The transcriptional responses of cultured wound cells to the excretions and secretions of medicinal *Lucilia sericata* larvae

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Abstract

Maggots, through their excretions and secretions (ES), promote wound healing by removing necrotic tissue, counter bacterial infection, and activate wound associated cells. We investigated the effects of a physiological dose of maggot ES on four wound-associated cell types *in vitro* with Affymetrix gene expression arrays; keratinocytes, endothelial cells, fibroblasts and monocytes. Keratinocytes showed the fewest (n=5; $p<0.05$, fold-change ±2) and smallest fold-changes (up to 2.32x) in gene expression and conversely THP1 monocytes had the most (n=233) and greatest magnitude (up to 44.3x). There were no genes that were altered in all four cell-lines. Gene pathway analysis identified an enrichment of immune response pathways in three of the treated cell-lines. Analyses by quantitative RT-PCR found many genes dynamically expressed in ES dose dependent manner during the three day treatments. Phenotype analyses however found no effects of ES on cell viability, proliferation, migration and angiogenesis. ES was 100x less potent at triggering IL-8 secretion than fibroblasts treated with purified bacterial lipopolysaccharide (LPS; in equivalent amounts to that found in ES; ~40EU/mL). Furthermore, co-treatment with LPS and ES decreased the LPS-alone triggered IL-8 secretion by 13%. Although ES had no direct effect on wound cell phenotypes it did partially reduce the immune response to bacterial LPS exposure. These observations were consistent with the profile of transcriptional responses that were dominated by modulation of immune response genes. Maggot therapy may therefore improve wound healing through the secondary effects of these gene changes in the wound cells.

Introduction

Chronic wounds are characterised by delayed healing and they affect over 2% of the global population (1). Common examples include venous leg ulcers, diabetic foot ulcers and pressure ulcers but can also result from other traumatic aetiologies such as burns and chronically infected wounds. Chronic wounds are arrested in the inflammatory stage of healing, partly due to the presence of necrotic tissue, low oxygen and presence of bacterial biofilms (2). The wound cells require appropriate signals to enter into the proliferative and remodelling phases of wound healing. At each stage in the healing process individual cell types play different and important roles. In the proliferative phase, for example, endothelial cells form new blood vessels via angiogenesis, dermal fibroblasts secrete a new extracellular matrix and keratinocytes migrate to cover the wound with a new epidermal layer. At the later inflammatory and remodelling phases of healing these cell types remain key to successful resolution of a wound.

Maggots have been used to treat wounds since before the Middle Ages, originating from observation of the beneficial effects seen from natural myiasis. The history of maggot therapy is well reviewed by Whitaker *et al* (3) who report the first officially documented wound treatment was during the American Civil War from surgeon John Forney Zacharias, with application of the green bottle blow-fly larvae, particularly of the *Lucilia* species, placed on open wounds. There is no doubt maggots debride infected and necrotic wounds, but formal investigation of their influence on wound healing has been limited. Recent publications, including a randomised controlled trial and meta-analysis, have demonstrated a reduction in healing time and the effective removal of necrotic tissue (4-6).

There are several ways that maggots act on wounds. The secretion of proteins helps to break down necrotic tissue aided by chewing and movement in the wound (7, 8). These actions are also antibacterial removing invading pathogens (9, 10). Interestingly, maggot excretions and secretions (ES) have also been proposed to initiate wound healing by directly signalling to wound cells. It is thought that they assist in bypassing the pro-inflammatory stage block that is characteristic of chronic wounds. For example, ES stimulates fibroblasts to increase cell proliferation (11) and promote their migration on fibronectin and collagen coated surfaces (12, 13). Other publications show that the ES regulates immune cell phenotypes, for example inhibiting neutrophil chemotaxis (14) and skewing monocyte differentiation into wound-healing promoting M2 macrophages (15).

Many individual molecules, including peptides, proteins and fatty acids, have been identified as 'active' in maggot ES (16, 17). Little is known about which molecules are most important and which combination has the most positive influence on wound healing. The positive effect of ES on wound healing may require a combination of all of these active molecules. To date, no study has profiled the transcriptional response to whole maggot ES treatment of *in vitro* cultured wound related cells. The aim of this study was to report Affymetrix gene expression profiles in four cell types in response to ES treatment and correlate those to phenotypic assays in order to better understand the mechanisms of bioactive *Lucilia sericata* larval secretions.

Materials and Methods

Cell culture

Human cells line HaCaT (immortalised keratinocytes), THP-1 (monocyte from acute monocytic leukaemia) were received from researchers at the University of Auckland, HMEC-1 (human microvascular endothelial cells CRL-3243) from American Type Culture Collection (ATCC) and HDFa (Human dermal fibroblasts) from ThermoFisher Scientific. HaCaT and HDFa were grown in DMEM high glucose (Cat#11965 ThermoFisher), and THP-1 in RPMI (Cat#11875 ThermoFisher), all supplemented with 10% fetal bovine serum. HMEC-1 were cultured in MCDB-131 (Cat#10372 ThermoFisher) supplemented with 10% FBS, 0.8 µg/mL hydrocortisone and 4 mM L-glutamine.

