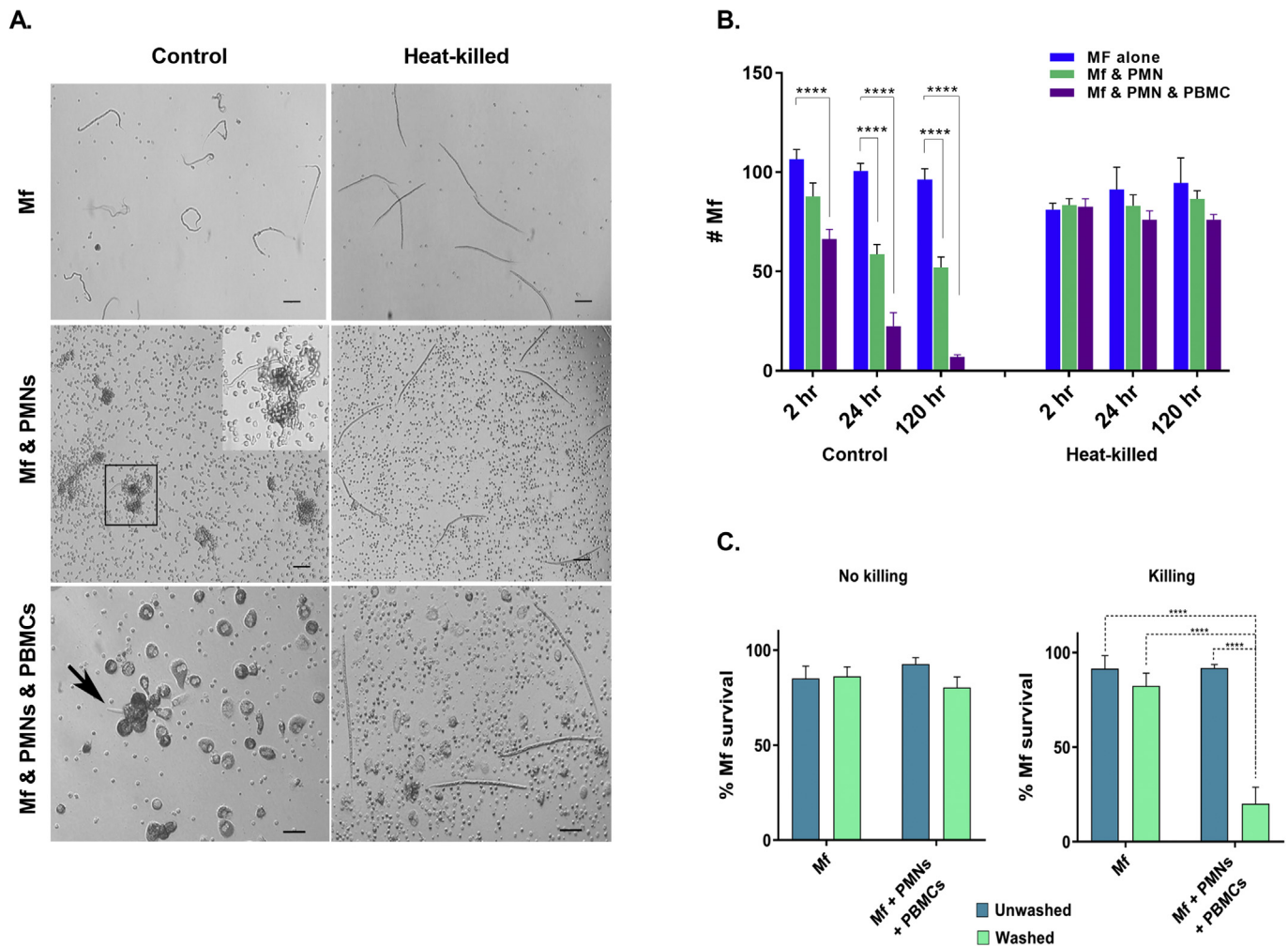


Fig. 2. Mf condition affects interactions with immune cells. A) Micrographs depicting control Mf (left panels) and heat-killed (HK) Mf (right panels) incubated without cells (top panels) or with PMNs (middle panel) or PMNs and PBMCs (bottom panel) for 120 h. The inset in Mf & PMNs shows the degree of association of immune cells with Mf. The arrow in Mf & PMNs & PBMCs indicates a portion of one Mf, the rest being covered by cells. Bar = 50 μm. B) Numbers of control Mf decreased in the presence of PMNs or PMNs and PBMCs at 2, 24 and 120 h versus Mf alone (‘control’), ****p ≤ 0.0001 using two way ANOVA, N = 3, each sample was assayed in triplicate. The number of heat-killed Mf without cells was not statistically different to those incubated with either PMNs or PBMCs at any time point (‘heat-killed’). Error bars represent standard error of the mean. C) The percent survival at 120 h versus 2 h in ‘no-killing’ experiments comparing Mf alone to Mf & PMNs & PBMCs was not statistically different whether Mf were washed according to the standard protocol (green) as described in Materials and Methods, or whether they were added without washing and filtering (blue). In ‘killing’ experiments (left panel), the percent of Mf survival decreased at 120 h in the presence of PMNs and PBMCs (green bars) ****p ≤ 0.0001 using two way ANOVA; in contrast when Mf were not washed, there was no statistical difference between ‘no cell’ controls and addition of PMNs and PBMCs (blue bars). Error bars represent standard error of the mean. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

One proposal for the mechanism that enables Mf to evade the host immune system is the secretion of immunomodulatory molecules through the excretory-secretory (ES) pore (Falcone et al., 2001; Moreno et al., 2010; Harnett, 2014; Pineda et al., 2014). In our previous experiments, Mf were washed several times with PBS and then filtered before resuspension in fresh RPMI prior to incubation with PMNs and PBMCs. This procedure could remove any such molecules prior to addition. To investigate whether this washing procedure affected immune cell recognition and killing, Mf were divided into two samples, one treated as previously with two PBS washes, filtering on day 1 and another filtering on day 2 prior to resuspension in RPMI. The other sample was not subjected to further processing and were added to the assay in the culture medium they had