RESEARCH REPORT

Conservation of cytokine-mediated responses in innate immunity: a flow cytometric study investigating the effects of human proinflammatory cytokines on phagocytosis in the earthworm *Eisenia hortensis*

SL Fuller-Espie[1], L Goodfield[1], K Hill[1], K Grant[2], N DeRogatis[3]

[1] Science Department, Cabrini College, Radnor, Pennsylvania, USA
[2] College of Graduate Studies, Thomas Jefferson University, Philadelphia, Pennsylvania, USA
[3] The Children's Hospital of Philadelphia, Department of Pathology and Laboratory Medicine, Philadelphia, Pennsylvania, USA

Abstract

This study was aimed at determining the influence of human proinflammatory cytokines on innate immune responses in the earthworm *Eisenia hortensis*. Preincubation of earthworm coelomocytes *in vitro* with either interleukin-1 beta (IL-1 beta), granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-2 (IL-2), or tumor necrosis factor-alpha (TNF-alpha) followed by subsequent bacterial challenge was carried out to investigate whether human proinflammatory cytokines would induce a state of enhanced responsiveness in phagocytic cells derived from the coelomic cavity of *E. hortensis*. The effect on phagocytosis by large coelomocytes (hyaline amebocytes) was evaluated using flow cytometry where the uptake of *Escherichia coli* expressing green fluorescence protein in the presence or absence of pretreatment with proinflammatory cytokines was measured. Our results show that proinflammatory cytokines enhanced phagocytosis to a statistically significant ($p \leq 0.05$) degree in 10-18 % of earthworms tested for IL-1 beta, 20 % for GM-CSF, 20-27 % for IL-2, and 27-30 % for TNF-alpha, depending on the cytokine concentration used. Our results favor the suggestion that receptor-coding genes have been conserved through evolution between vertebrates and invertebrates.

Key words: *Eisenia hortensis*; proinflammatory cytokines; phagocytosis; innate immunity; flow cytometry; hyaline amebocytes

Introduction

Invertebrates possessing a coelomic cavity utilize innate immune responses consisting of highly effective cellular and humoral components (Roch, 1996; Cooper *et al.*, 2002). For example, the coelomic fluid in earthworms consists of lytic and antimicrobial components such as eiseniapore (Lange *et al.*, 1997; Lange *et al.,* 1999), the cytokine-like cytolytic protein known as coelomic cytolytic factor 1 (CCF-1), and the highly homologous proteins fetidin and lysenin (Bruhn *et al.*, 2006; Procházková *et al.*, 2006). CCF-1 is functionally analogous with the mammalian cytokine tumor necrosis factor (Bilej *et al.*, 1995). When earthworms

were injected intracoelomically with the endotoxin lipopolysaccharide (LPS) both humoral and cellular levels of CCF-1 expression increased significantly (Bilej *et al.,* 1998), an effect also observed with TNF when mammals were injected with LPS (Carswell *et al.*, 1975). Despite functional analogy between CCF-1 and TNF, it is interesting to note the absence of gene homology between the two (Beschin *et al.*, 2004). It has been suggested that CCF-1 is representative of a primitive inflammatory cytokine-like factor which participates in humoral and cellular inflammatory responses including the activation of prophenoloxidase activity (Bilej *et al.*, 1998; Beschin *et al.*, 1999).

Cytokine-like factors have also been reported in other invertebrates. Prendergast *et al.* (1983), working with the starfish, *Asterias forbesi*, isolated sea star factor (SSF) and demonstrated its ability to function across species by stimulating chemotaxis and macrophage activation in mammals.

*Corresponding autho*r:
Sheryl L Fuller-Espie
Science Department
Cabrini College, 610 King of Prussia Road
Radnor, Pennsylvania 19087-3698, USA
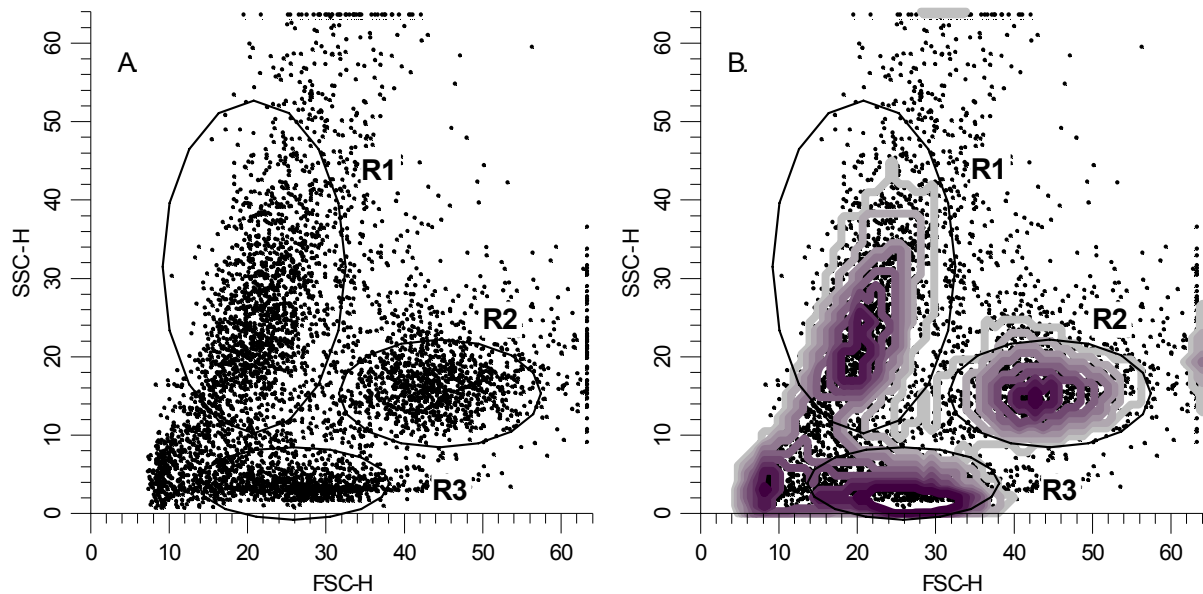Email: sfuller-espie@cabrini.edu

Fig. 1 Typical scatter profile of earthworm coelomocytes using flow cytometry. A two parameter dot plot measuring forward scatter (FSC) versus side scatter (SSC) of a typical earthworm coelomocyte population after extrusion and incubation overnight in SDMEM medium is shown. This data shows the results obtained from EW14. Three distinct populations are indicated; region 1 (R1) represents the eleocytes, region 2 (R2) represents the large coelomocytes (LC), and region 3 (R3) represents the small coelomocytes (SC). Dot plots are shown without (A) and with (B) contour marks to emphasize densities of subpopulations.

