Maggot therapy´s modes of action : effect of maggot secretions on microbiological, haematological and immunological processes

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CHAPTER SIX

Maggot secretions suppress pro-inflammatory responses of human monocytes through elevation of cAMP

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Abstract

Aims Maggots of the blowfly *Lucilia sericata* are used for the treatment of chronic wounds. As monocytes may contribute to the excessive inflammatory responses in such wounds, this study focussed on the effects of maggot secretions on the pro-inflammatory activities of these cells.

Methods Freshly isolated monocytes were incubated with a range of secretions for 1 h and then stimulated with LPS (range 0-100 ng/mL) or LTA (range 0-5 µg/mL) for 18 h. The expression of cell surface molecules, cytokine and chemokine levels in supernatants, cell viability, chemotaxis and phagocytosis and killing of *Staphylococcus aureus* were measured.

Results Maggot secretions dose-dependently inhibited the production of the pro-inflammatory cytokines TNF-α, IL-12p40 and MIF by LPS- and LTA-stimulated monocytes while enhancing the production of the anti-inflammatory cytokine IL-10. Expression of cell surface receptors involved in pathogen recognition remained unaffected by secretions. In addition, maggot secretions altered the chemokine profile of monocytes by down-regulating MIP-1β and up-regulating MCP-1 and IL-8. Nevertheless, chemotactic responses of monocytes were inhibited by secretions. Furthermore, maggot secretions did not affect phagocytosis and intracellular killing of *S. aureus* by human monocytes. Finally, secretions induced a transient rise in the intracellular cyclic AMP concentration in monocytes and Rp-cAMPS inhibited the effects of secretions.

Conclusion Maggot secretions inhibit the pro-inflammatory responses of human monocytes through a cAMP-dependent mechanism. Regulation of the inflammatory processes by maggots contributes to their beneficial effects on chronic wounds.

Introduction

Chronic wounds are common in patients with vascular insufficiencies and underlying chronic conditions such as diabetes mellitus, as well as patients suffering from acute, extended trauma[1]. Of the patients with diabetes, up to 15% of the more than 200 million patients worldwide develop a foot ulcer at some stage, leading to over 1 million amputations every year[2]. The importance of chronic wounds in the pathway to lower limb amputation is paramount as 84% of amputations are preceded by a diabetic foot ulcer[3]. Chronic wounds and amputations in persons with diabetes often result in decreased physical, emotional and social function of patients, a reduced quality of life and major economic costs for both the patients, their families and society[4,5].

Sterile larvae -maggots- of the blowfly *Lucilia sericata* are used for the treatment of different types of wounds including diabetic foot ulcers[6-9]. The success rate of this therapy is around 68% for wounds unresponsive to conventional therapies although some characteristics (chronic limb ischaemia, wound depth, and age) may negatively influence the outcome[8]. Besides the removal of necrotized tissue and infectious microorganisms, maggots potently inhibit the pro-inflammatory responses of human neutrophils without affecting their antimicrobial activities[10]. Another prominent type of phagocyte in wounds is the monocyte. In response to chemotactic substances these cells migrate from the blood into the infected tissue to combat invading micro-organisms. In addition, monocytes regulate the inflammatory process by secreting cytokines and growth factors thereby recruiting more inflammatory cells and by antigen processing/presentation and lymphocyte activation.

In contrast to acute wound healing, chronic wounds are marked by a prolonged and dysregulated inflammatory phase. Inflammatory cells like neutrophils, monocytes and macrophages are not only present in excess numbers[11-13], they also have an enhanced production and release of pro-inflammatory cytokines, proteases and reactive oxygen species leading to growth factor inactivation and tissue destruction[14]. Therefore, inhibition of the pro-inflammatory responses of these cells could restrict their deleterious effects and thus contribute to healing processes. To obtain more insight in the mechanisms underlying the beneficial effects of medicinal maggots, this study focussed on the effects of maggot excretions and/or secretions on the pro-inflammatory activities of human monocytes.

Material and Methods

Maggots and their excretions/secretions

Sterile second- and third-instar larvae of *L. sericata* were a kind gift from BioMonde GmbH (Barsbüttel, Germany). Maggot excretions/secretions (ES) were collected as previously described[10]. Next, maggots were incubated for 1 h in H_2O to remove their excretions,

washed and then their secretions (S) were collected as described for ES. In the assays ES and S preparations were tested simultaneously, which is indicated as ES/S.