been incubated in for 2 days. Each of these experiments were carried out with one individual cell donor and a different batch of Mf. Results of these experiments varied with the ability of the cells to recognize the Mf, with some weeks being ‘killing weeks’ and others ‘non-killing’, as previously described (Fig. 1 and (McCoy et al., 2017)). If no killing occurred, no significant difference was observed between experiments using washed and unwashed Mf. However, in experiments when killing of the washed worms did occur, there was no significant killing of the unwashed worms (Fig. 2C). These findings support the hypothesis that there are either secreted or surface factors inhibiting recognition and killing of Mf by PMNs and PBMCs which can be eliminated by thorough washing.

3.3. Effects of IVM and DEC on Mf survival in the presence of immune cells

IVM and DEC clear Mf rapidly from human subjects. Our survival assay allowed us to investigate whether IVM and DEC could increase the ability of immune cells to attach to and kill Mf *in vitro*. The differences in survival outcome week to week allowed us to test whether the drugs could either enhance a “killing” scenario or alternatively, induce killing when Mf survived even in the presence of PMNs and PBMCs – the “non-killing” experiments. In addition to a no drug control containing Mf alone, Mf plus PMNs or Mf plus PMNs and PBMCs, either 1 μM IVM or 200 μM DEC was added, and the Mf counted at 2, 24 and 120 h post co-incubation (Fig. 3A). These drug concentrations are considerably higher than those reported to be present in the plasma of treated patients so we would expect to be able to detect any pharmacologically relevant effect of the drugs. In experiments where killing was observed, there was no significant difference in Mf survival between control (no drug) and IVM or DEC at 24 or 120 h. At 2 h post-incubation there was a slight but statistically significant reduction in survival between the DEC treated samples and the no drug control ($p \leq 0.05$). In “non-killing” experiments, neither IVM nor DEC acted to enhance killing; no significant difference between control and drug treated samples was observed (Fig. 3B). Even in the absence of cells, neither IVM nor DEC had any effect on Mf survival corroborating previous findings that these drugs do not kill Mf directly at these concentrations (Court et al., 1986; Gunawardena et al., 2005; Tompkins et al., 2010; Storey et al., 2014).