The fact that earthworms possess cytokine-like factors such as CCF-1 has prompted much attention on the biochemical activities of these cytokine-like factors, their evolution and conservation, and cross-species activity using mammalian-derived cytokines. The studies of Renzelli-Cain *et al.* (1995) demostrated that mammalian interleukin-1 alpha (IL-1 alpha) enhanced phagocytic activity of opioid-treated amebocytes of the earthworm *Lumbricus terrestris*, and also influenced their aggregation and conformation. Interleukin-8 (IL-8) has been shown to mediate alterations in cell shape and increase chemotaxis and phagocytic activity in the mussel *Mytilus galloprovincialis* (Ottaviani *et al.*, 2000). In the freshwater snails *Planorbarius corneus* and *Viviparus ater*, preincubation with IL-1 and TNF-alpha caused a decrease in the release of biogenic amines when subsequently exposed to corticotrophin-releasing factor, a proto-type stress response inducer (Ottaviani *et al.*, 1995). In addition, the natural killer (NK)-like activity of *P. corneus* hemocytes was preserved *in vitro* when incubated with interleukin-2 (IL-2) (Franceschi *et al.*, 1991). Franchini *et al.* (2000; 2006) have also demonstrated that the giant leopard slug *Limax maximus* had accelerated tissue repair when platelet-derived growth factor-AB (PDGF-AB) and transforming growth factor-beta (TGF-beta) were applied to wounds.

Investigation of the cellular immune responses of earthworms has resulted in the categorization of coelomocytes (leukocytes) which reside in the coelomic cavity into three major subpopulations, hyaline amebocytes (large coelomocytes), granular amebocytes (small coelomocytes), and chloragocytes (eleocytes). The coelomocytes are easily harvested from experimentally-induced earthworms following

extrusion of coelomic fluid from the dorsal pores of the body wall, and can be manipulated *in vitro* to study their immune functions. The large coelomocytes (LC) are the major phagocytic cells, the small coelomocytes (SC) constitute the population exhibiting NK-like activity, and the eleocytes contain chloragosomes and do not participate in either phagocytic or NK-like activities (Cooper, 1996; Cossarizza *et al.,* 1996; Adamowicz *et al.,* 2001; Engelmann *et al.,* 2002; Engelmann *et al.,* 2005).

Owing to the physical differences between coelomocyes including their granularity and size, flow cytometry permits LC, SC and eleocytes to be distinguished based on forward light scatter (FSC) and side light scatter (SSC) properties (Cossarizza *et al.,* 1996; Engelmann *et al.,* 2004; Cossarizza *et al.,* 2005; Patel *et al.,* 2007). In addition, flow cytometry can be used to selectively analyze immune functions of the subpopulations by drawing regions around and gating only those cells desired for investigative purposes. Although light microscopy has been used by researchers to study phagocytosis in earthworms (Adamowicz *et al.,* 2001; Kalaç *et al.,* 2002), this approach lends itself to subjectivity, and places restrictions on the number of cells that can be included in quantitative assays based on time constraints. Flow cytometry, in contrast, is an objective quantitative methodology which is capable of analyzing several thousands of cells per second, and limiting analysis to predetermined subpopulations.

The selected examples cited above illustrate that mammalian cytokines can trigger immune responses in invertebrate models. We sought to investigate the effects of four proinflammatory cytokines, namely IL-1 beta, GM-CSF, IL-2 and TNF-alpha, on the phagocytic activity of

coelomocytes isolated from the earthworm *Eisenia hortensis*. Our study used flow cytometry to follow the uptake of *Escherichia coli* expressing green fluorescent protein after gating on coelomocytes that satisfied the FSC and SSC parameters characteristic of LC. Our results show that preincubation of LC with the proinflammatory cytokines IL-1 beta, GM-CSF, IL-2 and TNF-alpha induced a stimulatory effect on *in vitro* phagocytosis in 10-30 % of the earthworms tested in this study.

Materials and methods

Reagents

General laboratory reagents and plasticware were purchased from Fisher Scientific unless otherwise noted.

Cell culture

All cell culture reagents and phosphate-buffered saline (PBS) were purchased from Invitrogen unless otherwise noted. Dulbecco's Modified Eagle Medium (DMEM) was supplemented with 10 % fetal calf serum, 100 μg/ml ampicillin (Shelton Scientific), 10 μg/ml kanamycin (Shelton Scientific), 10 μg/ml tetracycline, 5 μg/ml chloramphenicol (Fluka Biochemika), 1x penicillin/streptomycin/amphotericin B, 1x nonessential amino acids and 1x L-glutamine (Gibco) to comprise Super DMEM (SDMEM).

Earthworm husbandry

Eisenia hortensis (European nightcrawlers) were purchased from Vermitechnology Unlimited, Orange Lake, Florida, USA, who import *E. hortensis* from Star Food, Holland, Scherpenzeelseweg 95, 3772ME Barneveld, The Netherlands. Species identity was determined by the United States Department of Agriculture, USDA Permit #52262 (Vermitechnology, personal communication). Short-term colonies were maintained at room temperature (RT) in the dark on autoclaved shredded paper moistened with water and Single Grain Rice Cereal or Rice with Bananas Cereal (Gerber) until use. Shredded paper was changed twice weekly.