Both HMEC-1 and HDFa cells were used for experiments at passages lower than 15. Plating densities for each cell-line were the following: HDFa ~ 8500 cells/cm^2, THP1 (suspension cells) ~160000 cells/ cm^2, HMEC-1 ~25000 cells/cm^2, HaCaT ~25000 cells/cm^2.

Excretion and secretions collection

Consultant Entomologist Dallas Bishop, Upper Hutt, New Zealand provided L. Sericata eggs for sterilisation. The eggs were initially laid onto bovine liver, then removed and sent by overnight courier to our laboratory with an ice-pack to delay hatching. Eggs were surface sterilised as previously reported (18). Briefly this consisted of a 5 minute immersion in 1% sodium hypochlorite followed by 5 minutes in 70% ethanol followed by a final rinse in ddH_20. The eggs were hatched overnight in the absence of food and then the larvae used for collection of ES. Larvae were washed in 70% ethanol and then with ddH_20. Approximately

100 larvae were submerged in 200µL PBS for 1 hour before the supernatant, the ES, was collected by pipette and strained through a 0.22µm syringe filter to remove particulates and remaining bacteria. To ensure homogeneity in the ES samples, multiple collections were pooled prior to use in this study. ES concentration was determined for total protein content using a standard bicinchoninic acid (BCA) assay (ThermoFisher Scientific).

Treatments and RNA isolation

Target cells were plated in 6-well plates and treated in triplicate with one of three concentrations of ES (0.5, 2, and 8 µg/mL; total protein) or PBS control for three time frames 24, 48, 72 hrs without removal of the treatment media. These concentrations were chosen as we have found that one 200µL ES sample derived from ~100 instar 1 larvae, is similar to the number of larvae that would be applied to a chronic wound, and this volume yields ~50ng/µL or <10µg of protein.

Total RNA was isolated from treated cells using a standard miRvana total RNA isolation method (ThermoFisher). RNA quantity was assessed by Qubit fluorometry and quality by Tapestation (Agilent).

Gene expression arrays and analysis

For each of the cell-lines, triplicate RNA isolations from the maximal 8µg/mL ES or PBS control at the T48 hour time-point were assessed for transcriptional changes using Affymetrix expression arrays. Affymetrix GeneChip 1.0 arrays were used for HaCaT and HMEC-1 and Affymetrix Primeview arrays for THP-1 and HDFa, each with a very similar

gene content. Complementary RNA was made from 300ng input RNA and hybridisation, wash and staining provided as a service by New Zealand Genomics Ltd.

CEL files were normalised in Affymetrix gene expression console with RMA normalisation and differential expression determined in transcription analysis console applying cut-offs for pair-wise differences set at ANOVA $p<0.05$ (not FDR) and fold-change greater than 2 between treated and untreated replicates. Samples were normalised in batches within cell-lines. Data are freely available from Gene Expression Omnibus study number GSE79765. Pathway analysis of the significantly modulated RNAs in each cell-line was performed in GATHER (19) to identify gene signatures that are affected by ES treatments in each cell type.

Quantitative RT-PCR

Primers to gene candidates were designed using Primer3 (Table S1). Complementary DNA was synthesised from 200-500ng RNA from treated cells using qScript cDNA supermix (Quanta Biosciences) as per the manufacturer's instructions. This was diluted 1:4 before use. PCR was performed using 5 µL PerfeCTa SYBR Green Fastmix ROX in a 10µL reaction volume containing 0.25 µL of each 10 µM forward and reverse primer, 2.5 µL of water, and 2µL diluted cDNA. Cycling conditions were (2 minutes at 95^{o}C, 40 cycles of 15 seconds at 95^{o}C and 45 seconds at 60^{o}C). Samples were run in triplicate wells and genes were normalised to ACTB for RNA loading purposes using a dCT method.

Cell viability, proliferation and migration assays

Cell viability and proliferation were determined using PrestoBlue and CyQuant respectively (ThermoFisher Scientific) using the manufacturers instructions and read on an EnSpire 2300 (PerkinElmer) plate reader for absorbance and fluorescence respectively. Cells were plated in 4 replicates in 96 well plates and treated as before at three ES concentrations and timepoints. Viability after treatment with ES was determined for all four cell-lines and proliferation using CyQuant in two cell-lines.

Cell migration was assessed for HDFa cells using standard scratch assays. Cells were plated in triplicate into 6 well plates and grown to confluence over night. The confluent cell monolayer was scratched with a p10 pipette tip prior to washing well with PBS well to clear the scratch of loose debris and replacing with serum-free media.. Cells were treated during healing or pre-treated with 8µg/mL ES and visualised for wound closure at T0, T4, T8, T24 using a Nikon Eclipse T*i*-U microscope with a 4X magnification objective. Percentage of covered wound area compared to T0 was measured with software Image J v1.49.