One possibility for the lack of effect when co-incubating Mf with immune cells and drug at the same time is that the Mf may be able to abrogate the effects of the drugs with immediate effect whereas immune cells may need to be ‘activated’ prior to addition in order to enhance killing. To test this hypothesis we pre-incubated immune cells for either 1 h or 24 h prior to addition of the Mf. After either a 1 h or 24 h pre-incubation the number of Mf in controls versus either IVM or DEC treatment mirrored the effects without pre-incubation; there was no statistically significant difference in the number of Mf between no drug and drug treated samples (Figure S1).

3.4. Effects of IVM and DEC on PMN and PBMC gene expression

Findings described in the previous experiments suggest IVM and DEC are not acting directly on immune cells to enhance Mf killing. The

immune system, however, relies on diverse molecules and cells that act together to ultimately exert their effect (Bergthaler and Menche, 2017). Therefore treatment with either DEC or IVM could act by altering gene expression in PMNs or PBMCs leading to the production of factors which target additional immune components not present in our *in vitro* assay. To address this possibility we incubated PMNs and PBMCs with either 0.1% DMSO as a control, 50 μM DEC or 100 nM IVM in RPMI containing 5% autologous serum for either 1 h or 16 h respectively prior to extracting RNA and analysis by RNA-Seq.

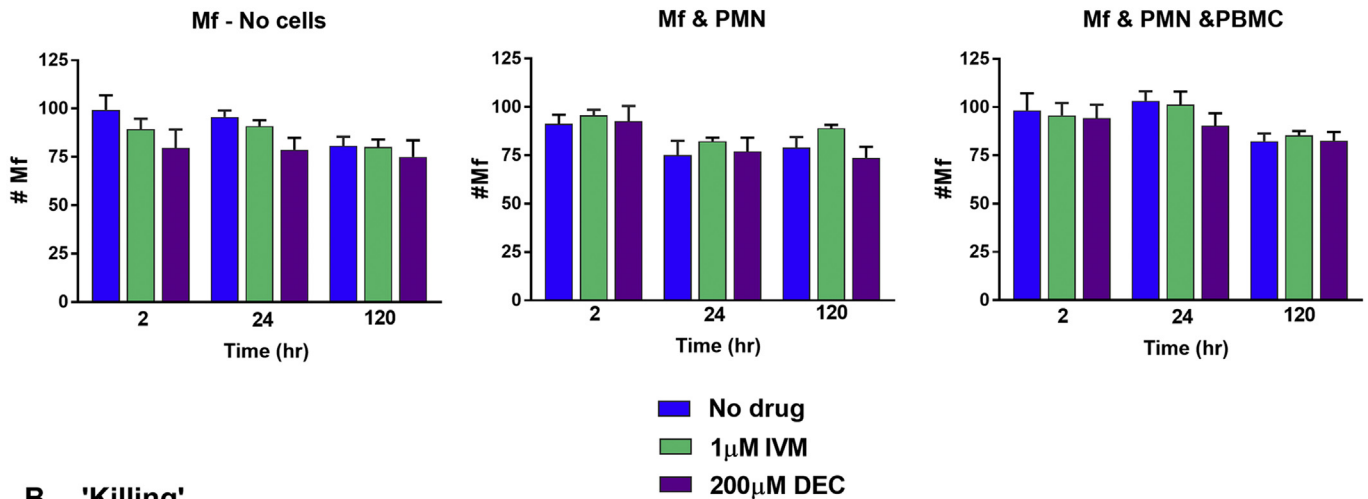
Results from these experiments showed an absence of differentially expressed genes in either PMNs or PBMCs treated with DEC or IVM. Principal component analysis (PCA) of the transcriptomic data from single determinations of PBMCs from 4 individual donors (5.1–8.1) and two determinations from one donor (4.1 and 4.2) (Fig. 4) demonstrates that the data collected from all treatment groups associated with one individual tended to be more similar to each other compared to those from the same treatment groups. Although this data does not preclude that IVM or DEC induced subtle changes in PMN or PBMC gene expression can affect Mf survival, the inter-individual variation may mask this effect.