Extrusion of coelomocytes

Prior to experimentation, earthworms were chosen based on their color and activity; earthworms with healthy deep coloration, lacking yellow appearance, and with high activity were placed overnight on moist paper towels saturated with 2.5 μg/ml Fungizone (Fisher Scientific) to reduce the level of fecal material and other surface contaminants.

To collect coelomocytes from an earthworm, earthworms were extruded according to Engelmann *et al.* (2004) with minor modifications. Briefly, earthworms were placed in a 100 mm Petri dish containing 3 ml ice cold extrusion buffer (71.2 mM NaCl, 5 % v/v ethanol, 50.4 mM guaiacol-glyceryl-ether, 5 mM EGTA, pH 7.3). The coelomocytes were then transferred to 1 ml Accumax (Innovative Cell Technology) for a 5 min incubation period at RT. Then the cells were washed with 5 ml Lubricus Balanced Salt Solution (LBSS, 71.5 mM NaCl, 0.3 mM NaH$_2$PO$_4$, 4.2 mM NaHCO$_3$,4.8 mM KCl, 0.4 mM KH$_2$PO$_4$, 1.1 mM MgSO$_4$ x 7 H$_2$O, pH 7.3), prior to centrifugation at 150xg for 5 min at 4 ^{o}C.

Coelomocytes were resuspended in 1 ml SDMEM and enumerated using a hemocytometer. Only large coelomocytes (LC) and small coelomocytes (SC) were included in the cell count; eleocytes were not counted but did factor into a quality score. Samples with large numbers of eleocytes compared to LC and SC were not used in phagocytosis assays.

Cytokines

All cytokines were reconstituted and stored according to vendors' recommendations. Recombinant human IL-1 beta was purchased from R & D Systems (201-LB). Recombinant human GM-CSF, recombinant human IL-2, and recombinant human TNF-alpha were purchased from ProSpec-Tany TechnoGene (CYT-221A, CYT-209 and CYT-223, respectively). In all cases purity was greater than 97 % and endotoxin levels were less than 1.0 EU/μg. IL-1 beta was used at 20 ng/ml and 40 ng/ml; GM-CSF was used at 2 ng/ml and 4 ng/ml; IL-2 was used at 12.5 ng/ml and 25 ng/ml; and TNF-alpha was used at 2.5 ng/ml and 5 ng/ml.

Bacteria

Escherichia coli HB101 transformed with pGLO (BioRad) and expressing green fluorescent protein (GFP) were grown on tryptic soy agar containing 100 μg/ml ampicillin and 0.2 % arabinose at 32 $^\circ$C for 24 h. Cells were chemically fixed using 4 % paraformaldehyde for 1 h at RT, and then washed three times with PBS. Centrifugation was carried out at 3273xg for 5 min at 4 ^{o}C, cells were resuspended in PBS, enumerated using a hemocytometer, and stored in the dark at 4 ^{o}C until used. These cells hereafter are referred to as *E. coli*-GFP.

Phagocytosis Assay

Phagocytosis assays were carried out in SDMEM. Coelomocytes [20,000 per well for earthworm (EW) 1 and EW2; 50,000 per well for all others] were pretreated with individual cytokines at concentrations indicated above for 20 h, 5 % CO$_2$ at 20 ^{o}C in 96-well, V-bottom plates in 100 μl. Depending on coelomocyte yield and scope of each experiment, the number of replicates differed between assays affecting the degrees of freedom in the statistical analyses. Experiments were set up in duplicates for EW5 and EW15, in triplicates for EW1, EW4, EW6, EW8-14, and EW16, and quadruplicates for EW2, EW3, and EW5. The CO$_2$ incubator was placed in a 4 ^{o}C walk-in cold room in order to obtain these conditions. Following cytokine pretreatment, *E. coli*-GFP was added to each well at a multiplicity of infection of 1000 bacteria: 1 coelomocyte in 200 μl final volume per well. To control for non-specific binding of *E. coli* to the external surface of coelomocytes, 5 μM cytochalasin B was added to control wells 30 min before the addition of *E.coli*-GFP. Incubation times for *E. coli*-GFP uptake ranged from 1-4 h at 30 ^{o}C.

Following *E. coli*-GFP uptake, trypan blue was used at a final concentration of 0.02 % for 30 min at room temperature in the dark, for quenching purposes to reduce background fluorescence (Mosiman *et al.,*1997). The cells were centrifuged at 150xg for 5 min at 4 ^{o}C, washed once with PBS, and then resuspended in FACS Flow buffer (BD Bioscience) for flow cytometry analysis.

Cytokine (concentration)	Number of earthworms treated with cytokine	Number of responding earthworms (p ≤ 0.05) (EW identity number)	Statistically significant response rate to cytokine (p ≤ 0.05)
IL-1 beta (40ng/ml)	28	5 (EW 4,5,8,12,15)	18 %
IL-1 beta (20ng/ml)	20	2 (EW 3,5)	10 %
GM-CSF (4ng/ml)	10	2 (EW 4,5)	20 %
GM-CSF (2ng/ml)	50	10 (EW 1,2,5,6,8,9,10,11,14,16)	20 %
IL-2 (25ng/ml)	22	6 (EW 4,5,9,11,13,16)	27 %
IL-2 (12.5ng/ml)	10	2 (EW 4,5)	20 %
TNF-alpha (5ng/ml)	22	6 (EW 4,5,9,11,13,14)	27 %
TNF-alpha (2.5ng/ml)	10	3 (EW 5,6,7)	30 %

Table 1 Summary of total number of earthworms used in this study and percent responders. The four cytokines and the concentrations used are indicated. The total number of earthworms pretreated with the cytokines, the number responding with p ≤ 0.05 to each cytokine, the identity of the earthworm responders, and the percentage of statistically significant responses are shown.