Angiogenesis tube formation assays

HMEC-1 cells, treated as above with 8µg/mL ES for 72 hours in 6 well plate, were then trypsinised and plated at $1x10^4$ cells per well onto growth factor reduced Matrigel® in µ-Slides Angiogenesis, ibiTreat. Tube formation and branching were visualised after 4 hours and quantitated by Wimtube software. Repeated in triplicate wells.

LPS detection and assays

LPS was quantified by EndoZyme® recombinant Factor C Endotoxin Detection Assay (Hyglos GmbH, Germany). The procedure was performed as per manufacturers instructions and read on a PerkinElmer EnSpire 2300 plate reader.

IL-8 ELISA

Detection of secreted IL-8 protein was performed using Human IL-8 ELISA Set (BD Biosciences OptEIA) in 96 well microplates (BD Biosciences). HDFa cells were plated at density of 8000 cells /cm2 on a 96 well plate and after a 24 hour treatment with ES (0.5, 2 and 8 µg/mL), or LPS (Ultrapure LPS from InvivoGen; 0.4, 4 and 40 EU/mL), the spent medium was removed and stored at -80^{0}C until use. This assay was performed in duplicate wells.

Statistical analyses

Significance was determined in the phenotype assays using two-tailed t-test to compare treated and untreated samples from the same timepoints in a pairwise manner.

Results

Effects of ES on transcription in wound cell lines

HaCaT: There were only 5 annotated genes modulated when keratinocytes were treated with 8μg/mL ES for 48 hours (P<0.05; Fold-change (FC) >2; Table S2), 2 down with ES treatment (AMTN, CXCL10) and 3 up (S100A8, RIMKLBP1, ANKRD20A4). The fold-changes were small with 2.32 maximum. After gene function enrichment analysis using GATHER (19) no enrichment of pathways or features were seen in these five genes (Table 1).

HMEC-1: Endothelial cells responded dramatically to the ES treatment at the transcriptional level with 72 annotated genes modulated between PBS and ES treated cells at T48. Only 11 were repressed with the remainder (n=61) showing an overwhelming induction upon ES treatment relative to the PBS control (FC 2 to 14.2). When stringently analysed with application of a false discovery rate (FDR) two interferon-induced antiviral RNA-binding protein genes were upregulated, IFIT1 and IFI44. Gene pathway analysis in HMEC-1 cells identified many enriched pathways in the ES-upregulated genes including 'immune response', 'response to biotic stimulus' and 'defense response' (Table 1). This analysis also predicted that the modulated genes produced proteins that bind to the protein receptors IL-8RA and IL-8RB and predicted that 30 of the genes are transcriptionally regulated by ICSBP (interferon regulatory factor 8) transcription factor.

HDFa: Fibroblasts also responded strongly to ES treatment. As for the HMEC-1 cells, most of the 82 identified regulated genes were upregulated upon ES treatment (n=74, FC 2 to 24.1). With application of FDR to the analysis, there were 6 upregulated genes; CFB, CXCL3,

IL-8, IFI6, CCL8 and CXCL1. Again, GATHER analyses (Table 1) identified 'immune response' pathways as a common function of the modulated genes and as for HMEC-1 cells predicted they may be transcriptionally regulated by the interferon transcription factor. However, in contrast to HMEC-1 and THP-1 modulated genes (see below), the RNAs modulated in HDFa cells were not significantly enriched for transcriptional regulation by the interferon regulated transcription factors, instead CREB and E2F1 were predicted.

THP-1: The inactivated monocytes showed the largest transcriptional response to ES of all four cell-lines assayed. Again, upregulation of genes (156 of 233 total), was more common than down regulation (77 of 233) and fold changes were generally greater in these upregulated genes (FC 2 to 44.3). Use of FDR only identified one significant gene expression change for TFAP2C, suggesting that the replicates were more varied for this cell-line than for the other three tested. Indeed the THP-1 cells are liable to loss of cells during handling due to being a suspension culture that requires gentle centrifugation. As for the previous cell-lines GATHER analysis again identified enriched immune and inflammatory response pathways and alongside the predicted interferon transcription factor regulation, we also identified a possible role for NF-KB in the monocytes (Table 1).

Comparison in transcriptional changes with ES treatment between cell-lines identified that many of the modulated genes were unique to the individual cell-lines (Figure 1). All of the genes found in two or more of the cell lines can be annotated as 'interferon response genes' that can be triggered by multiple stimuli and signalling pathways including Interferon, TNFa and LPS. There were no genes that were altered in all four cell-lines and only nine

that were altered between THP-1, HDFa and HMEC-1, the cell-lines with the most modulated genes in total. GATHER functional analysis shows that these nine are again associated with the biological processes of 'inflammatory response', 'response to pest pathogen or parasite' and 'response to external biotic stimulus'.