4. Discussion

Filarid nematode infections typically result in the appearance and survival of very large numbers of microfilariae in the host. These Mf are capable of living for a considerable period of time, showing that they must be able to evade or avoid the host immune system (Harnett, 2014; Harnett and Harnett, 2006a; b). Treatment of people infected with these parasites with anthelmintic drugs such as DEC or ivermectin results in a rapid and long-lasting clearance of the Mf from the circulation (Kwarteng et al., 2016; Ottesen, 2006). The long-lasting suppression of Mf numbers following ivermectin treatment is due to the suppression of new Mf production by adult females, possibly mediated via the expression of glutamate-gated chloride channels, the proposed targets of ivermectin in parasitic nematodes (Wolstenholme, 2012), in the female reproductive organs (Li et al., 2014). However, this cannot explain the rapid clearance of Mf, which happens within hours. *In vitro*, ivermectin and DEC have limited effects on Mf at pharmacologically relevant concentrations, suggesting that their rapid removal is not due to direct killing of the parasites by the drugs. This has led to suggestions over many years that the anthelmintic effects of DEC and ivermectin may be mediated through the host immune system, either directly or by affecting the parasites’ ability to evade detection and destruction (Maizels and Denham, 1992; Kwarteng et al., 2016; Wolstenholme et al., 2016). Ivermectin inhibits secretion by *B. malayi*, which provides a possible mechanism for this inhibition of their ability to modulate the immune system (Moreno et al., 2010; Harischandra et al., 2018).

Our recent finding that ivermectin can increase the attachment of canine PMNs and PBMCs to *Dirofilaria immitis* Mf *in vitro* (Vatta et al., 2014) supports this suggestion and the development of an *in vitro* assay to measure attachment to, and killing of, *B. malayi* Mf by human cells (McCoy et al., 2017) allowed us to test these suggestions for a human parasite. However, some aspects of the *B. malayi*/human leukocyte assay required clarification. We observed considerable variation in the amount of attachment and parasite killing between experiments; each experiment used cells isolated from a different human donor and parasites isolated from a different gerbil. The experiments in Fig. 1 tested whether it was the human cells or the parasite that was responsible for this variation and clearly show that the largest effect was given by the Mf, though we did see some variation between the human donors as well. This was despite our best efforts to keep the conditions during the isolation of the Mf from the gerbils as consistent as possible. Since the Mf were obtained from a third party (FR3), and the experiments were carried out over several months, it was unavoidable that the Mf were isolated from gerbils infected with different batches of L3 at different times, and we were unable to test the effect of this. The