Flow Cytometry

Fluorescence was measured using a FACSCalibur flow cytometer and Cell Quest Software (BD Biosciences) with the following specifications for all experiments described in this study: Forward Light Scatter (FSC) E00-Linear, Side Light Scatter (SSC) 332v-Linear, green fluorescent protein fluorescence (FL-1) 312v-Log. LISTMODE data was acquired with Cell Quest Software, and analysis of data was carried out using WinList 5.0 (Verity Software House, Inc.). Only coelomocytes with the appropriate granularity and size corresponding to the large coelomocyte population were gated for analysis in two-dimensional dot plots of FSC vs FL-1. Percent specific phagocytosis was determined by subtracting the autofluorescent background of the controls (coelomocytes incubated with cytochalasin B and *E. coli*-GFP) from the sample fluorescence measured by the FL-1-

detector. All histograms and dot plots were created using WinList 5.0. See Figs 1-2 for representation of data acquisition and analysis.

Statistical Analysis

All graphs and data analysis were created and processed using Microsoft Excel 2007. Only statistically relevant results with p ≤ 0.05 (as defined by Student's t-test) comparing untreated versus cytokine-treated samples are shown in Figs 3-6.

Results

Enhanced phagocytosis was detected when coelomocytes were preincubated with the proinflammatory cytokines IL-1 beta, GM-CSF, IL-2 and TNF-alpha

Fig. 1 illustrates a typical profile of earthworm coelomocytes obtained when measuring forward light
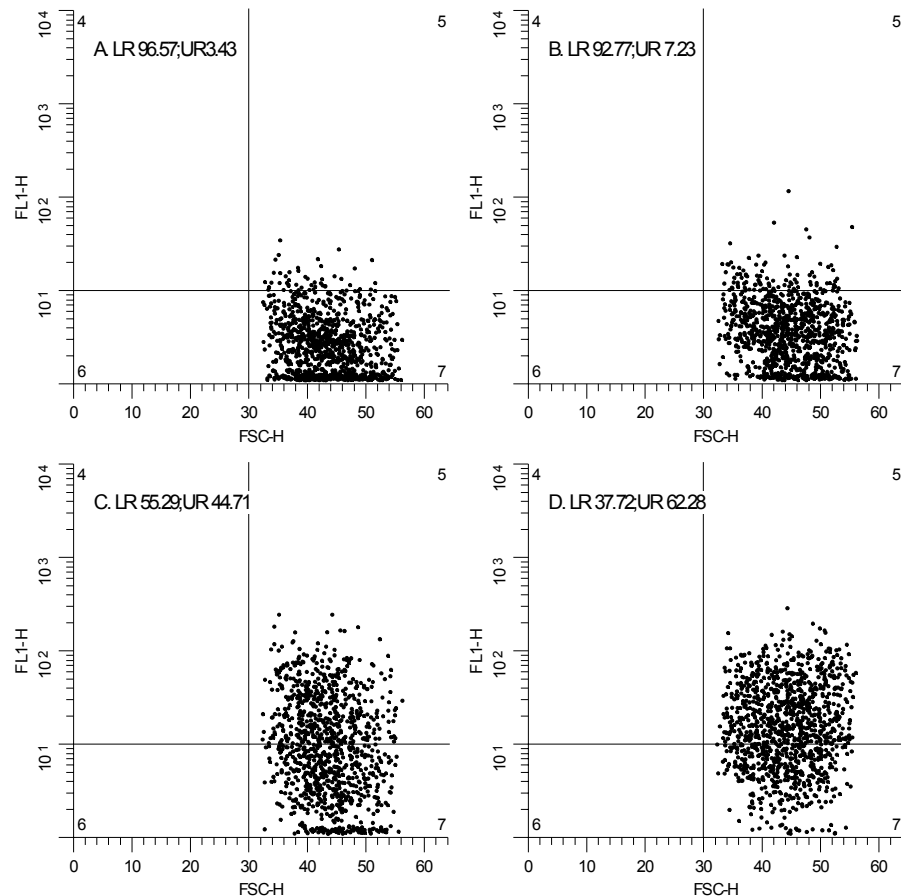
Fig. 2 Characteristic profile of large coelomocytes (LC) before and after phagocytosis and proinflammatory cytokine treatment. Two dimensional dot plots measuring forward scatter (FSC) on the x-axis versus green fluorescence (FL-1) on the y-axis were generated after gating on the R2 subpopulation (LC) as indicated in Fig. 1. This data shows the results obtained for EW11. LR = % gated LC in lower right quadrant; UR = % gated LC in upper right quadrant. A. LC illustrating the level of autofluorescent background. B. LC in the presence of *E. coli*-GFP plus cytochalasin B, an antibiotic which interferes with microfilament activity and inhibits phagocytosis. C. LC incubated with *E. coli*-GFP. D. LC preincubated with IL-2 (25 ng/ml) prior to the addition of *E. coli*-GFP (p = 0.016). *E. coli*-GFP control was not detected using the FSC, SSC and FL-1 settings employed (data not shown).

scatter (FSC) versus side light scatter (SSC) properties. Coelomocytes from EW14 are depicted. Three characteristic populations were routinely observed and three regions, R1, R2, and R3, were drawn around the subpopulations corresponding to the eleocytes, large coelomocytes (LC) and small coelomocytes (SC), respectively. A dot plot represents the total population acquired (Fig. 1A) while a contour plot shows the relative densities of each of the three subpopulations (Fig. 1B).