Quantitative RT-PCR validation of gene expression changes in ES treated cells

Eighteen genes were chosen for qRT-PCR validation on the full treatment cohorts of ES concentrations (0.5, 2, 8μg/mL) and time points (24, 48, 72 hours), based on the array data as differentially expressed candidates in each cell-line. Genes that were modulated in more than one cell-line are shown in Figure 2 and those that were unique to a single cell line are presented in Figure 3. All qRT-PCR data is summarised in Table S3 for fold-change and p-values. This qRT-PCR validation confirmed many of the changes reported by array.

HaCaT cells showed the fewest and smallest expression changes and none of the genes responded in an ES dose related manner. We also found that all of the genes assayed naturally change with proliferation and confluence in HaCaT (>2x fold-change). For example S100A8 increased 84-fold from 24 to 72 hours in the PBS control cells.

In contrast to HaCaT, in the three other cell-lines, most of the genes assayed did respond in a dose-responsive manner to the ES concentration and the final expression induction could be both rapid and large in quantity. For example in HMEC-1 cells, IL-8 was induced 76-fold in the 8μg/mL ES cells relative to controls at 24 hours ($p=0.005$) and dropped to 9-fold at 72 hours ($p=0.064$). The early induction in IL-8 was also seen in HDFa and THP1 cells. This

pattern was also seen for CXCL10, DDX58, MX2 and IFIT1 in HMEC-1. A time-related induction pattern from 24 to 72hrs, and thus later responders to the ES treatment, was seen for C3, CCL8 and CFB (Figure 2). Although the major transcriptional response to ES treatment was an induction of gene expression, we have also validated small non-dose-related decreases in expression of SMOC2 in HDFa (8μg/mL, 48hr, 5-fold; Figure 3) and FN in HaCaT (0.5μg, 48hr, 2-fold; Figure 2).

Phenotypic effects of ES treatments on cultured wound cells

Cell viability and proliferation

We tested the effects of ES on cell viability and proliferation over the course of a 72 hr treatment. However, even at the maximal 8μg/mL ES treatment, no significant changes to these phenotype tests were detected in any of the four cell-lines assessed (Supplemental Figure S1). This data highlights that although rapid, dynamic and sometimes striking transcriptional changes were caused by ES treatment of cells, even at the maximal treatment concentration it was not cytotoxic to cells.

Fibroblast migration

Due to the proposed effects of ES on cell migration, we next tested the effects of ES treatment on the movement of HDFa cells using scratch migration assays. Fibroblasts were tested for scratch wound healing in the presence of 8μg/mL ES, or when pre-treated with the same quantity of ES for 24 hours (Supplemental Figure S2). These assays did not find any significant differences in migration rate between PBS control and 8μg/mL ES treated cells.

Endothelial angiogenesis

Many of the chemokines we identified as upregulated in HMEC-1 cells by Affymetrix array analyses, such as IL-8, CXCL1 and CXCL10 have been linked with angiogenesis. These genes have opposite roles in the regulation of angiogenesis with IL-8 and CXCL1 being potent promoters and CXCL10 (IP-10) an inhibitor (20). Therefore we assayed the effect of 8μg/mL ES on HMEC-1 tube formation and branching. However there were no significant changes to tube formation (Supplemental Figure S2) from a 24 hour ES pre-treatment compared to PBS controls.

Comparison of effects of ES with bacterial lipopolysaccharide

Finally, many of the transcriptional changes identified by the array analyses highlighted an upregulation of immune response pathways which are also known to be triggered by bacterial lipopolysaccharide (LPS). We therefore theorised that the effects of ES on gene transcription may be due to its LPS content. Indeed when assayed we found that one sample of 100μg ES contained 500EU of LPS meaning that our 8μg/mL maximal treatment would contain ~40EU LPS.

We first confirmed that the induction of transcription of IL-8 seen by array in fibroblasts by ES was also associated with an induction of secretion of the protein product into the culture media by ELISA (Figure 4A). The protein secretion was also dose related. To test whether the ES triggers IL-8 secretion to a greater extent than LPS alone we treated cells for 24 hours with ES or a range of LPS concentrations (0.001-40 EU/mL). Our hypothesis

that ES would trigger more IL-8 secretion than an equivalent amount of LPS alone (40EU/mL) was not supported as we found that less IL-8 was actually released (p=0.001). Therefore, even though the ES contained an equal amount in terms of EU of LPS, it had a lesser effect on IL-8 secretion from treated fibroblasts (Figure 4B). To test whether the effects of the LPS on IL-8 secretion was inhibited by ES we co-treated with 8μg/mL ES and 4EU/mL LPS. There was a trend toward a reduction of IL-8 secretion by 13% relative to LPS alone (Figure 4B; p=0.073).