A. 'Non-killing'



B. 'Killing'

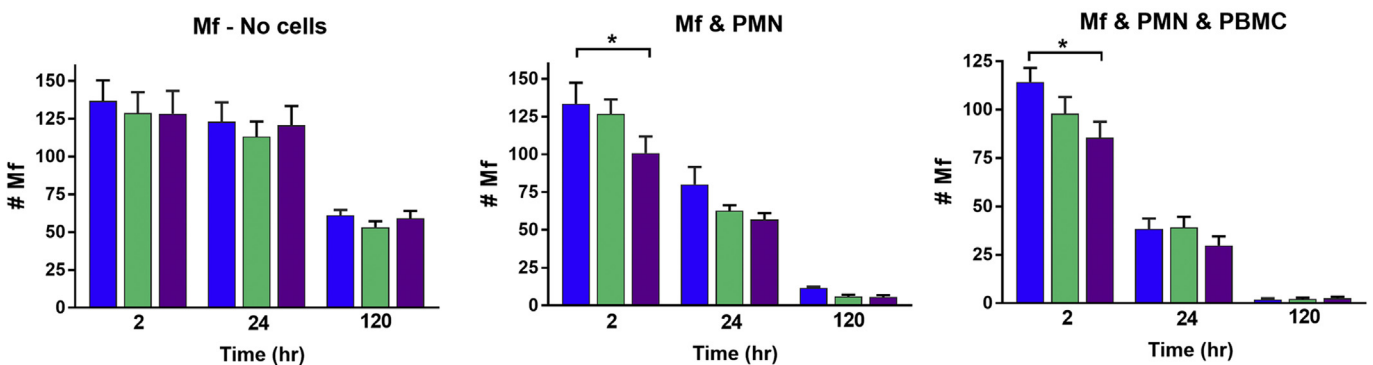


Fig. 3. Effect of IVM and DEC on Mf survival in the presence of PMNs and PBMCs. **A)** 'Non-killing'. Either Mf alone (left panel), Mf & PMN (middle panel) or Mf & PMN & PBMCs (right panel) were incubated either with no drug (blue bar), 1 μM IVM (green bars) or 200 μM DEC (purple bars) for 2, 24 or 120 h as indicated. In these experiments there was no significant difference in the number of Mf surviving at either 24 or 120 h ('non-killing') in the presence of immune cells and no significant difference between no drug and drug treated samples. **B)** 'Killing'. Either Mf alone (left panel), Mf & PMN (middle panel) or Mf & PMN & PBMCs (right panel) were incubated with no drug (blue bars), 1 μM IVM (green bars) or 200 μM DEC (purple bars) for 2, 24 or 120 h as indicated. In these experiments there were significant differences in the number of Mf surviving at both 24 as well as 120 h versus 2 h when Mf were incubated with PMN alone or PMN & PBMC ('killing') (blue bars middle and right panels), $p \leq 0.0001$ by two way ANOVA (significance levels have not been included in the graph for these comparisons for clarity); however the only significant difference between control and drug treated was between no drug and DEC at 2 h post incubation $*p \leq 0.05$ (blue bars versus green or purple). $N = 5$, each sample assayed in triplicate. Error bars represent standard error of the mean. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

possible source of the variation between Mf batches will be investigated further. We did note some differences in the presence and density of granuloma formation in the gerbils and did attempt to correlate this with Mf killing, however the number of experiments for which this information was available precluded a definitive conclusion.

Heat killing the Mf prior to exposing them to PMNs and PBMCs abolished leukocyte recognition and destruction of the worms. This was in contrast to live Mf, which were quickly removed after cell attachment (Fig. 2). The heat treatment clearly destroyed something that the cells recognize; this may be a protein or other parasite product, or it may be that the motility of the Mf is required for cell attachment. With *Pseudomonas aeruginosa* neutrophil extracellular trap formation requires that the bacteria are motile (Floyd et al., 2016) and *B. malayi* Mf do induce the formation of such traps (McCoy et al., 2017). An alternative approach was taken by omitting the PBS-washing steps during Mf isolation, as we hypothesized that these might remove a protective factor from the parasite surface. The data in Fig. 2C provide some support for that hypothesis, in that omitting the washing steps did abolish cell binding and killing in those experiments where it occurred; however, this does not explain why the same procedure only makes the Mf

susceptible to the cells some of the time. Taken together, the heat-killing and washing experiments suggest that multiple factors affect the Mf-leukocyte interaction. A heat-labile factor is required for human leukocytes to recognize and consume Mf, but the parasites also produce factors, which may be secreted into medium or remain associated with the worms' surface, that inhibit this recognition. *B. malayi* and other filarids produce many potential immunomodulatory molecules (Hewitson et al., 2008; Harnett, 2014); the identification of the exact ones involved here will be the subject of future investigations.

Our assay permitted us to test the effects of adding either DEC or ivermectin on leukocyte attachment and killing, both under conditions where these were taking place in the absence of the drug and also when they were not. Ivermectin did not have any significant effect on Mf survival in the presence of the human PMNs or PBMCs (Fig. 3), regardless of when it was added. This is in contrast to our data with *D. immitis*, where the drug increased canine PMN and PBMC attachment (Vatta et al., 2014); no Mf killing is seen in the dog/*D. immitis* experiment, whereas human cell attachment to *B. malayi* Mf always results in the destruction of the parasites. It is also in contrast to observations made with other filarial nematodes, such as *Acanthocheilonema viteae*