Percent specific phagocytosis was determined for each earthworm treated with IL-1 beta, GM-CSF, IL-2 and TNF-alpha. Fig. 2 represents a typical profile of responding earthworm coelomocytes measuring forward scatter (FSC) versus GFP fluorescence (FL-1) before and after treatment with IL-2 (25 ng/ml) (p = 0.016). Coelomocytes from EW11 are depicted. FSC versus FL-1 dot plots were gated on the R2 subpopulation (LC) as illustrated in Fig. 1. Each dot plot was divided into four quadrants, upper left (UL) (4), upper right (UR) (5), lower left (LL) (6), and lower right (LR) (7). Since

analyses included R2-gated cells, events fall only in the UR and the LR quadrants. The LR quadrant corresponded to coelomocytes which did not phagocytose *E. coli*-GFP (i.e. nonfluorescent), and the UR corresponded to those coelomocytes which did, exhibited by the higher relative fluorescence intensity (FL-1) compared to controls. Fig. 2A shows the large coelomocytes of EW11 incubated in the absence of *E. coli*-GFP illustrating the level of background autofluorescence observed. Nonfluorescent cells comprised 96.57 % of the total gated LC population (LR), while autofluorescent cells comprised 3.43 % (UR). The LC had relatively low levels of autofluorescence in all samples analyzed, while the eleocytes had significantly higher levels, but the eleocytes were excluded from the analysis. Fig. 2B shows the LC of EW11 incubated with *E. coli*-GFP together with cytochalasin B, an antibiotic which interferes with microfilament activity and thereby inhibits phagocytosis (Axline *et al.*, 1974). This control was important to exclude the possibility of nonspecific

Earthworm	IL-1β 40ng/ml	IL-1β 20ng/ml	GM-CSF 4ng/ml	GM-CSF 2ng/ml	IL-2 25ng/ml	IL-2 12.5ng/ml	TNF-α 5ng/ml	TNF-α 2.5ng/ml
EW1		T		T/R				
EW2		T		T/R				
EW3		T/R		T				
EW4	T/R	T	T/R	T	T/R	T/R	T/R	T
EW5	T/R	T/R	T/R	T/R	T/R	T/R	T/R	T/R
EW6	T	T	T	T/R	T	T	T	T/R
EW7	T	T	T	T	T	T	T	T/R
EW8	T/R			T/R	T		T	
EW9	T			T/R	T/R		T/R	
EW10				T/R				
EW11	T			T/R	T/R		T/R	
EW12	T/R			T	T		T	
EW13	T			T	T/R		T/R	
EW14	T			T/R	T		T/R	
EW15	T/R			T	T		T	
EW16	T			T/R	T/R		T	

Table 2 Summary of earthworms used in this study exhibiting a statistically significant response to at least one of the cytokines used in pretreatment. Only worms exhibiting statistically significant responses to at least one of the cytokine treatments are shown. For each earthworm shown, the cytokines used are indicated. T = earthworm treated with cytokine at indicated concentration; T/R = earthworm treated with cytokine at indicated concentration and exhibiting a statistically significant response (p ≤ 0.05).

binding of *E. coli*-GFP to the cell surface of the large coelomocytes and provided background values which were subtracted from the experimental values to obtain percent specific phagocytosis. In this example nonfluorescent cells comprised 92.77 % of the population (LR), while fluorescent cells made up 7.23 % (UR). Percent specific phagocytosis was calculated by first subtracting the fluorescent background of the averaged cytochalasin B control samples (i.e., 7.23 %) from the fluorescence observed
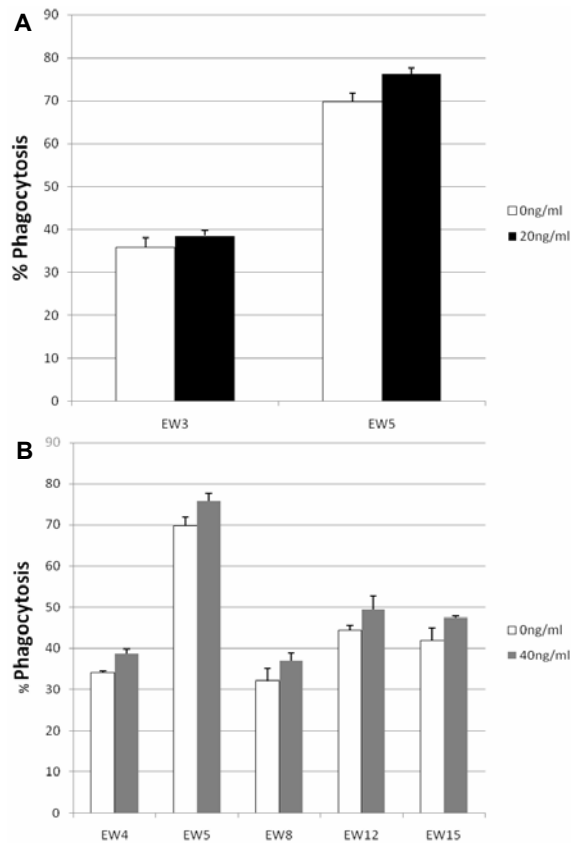
Fig. 3 Effects of IL-1 beta on phagocytosis in *Eisenia hortensis* large coelomocytes. Percent specific phagocytosis is shown for large coelomocytes incubated with *E. coli*-GFP in the absence (white) or presence of cytokine at (A) 20 ng/ml (black) or (B) 40 ng/ml (gray). Percent specific phagocytosis represents cells exhibiting fluorescence above background autofluorescence levels. Error bars represent ± SD. Only earthworms with p ≤ 0.05 are shown.

with *E. coli*-GFP only (Fig. 2C, UR quadrant), or treated with *E. coli*-GFP plus cytokine (Fig. 2D, UR quadrant). The % specific phagocytosis in this example for *E. coli*-GFP is 44.71 % - 7.23 % = 37.48 %, and the % specific phagocytosis for *E. coli*-GFP plus IL-2 is 62.28 % - 7.23 % = 55.05 %. Then statistical analyses were performed to determine if the differences between the averaged untreated and cytokine-treated samples were statistically significant. Using these criteria analyses were carried out for assays investigating the effects of IL-1 beta, GM-CSF, IL-2 and TNF-alpha.