Discussion

Here we report the first comprehensive transcriptome analysis of the effects of maggot ES on cultured naïve wound-related cells. The data shows that ES caused transcriptional changes in the cells and that the modulated genes were mostly unique to each individual cell-line. Pathway analysis of these genes did not point to the modulation of phenotypes such as motility, survival or angiogenesis, and this was validated in our phenotype assays. In contrast clear overlap between cell-lines was however seen for the highly upregulated 'immune response' cytokine genes such as those encoding IL-8.

The largest published transcriptional analysis evaluated just 87 wound healing associated genes in response to ES treatment in a rat skin wound model (21). There was no overlap between their findings and our cell specific gene expression changes. This is likely due to the limitations of their minimal gene analysis and also use of a more complex *in vivo* model and mixed whole tissue samples.

Published analyses on the effects of ES on cellular characteristics have assessed either single cell types, specific phenotypes or specific signalling pathways. We also note that it can be difficult to compare between studies due to differences in their methodology used in ES isolation and the use of varied and wide-ranging amounts of ES (0.1-100,000μg/mL) in experiments. Furthermore, studies with immune cells tend to pre-treat naïve cells with LPS to mimic an activated state as would be present in an infected chronic wound. As an example of this experimental complexity, the only study to date using keratinocytes (22) used a whole maggot lysate rather than standard ES and found that 200μg/mL of this product was toxic to HaCaT cells. In contrast we found that using a lower 8μg/mL dose of 'pure' ES had only limited effects on transcription and no detectable effects on phenotype. The few modulated genes identified in HaCaT cells analysis studies, such as S100A8 and FN1, are however associated with cell confluency rather than proliferation and suggesting that the ES may promote a differentiated state (23).

In this study, endothelial cells, fibroblasts and monocytes were more responsive at the transcriptional level to the 8μg/mL ES treatment than the keratinocytes, with each showing more than 70 genes regulated by array analysis. All three cell-lines showed an enrichment in induced genes that are linked to immune response pathways. Quantitative RT-PCR validations confirmed that these immune related genes, such as IL-8, were affected in a dose responsive manner in each of these three cell-lines. Many of the validated genes were also dynamically expressed through the three day time-course.

HMEC-1 cells have been reported previously to show induced migration by scratch assay after treatment for 24 hours with 10μg/mL ES from Instar 3 larvae ((24); 34 % reduction in wound length compared to control), conditions very similar to those used in this study, but with no effect on cell viability. This migration was linked to rapid AKT1 phosphorylation and PI3K pathway signalling. We confirmed no effect of ES on HMEC-1 cell viability and proliferation but could not corroborate the effects of this previous report on induction of cell migration. There is no clear explanation for the difference here but it may relate to the different larval instar, ES collection and storage protocols. The absence of a change in migration phenotype in the endothelial cells in our study was corroborated by our transcriptional findings that supported a more molecular immune signalling response than change in motility profile.

Interferon stimulated gene and chemokine transcription was also triggered by ES in fibroblasts and monocytes. Fibroblasts have been shown to respond to maggot ES by increases in growth (11), metabolism and translation (25) and migration on collagen and fibronectin coated surfaces (12, 13). Their migration is thought to be triggered by serine proteases in the ES that degrade fibronectin and remodel the extracellular matrix. Our studies used simple hydrophilic plastic with no fibronectin coating and showed no effect of the dose of 8μg/mL ES on fibroblast migration. In our qRT-PCR validation, we found fibronectin expression was decreased by up to 1.9x in keratinocytes and 1.6x in endothelial cells and fibroblasts by ES treatment. Therefore, not only do proteases in ES digest the fibronectin, but ES treated wound cells may also transcribe less.

The effect of maggot ES on chronic wounds is considered to relieve the pro-inflammatory state of the wound to induce an anti-inflammatory, pro-angiogenic environment to allow the stages of wound healing to progress. Based on this proposed action, multiple studies have been performed using immune cells (14, 15, 26). In neutrophils, 5-50μg/mL ES inhibited neutrophil chemotaxis, and stimulated endothelial protein anchors on the their surface (14). In monocytes, ES causes skewing of their differentiation into macrophages, promoting wound healing associated M2 type cells and repressing HLA-DR mRNA expression (15). Our array analyses of treated THP-1 cells also found repression of HLA-DRA and HLA-DRB1 gene expression. It has been reported by others that pre-treated monocytes with ES prior to activation with LPS and found modulation of chemokines including upregulation of MCP1 (CCL2) and IL-8 (26), two genes we also found to be upregulated in our monocytes, fibroblasts and endothelial cells.

The complement system, is a key regulator of the inflammatory response and has been investigated by treating patient serum samples with ES (27). It was reported that complement activation was reduced by ES due to the degradation of complement proteins C3 and C4. In contrast, we have found that C3 is induced at the transcriptional level by ES treatment of fibroblasts, monocytes and endothelial cells. We note again however, discrepancies between sample treatment amounts, whereby their minimum dose used was 250μg/mL, which was over thirty times more than our maximum dose. Caution is required in the interpretation and significance attributed to these supra-physiological exposures in terms of understanding the mechanism of action of ES in a chronic wound.