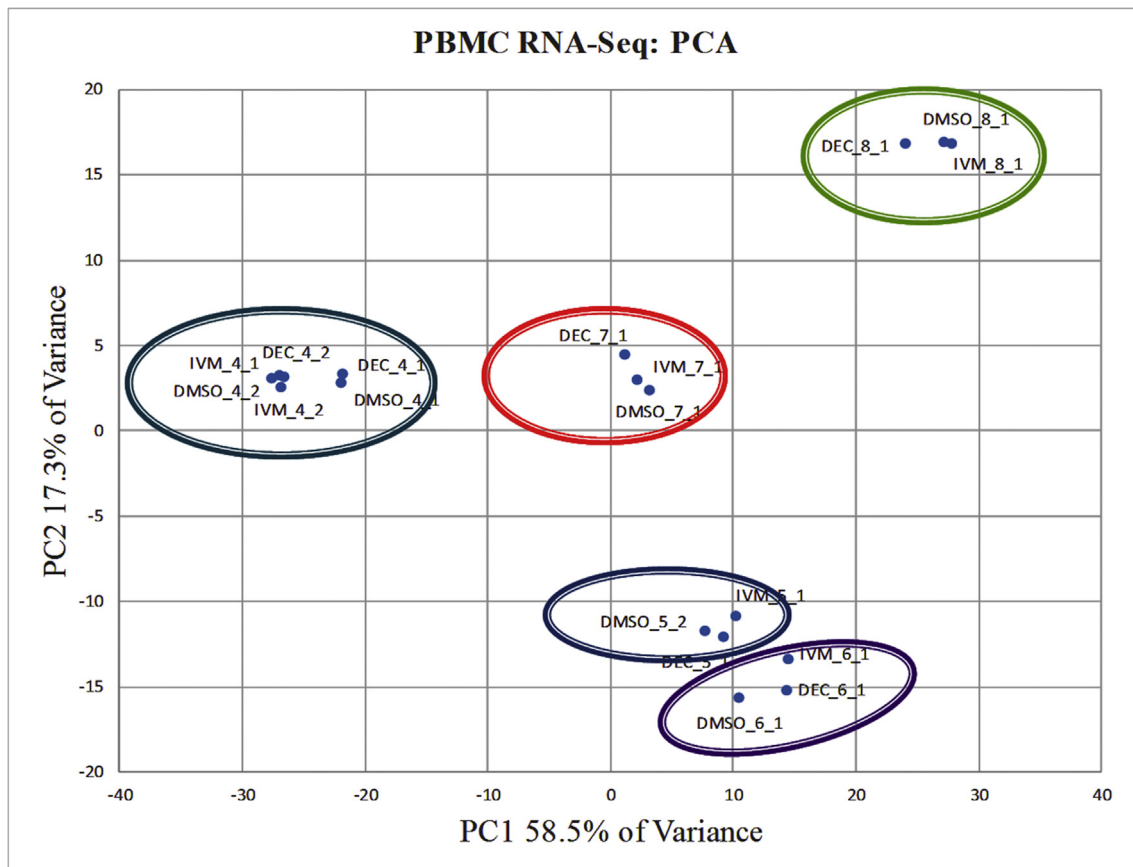


Fig. 4. PCA analysis of the effects of IVM and DEC on PBMC gene expression. PBMCs from 5 separate individuals were incubated with either 50 μ M DEC or 100 nM IVM prior to processing and analysis by RNA-seq. Each colored circle indicates results from one donor. For all individuals, no drug, IVM and DEC results clustered together indicating inter-individual variation masked any differences between control and drug treatment. Two separate samples for donor number 4 were processed and yielded similar results (left dark blue circle). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(Rao et al., 1987) or *Litomosoides carinii* (Zahner et al., 1997). DEC had a small effect on Mf survival early in the incubation (2 h) in those experiments where killing was observed, however this effect disappeared after 24 h. Neither drug had any effect on gene expression in PMNs or PBMCs, though small effects may have been masked by the high level of variation seen between the individual samples used in this experiment. These results provide only very limited support, none for ivermectin, for any interaction between DEC and the human immune cells used in this *in vitro* assay. At present, it is not possible to distinguish whether this is because such an interaction does not exist, or that this *in vitro* assay is not capable of detecting it. Any *in vitro* assay is obviously a very simplified version of the complex interactions that take place *in vivo*, so our data does not imply that there are no interactions between anthelmintic drugs and the host immune system, merely that our current experimental set-up did not detect them.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijpddr.2018.09.002>.

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