This study analyzed the response of the coelomocytes extruded from 50 earthworms to IL-1 beta, GM-CSF, IL-2 and TNF-alpha in 9 separate assays. Cytokines were used individually in all cases, and not in combination. Coelomocytes from 28 of these earthworms were treated with all four of these cytokines at one or both of the concentrations chosen for the assays. Coelomocytes from the remaining earthworms were treated with GM-CSF only, or GM-CSF and IL-1 beta. Table 1 illustrates the number of earthworms tested for each cytokine and the corresponding concentrations. Sixteen of

the 50 earthworms exhibited a statistically significant (p ≤ 0.05) response to at least one of the four cytokines used and only those earthworms were included in our final analyses. Table 2 lists the 16 responding earthworms, indicates which cytokines were used and the concentrations employed, and whether or not enhancement of phagocytosis was statistically significant.

Figs 3-6 show the results for only those earthworms which exhibited statistically significant responses (p ≤ 0.05) to the cytokines employed. Results for earthworms with p > 0.05 are not shown. When IL-1 beta was used at 20 ng/ml, 2 out of 20 (10 %) responded significantly (Fig. 3A). At the higher concentration of 40 ng/ml, 5 out of 28 (18 %) responded significantly (Fig. 3B). GM-CSF used at 2 ng/ml induced a 20 % response rate with 10 out of 50 earthworms responding significantly (Fig. 4A). At 4 ng/ml, 2 out of 10 (20 %) responded significantly. (Fig. 4B). IL-2 gave similar results with 2 out of 10 (20 %) responding at 12.5 ng/ml (Fig. 5A), and 6 out of 22 (27 %) responding at 25 ng/ml (Fig. 5B). Higher response rates were observed with TNF-alpha; 3 out of 10 (30 %) responded to 2.5 ng/ml (Fig. 6A), and 6 out of 22 (27 %) responded to 5 ng/ml (Fig. 6B). In summary, these results show an overall increase in phagocytosis in 10 - 30 % of earthworms subjected to proinflammatory cytokine treatment compared to untreated controls.
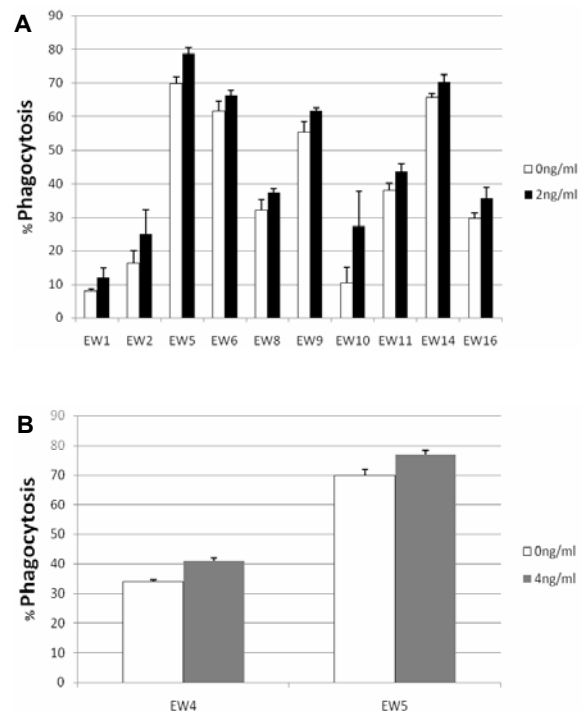

Fig. 4 Effects of GM-CSF on phagocytosis in *Eisenia hortensis* large coelomocytes. Percent specific phagocytosis is shown for large coelomocytes incubated with *E. coli*-GFP in the absence (white) or presence of cytokine at (A) 2 ng/ml (black) or (B) 4 ng/ml (gray). Percent specific phagocytosis represents cells exhibiting fluorescence above background autofluorescence levels. Error bars represent ± SD. Only earthworms with p ≤ 0.05 are shown.
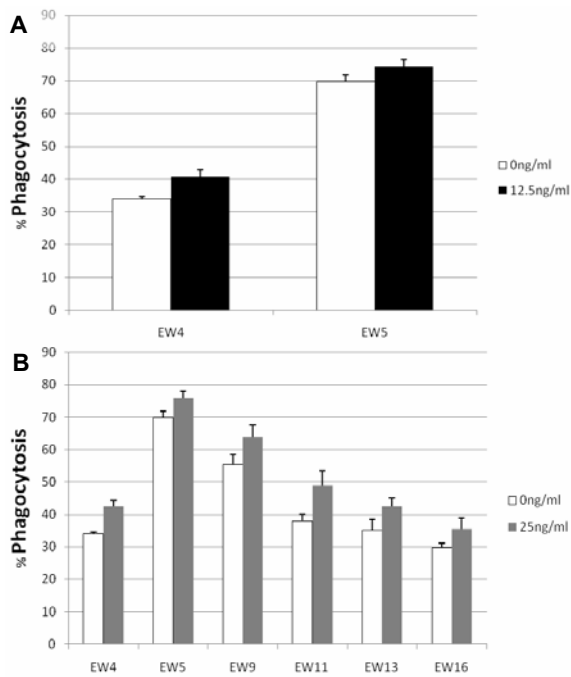
Fig. 5 Effects of IL-2 on phagocytosis in *Eisenia hortensis* large coelomocytes. Percent specific phagocytosis is shown for large coelomocytes incubated with *E. coli*-GFP in the absence (white) or presence of cytokine at (A) 12.5 ng/ml (black) or (B) 25 ng/ml (gray). Percent specific phagocytosis represents cells exhibiting fluorescence above background autofluorescence levels. Error bars represent ± SD. Only earthworms with p ≤ 0.05 are shown.