Finally, due to the gene expression changes in three of the cell-lines being linked to inflammatory/immune responses including those triggered by LPS we assessed the ES itself for LPS contamination. Although the eggs were surface sterilised, and larvae housed in sterile conditions it is possible that residual LPS was present and triggered the gene expression changes seen. Reports have highlighted that topical application of LPS itself could promote wound healing (28, 29). Indeed, low concentrations of LPS, at 10µg/mL (29) improved epithelial wound repair in a scratch assay. However, the LPS levels in each 8µg/mL dose of ES were 1000 x less than this being only equivalent to 40EU (4-40ng LPS/mL). By addition of LPS to HDFa cells we found that to induce equivalent amounts of IL-8 protein secretion as we saw for the 8µg/mL ES, a minimum of 0.4EU/mL LPS was required, which is still 100x less than that present in the ES itself. This means that our findings were not confounded by high LPS levels.

IL-8 is a pro inflammatory cytokine and is more abundant in chronic than acute wounds (30, 31) and thus repression of IL-8 expression could be beneficial for chronic wound healing. The co-treatment of LPS and ES in our cell model to mimic a wound bed showed that there was a definite trend towards ES repressing LPS triggered IL-8 secretion by 13% in fibroblasts. Interestingly a similar anti-inflammatory action of reducing IL-8 secretion has been reported when epithelial cells are co-cultured with secretions from both the LPS producing Gram negative *Pseudomonas* and from suppressive *Staphylococcus aureus* (32). The anti IL-8 secretion effects of *S. aureus* were proposed to be via triggering of the TLR1/2 pathway. This could be an interesting target for further investigation into the effects of maggot ES on IL-8 secretion repression.

The strengths of our study are the use of four different cell types to tease apart the effects on an individual cell type and the use of a more physiological amount of ES in our analyses. However we concede that *in vitro* wound healing models are not ideal and the findings that we present are limited to naïve cells grown separately in culture without the possibility for communication and at limited timepoints. A perfect chronic wound model is not however available *in vitro* or *in vivo* (33) and therefore a follow-up study could be to implement full transcriptome expression profiling on patients undergoing maggot debridement therapy.

In summary, we have found that a common transcriptional response to exposure to realistic physiological concentrations of ES on the representative wound related cell classes in a wound bed is predominantly the modulation of their immune response pathways, irrespective of cell-type. In addition the individual cell types did show unique responses. The gene expression changes detected by arrays and gene pathway analyses did not predict that there would be overt promotion of cellular changes in growth, viability, migration or angiogenesis, and this was supported by our subsequent phenotype analyses. Instead, the major effects of ES appeared to trigger a 'molecular phenotype', which was linked to the regulation of the immune response such as the secretion of cytokines. Thus ES may be causing beneficial effects on cell migration and angiogenesis in chronic wounds by an indirect effect through cytokine and immune modulation rather than by direct changes to wound cell gene expression for these pathways. In conclusion we have investigated the effect of exposure to 'physiological' concentrations of maggot ES on classes of cells found in chronic wounds. At these doses, ES causes a 'molecular phenotype' to enhance the gene

pathways for cell protection and/or alter the secreted inflammatory signals released by these cells which may in turn indirectly influence other resident cells in the wound environment.

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Abbreviations

ES, excretions and secretions; FBS, fetal bovine serum; ANOVA, analysis of variance; ATCC, American Type Culture Collection; LPS, lipopolysaccharide; EU, endotoxin units; ELISA, enzyme-linked immunosorbent assay; BCA, bicinchoninic acid assay; PBS, phosphate buffered saline; GATHER, gene annotation tool to help explain relationships.

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Table 1: GATHER pathway enrichment for ES-modulated genes in each cell-line

	HaCaT keratinocyte	HMEC-1 endothelial	HDFa fibroblasts	THP1 monocytes
Number of Genes	5	72	82	233
Gene ontology				
GO:0006954 inflammatory response	7*	10	55	21
GO:0006955 immune response	5	55	66	51
GO:0009607 response to biotic stimulus	4	53	64	25
GO:0006952 defense response	4	51	68	47
GO:0009613 response to pest, pathogen or parasite	5	22	62	27
GO:0043207 response to external biotic stimulus	5	21	63	25
GO:0009611 response to wounding	6	*7*	58	*19*
KEGG pathway				
path:hsa04060 cytokine-cytokine receptor interaction	2	8	13	16
Protein binding				
FY duffy blood group		17	11	17
CSPG2 chondroitin sulphate proteoglycan		9	7	
IL-8RA interleukin 8 receptor alpha		8	11	23
IL-8RB interleukin 8 receptor beta		7	9	21

CCBP2 chemokine binding protein 2		12	10
TRANSFAC transcription factor binding			
V$ICSBP_Q6		30	13
V$ISRE_01		15	9
V$IRF_Q6		16	4
V$CREB_Q2_01		8	
V$E2F1_Q6		7	
V$NFKB_C	1		15

*Bayes Factor determined by GATHER algorithm where a higher number represents a more significant enrichment of identified feature associated with the gene list tested

Figure legends

Figure 1. Overlap of differentially expressed genes between the four cell-lines after treatment with maggot ES. Gene symbols listed were found to be modulated after 48 hours treatment with 8μg/mL ES relative to PBS control cells ($p<0.01$ ANOVA, fold-change >2).