Discussion

Innate immune cells utilize pattern recognition receptors (PRRs) to recognize chemical entities that are common to different classes of pathogens. PRRs bind to pathogen-associated molecular patterns (PAMPs) and initiate cell-signaling pathways of innate immunity. One type of PRR is the evolutionarily conserved Toll/Toll-like receptor, which has the ability to recognize a broad spectrum of ligands. The Toll receptor was originally discovered in *Drosophila* during an investigation of dorsoventral patterning in embryonic development (reviewed in Janssens *et al.*, 2003). Homologues of insect Toll receptors have been identified in mammals and other animals and are known as Toll-like receptors (TLRs). Two examples of TLR signaling pathways are: 1) TLR2, which binds to peptidoglycan of both gram-positive and gram-negative bacteria, lipoteichoic acid of gram-positive bacteria, and zymosan of yeast; and 2) TLR4, which binds to LPS of gram-negative bacteria, and certain viruses (reviewed in Leulier *et al.*, 2008). It has been shown that Toll receptor and TLR signaling induces phagocytosis and the synthesis of anti-microbial compounds in vertebrates, insects, and the invertebrate *Caenorhabditis elegans*, but it is not yet known if they are needed in order for these mechanisms to occur in earthworms (reviewed in Cooper *et al.*, 2006).

Our results show that the proinflammatory cytokines, IL-1 beta, GM-CSF, IL-2, and TNF-alpha significantly increased the levels of phagocytosis of LC in 10-18 % of earthworms tested for IL-1 beta, 20 % for GM-CSF, 20 - 27 % for IL-2, and 27 - 30 % for TNF-alpha, depending on the cytokine concentration used in the assay. It is interesting to note that of the 28 earthworms that were treated with all four cytokines, 12 (43 %) responded significantly to at least one of the cytokines and in some cases to all four cytokines. Specifically, 3 responded to only one cytokine (EW7 to TNF-alpha; EW12 and EW15 to IL-1 beta), 5 responded to two cytokines (EW6 and EW14 to GM-CSF and TNF-alpha; EW8 to IL-1 beta and GM-CSF; EW13 to IL-2 and TNF-alpha; and EW16 to GM-CSF and IL-2), 2 responded the three cytokines (EW9 and EW11 to GM-CSF, IL-2 and TNF-alpha), and 2 responded to all four cytokines (EW4 and EW5) (Table 2). The overall responses to the cytokines used in our study reveal that the most efficient response was to TNF-alpha.

Possible reasons for the variation in sensitivity to IL-1 beta, GM-CSF, IL-2 and TNF-alpha could be related to different batches of earthworms. Our studies were carried out over a 7 month period and
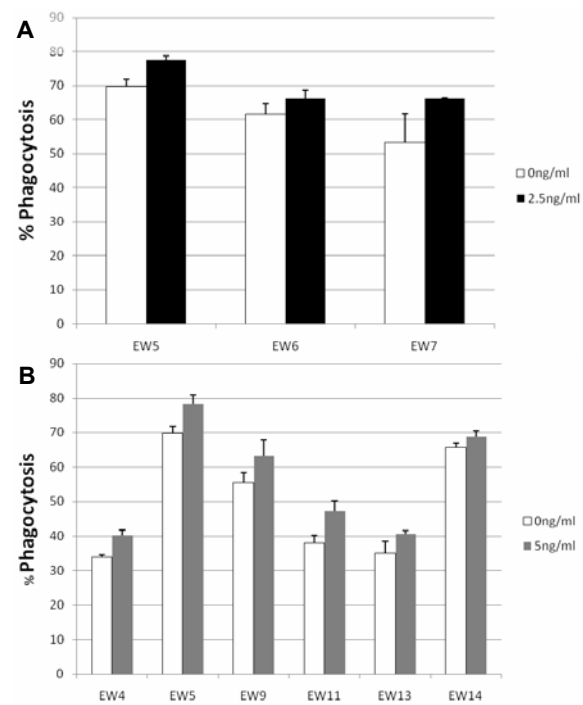

Fig. 6 Effects of TNF-alpha on phagocytosis in *Eisenia hortensis* large coelomocytes. Percent specific phagocytosis is shown for large coelomocytes incubated with *E. coli*-GFP in the absence (white) or presence of cytokine at (A) 2.5 ng/ml (black) or (B) 5 ng/ml (gray). Percent specific phagocytosis represents cells exhibiting fluorescence above background autofluorescence levels. Error bars represent ± SD. Only earthworms with p ≤ 0.05 are shown.

used different batches of earthworms. Seasonal variations may have also had an effect. In addition, the earthworm colony is an outbred population and genetic polymorphisms could contribute to cytokine sensitivities. It is also possible that the cytokine concentrations that were used in our studies were not in the appropriate range needed to stimulate immune responses in some of the earthworms. In addition, the fact that the cell populations were heterogeneous and included eleocytes, hyaline amebocytes and granular amebocytes, could explain variations due to different proportions of the three cellular subsets in each extruded sample. For example, a higher or lower number of eleocytes, known to contain riboflavin (Plytycz *et al.*, 2006) could influence the sensitivity of the amebocytes to the different cytokines used. Methodologies are needed to improve subset purification, while preserving cell yield, to enable these types of studies to be conducted with hyaline amebocytes in isolation.

Perhaps the enhancement of phagocytosis observed in our experiments is due to the binding of human cytokines to primitive earthworm receptors. This may then stimulate the upregulation of PRRs resulting in higher levels of bacteria being phagocytozed due to elevated levels of ligand receptors. Kurt-Jones *et al.* (2002) showed that GM-CSF induced upregulated expression of TLR2 and CD14 on the surface of human neutrophils and also increased IL-8 secretion and enhanced superoxide responses when GM-CSF-primed neutrophils were stimulated with TLR2 ligands. Further studies need to be conducted to determine whether a similar stimulatory pathway is induced by GM-CSF in our model.

Selective pressures operating on the different evolutionary lines of descent include the PAMPs that make up the common pathogenic burden shared by both lineages. Mandrioli *et al.* (2007), using a bioinformatic approach, suggested that invertebrate cytokine-like ancestral receptors may be promiscuous, and able to bind to different cytokines. Their results favor the model which supports the conservation of receptor-coding, rather than ligand-coding genes between vertebrates and invertebrates, and that present-day receptors are more similar to their ancestor counterparts than the ligands for those receptors. Gene duplications in vertebrates would permit receptor differentiation tailored for different ligands, with eventual specialization of a receptor towards a single ligand. The lack of gene duplication in invertebrates, however, would restrict receptor evolution, and favor the interaction of many ligands with a limited number of generalized receptors. This rationale would not only explain why molluscan immunocytes can bind to mammalian cytokines IL-1alpha, IL-1 beta, IL-2, TNF-alpha and TNF-beta (reviewed in Mandrioli *et al.*, 2007), but would also explain the findings reported in this study for earthworm coelomocytes. It is interesting to note that although not yet reported in *E. hortensis*, the existence of a gene encoding a putative helical cytokine in *Drosophila melanogaster* has been reported (Malagoli *et al.*, 2007) and shown to be upregulated following immune stimulation. These findings support the proposal that helical cytokines play a role in immune responses of invertebrates. Perhaps similar genes will be identified in earthworms and other invertebrates.

TNF-alpha, IL-1 and TLR ligands have in common the ability to bind to cell surface receptors which share a conserved intracellular signaling motif. Once engaged, the signal transduced culminates in the activation and nuclear translocation of nuclear factor-kappa B (NF-kB) and the subsequent upregulation of genes encoding TLRs, TNF-alpha, and IL-1 (O'Neill *et al.*, 2000; Muzio *et al.*, 2000; Haynes *et al.*, 2001; Hongxiu *et al.*, 2006). In contrast to the NF-kB signaling pathway initiated by TNF-alpha and IL-1 beta, GM-CSF and IL-2 stimulate the JAK-STAT pathway, which relies on Janus kinases (JAK) and signal transducers and activators of transcription (STAT). Interestingly, the JAK-STAT pathway components are molecularly and functionally conserved from the invertebrate *Drosophila* to humans to a high degree (Arbouzova *et al.*, 2006). The signaling pathways operating in earthworms require elucidation to establish whether these conserved signal transduction mediators and adaptors are also involved in the signal transduction of innate immune responses in annelids.

Although not presented in this paper, we tested the effects of GM-CSF in combination with IL-1 beta on phagocytosis and preliminary evidence suggests a synergistic effect as seen by enhanced phagocytosis. These results need to include a larger number of respondents to verify synergistic effects of proinflammatory cytokines on phagocytosis. It would also be worthwhile to employ a wider variety of cytokines and different combinations. Studies in our lab were also carried out to investigate whether IL-1 beta, GM-CSF, IL-2 or TNF-alpha would have an effect on cell proliferation *in vitro*. The coelomocytes were incubated *in vitro* with cytokine for 48 h in order to provide sufficient time for any effects to be observed. Cell proliferation was determined by measuring DNA content using propidium iodide and flow cytometry. In contrast to the results obtained with phagocytosis, there was little to no effect on cell proliferation when coelomocytes were treated with the proinflammatory cytokines; only 4.1 % of cases exhibited increased proliferation in response to TNF-alpha, and no increased proliferation was observed in response to IL-1 beta, IL-2, or GM-CSF (data not shown). Therefore, our preliminary results suggest that these proinflammatory cytokines do not enhance cell proliferation significantly in *E. hortensis* coelomocytes under the conditions used in this study. Problems with fungal and bacterial contamination, despite the inclusion of a large number of antimicrobials in the culture medium, restricted our incubation period to a maximum of 48 h, however, longer incubation periods or increased temperature may be necessary to observe proliferative effects *in vitro*. Enriching the different coelomocyte subpopulations may also lead to conditions warranting longer incubation periods needed to reveal proliferative responses to these cytokines.

Our lab also carried out preliminary experiments to study the *in vitro* effects of IL-1 beta

and GM-CSF on NK-like responses of small coelomocytes in *E. hortensis* using the non-radioactive flow cytometric procedure described by Patel *et al.* (2007). Interestingly the results obtained in these early experiments proved to be inhibitory, not stimulatory, to NK-activity with 3 out of 12 earthworms exhibiting statistically significant inhibition when pretreated with IL-1beta, and 1 out of 12 exhibiting statistically significant inhibition when pretreated with GM-CSF (data not shown). Perhaps this difference can be attributed to the observation that NK-like activity in earthworms is mediated by SC, not LC which carry out phagocytosis (Engelmann *et al.*, 2004; Salzet *et al.,* 2006). Perhaps these two different coelomocyte populations respond differently to proinflammatory cytokines through the use of distinct signal transduction pathways. It would be interesting to determine whether the signaling pathways associated with phagocytosis and proliferation in earthworms share signal transduction components which can be induced with similar cytokine or cytokine-like molecules.

It would be worthwhile and of great importance to carry out a microarray analysis, or generate a cDNA library to examine at the molecular level the changes occurring in earthworm coelomocytes in response to infection and exposure to human proinflammatory cytokines. For example, when high-density oligonucleotide microarrays were generated in *Drosophila* following microbial infection, 230 genes were induced and 170 were repressed, most of which had never before been associated with immune response, and many of the genes uncovered had unknown function (DeGregorio *et al.,* 2001), providing new leads for innate immune activities in invertebrates. There is clearly a need to understand the signaling pathways operating in earthworms and microarrays would provide an invaluable tool to decipher the humoral, cellular and molecular interactions regulating innate immunity defenses in annelids.

Acknowledgements

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