Figure 2. Quantitative RT-PCR validation of common genes in ES treated cells across 72 hours. Ct normalised against B-Actin (y-axes). Error bars are standard deviation of three replicates. Black triangle represents an increase of ES concentration from 0, PBS in media to, 0.5, 2 and 8μg/mL respectively.

Figure 3. Quantitative RT-PCR validation of unique genes in ES treated cells across 72 hours. Ct normalised against B-Actin (y-axes). Error bars are standard deviation of three replicates. Black triangle represents an increase of ES concentration from 0, PBS in media to, 0.5, 2 and 8μg/mL respectively.

Figure 4. Comparison of induction of IL-8 protein secretion between ES and LPS in HDFa cells. A. Effects of doses of ES and LPS on HDFa IL-8 protein secretion (pg/mL) after 24 hour treatment B. Effects of combined treatment with 8μg/mL ES and 4EU/mL LPS on HDFa IL-8 secretion relative to PBS control treatment. Error Bars are standard deviation. EU = Endotoxin units

Supplemental Files:

Table S1: Primer sequences for genes used in qRT-PCR validations

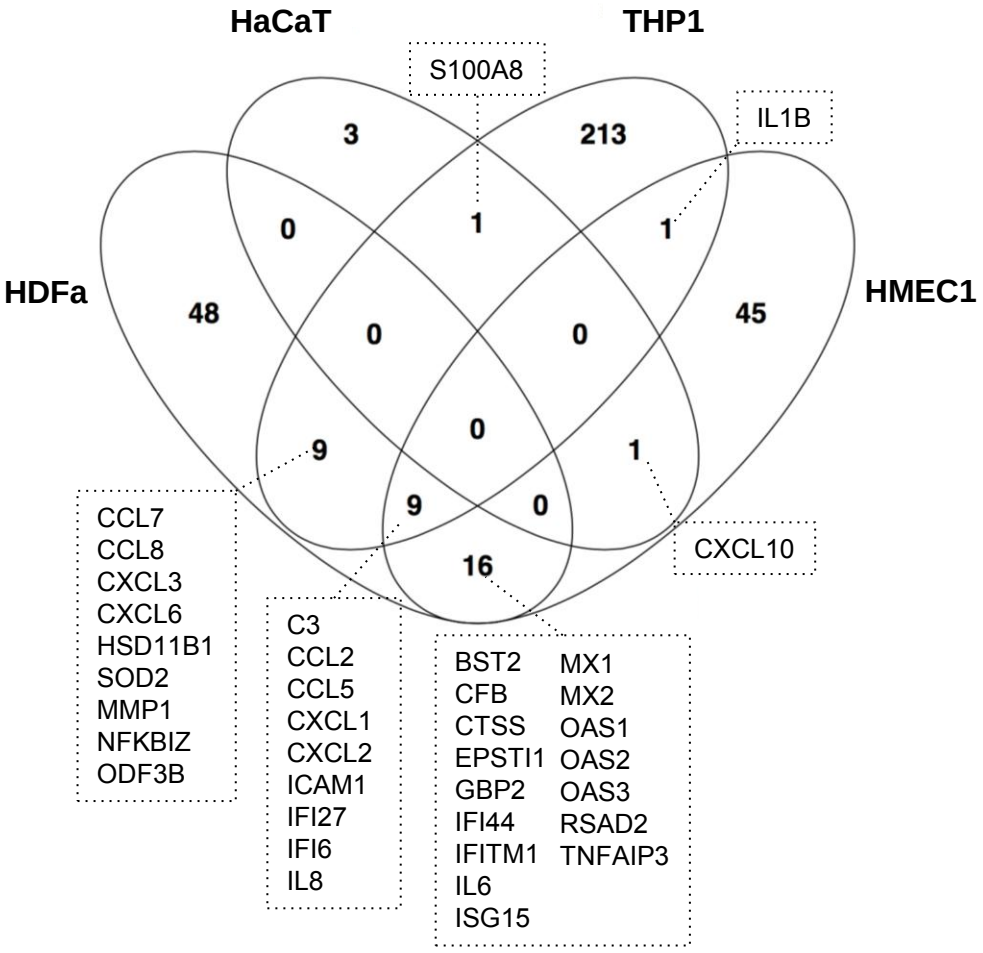
Table S2: Gene expression changes in cell-lines after 8μg/mL ES treatment at T48 Vs PBS control- List of up and down regulated probes/with gene names in each with $p<0.01$ and FC2 in each cell line.

Table S3: Full qRT-PCR validation data for each cell-line, all genes tested with p values (T-test)

Supplemental Figure S1. Viability and proliferation assays on ES treated cells. Cells treated with three concentrations of ES for up to 72 hours. Viability determined by Prestoblue, proliferation by CyQuant. A. HaCaT, B. HMEC-1, C. HDFa, D. THP-1. Average of samples run in three replicates, treatments presented relative to timepoint PBS control, error bars are standard deviation.

Supplemental Figure S2. Cell migration and angiogenesis in ES treated cells. Migration of HDFa fibroblasts assessed with treatment during scratch assay A. and prior to scratch assay B. PBS treatment used as a control. Presented as average of 3 replicates for the area of wound coverage as compared to 0 hr timepoint. Error bars are standard deviation. C. Angiogenesis tubule formation and branching assays of 8μg/mL ES treated HMEC-1 as determined by WimTube software, error bars are standard deviation. *$p<0.05$ t-test

Figure 1



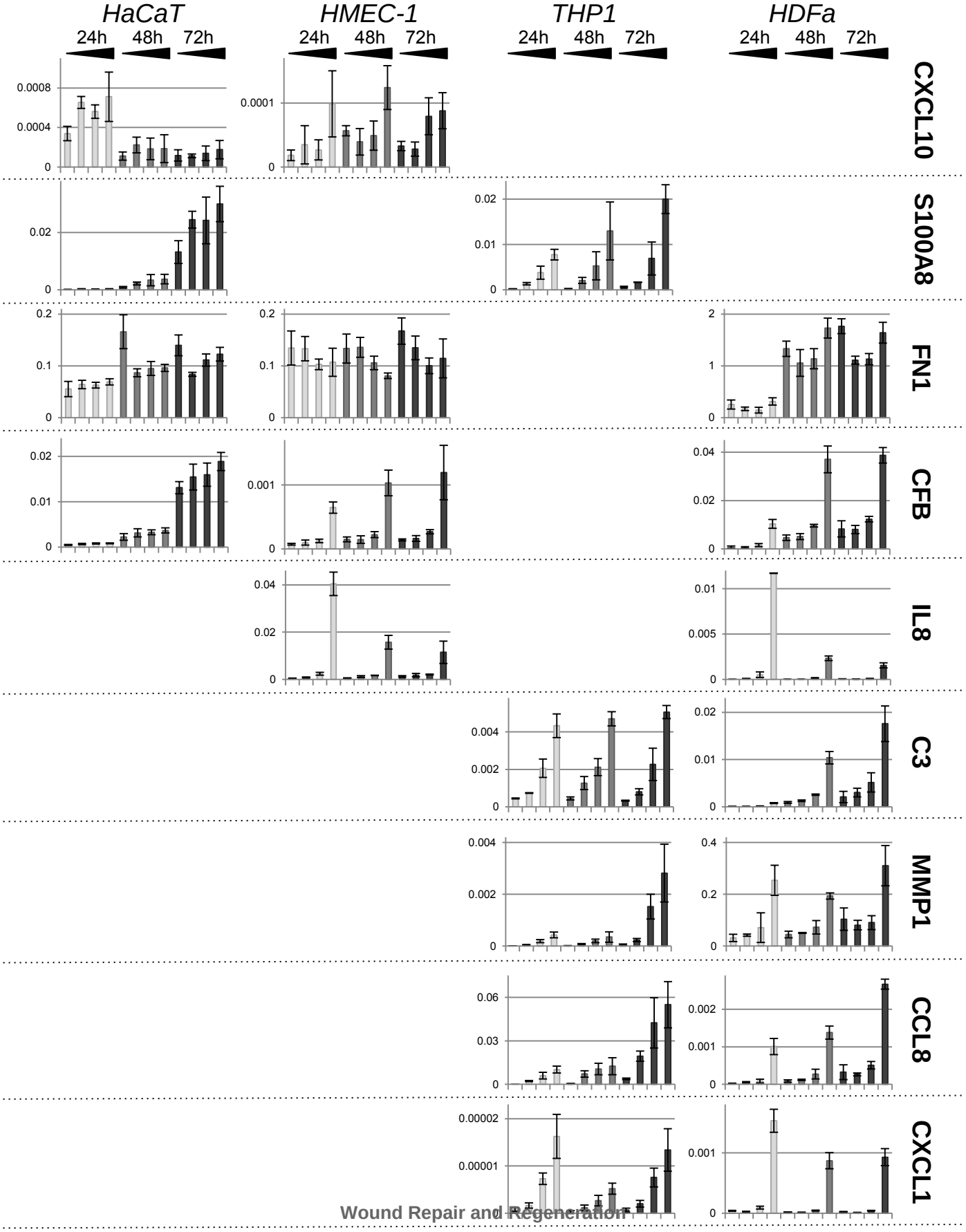


Figure 3