Isolation of human monocytes

PBMCs from healthy donors were isolated from buffy coats by Ficoll Amidotrizoate (ρ=1.077 g/mL) density centrifugation at 700xg for 20 min. Cells from the interphase were washed three times and monocytes were purified using anti-CD14 coated Microbeads (Miltenyi Biotec GmbH, Germany). When testing antimicrobial activities, cells in the interphase were used to avoid possible functional impairment of the monocytes by the interaction of CD14 with anti-CD14 coated microbeads. For the chemotaxis assay lymphocytes were removed from the interphase using anti-CD3 microbeads (Miltenyi) to avoid obstruction of the filters by large numbers of these cells.

Stimulation of monocytes

Approximately $1x10^6$ monocytes/mL of RPMI-1640 supplemented with 2 mM glutamax-I/glutamine, 2 mM penicillin/streptomycin and 10% inactivated foetal calf serum (standard medium) were transferred to wells of a 24-wells plate and incubated with ES/S or, as a control, H_2O for 1 h followed by stimulation with LPS (Sigma Chemical Co., St. Louis, MO, USA), LTA (Invivogen, Toulouse, France) or no stimulus. After 18-22 h incubation at 37°C and 5% CO_2, supernatants were collected and stored at -70°C.

Measurement of cytokine and chemokine levels

The cytokine and chemokine levels in the supernatants of the cell cultures were assessed using BioSource CytoSet™ (Biosource Europe, S.A., Belgium) and Bio-Plex kits (BIO-RAD, Hercules, CA, USA).

Chemotaxis

Migration of monocytes was measured as previously described[10] with the following modifications. The lower compartments contained a mixture of 25% HEPES buffer and 75% supernatants from monocyte cultures stimulated as described above. To test direct effects of ES/S on monocyte migration, 10 nM fMLP (Sigma) was added as well. In the upper compartment, 50 µL of $2x10^6$ monocytes/mL of RPMI-1640 were placed. Results are expressed as the number of cells counted in 2 $µm^2$ areas in 11 subsequent levels within each filter.

Phagocytosis assay

S*taphylococcus aureus* 42D were grown overnight in TSB at 37°C while shaking, then washed and resuspended ($1x10^7$/mL) in HBSS-0.1% (v/wt) gelatin. Equal volumes of this suspension and a freshly isolated or 18h (ES/S)-incubated monocyte suspension ($1x10^7$/mL

of HBSS-0.1% gelatin) were mixed and 10% AB-serum was added. Subsequently, 100 µL of this mixture were transferred to hydron-coated NUNCLON™ Surface plates (Nalge Nunc International, Rochester, NY, USA) containing ES/S or H_2O. At various intervals after incubation at 37°C while shaking, cells and bacteria were harvested in cold HBSS and centrifuged at 140xg for 6 min. Next, the number of non-cell-associated bacteria was determined microbiologically using serial dilutions which were plated onto agar plates. Phagocytosis is expressed as the percentage decrease of non-cell-associated *S. aureus*.

Intracellular killing assay

Opsonisation and intracellular killing of *S. aureus* were done as previously described[15] using hydron-coated NUNCLON™ plates. Disruption of monocytes was performed by harvesting these cells in H_2O supplemented with 0.01% (v/wt) BSA and then vortexing these suspensions for 60 s. Killing is expressed as the percentage decrease in the number of viable bacteria determined as described above.

Flow cytometry

Cells were incubated with FITC- or PE-conjugated monoclonal antibodies directed against CD11b, CD14, CD32, CD35, CD54, and CD64 (BD Pharmingen™, BD BioSciences, Erembodegem, Belgium), CD16 (EuroBioSciences GmbH, Friesoythe, Germany) and CD282 (TLR-2) and CD284 (TLR-4; Hycult Biotechnology, Uden, The Netherlands) in PBS containing 0.5% (w/v) BSA for 30 min on ice. Analyses were performed on the FACSCalibur (Becton&Dickinson, La Jolla, CA, USA) in combination with CellQuest™ Pro 4.0.2 software.

Cell viability

Monocytes were stimulated and incubated as described above and then incubated with fluorescently-labelled Annexin V (2.5 µg/mL, Sigma) and propidium iodide (1 µg/mL, Sigma) in 10 mM HEPES (pH 7.4) as previously described[16]. The mean fluorescence intensities of the cells were analyzed by flow cytometry.

Measurement of intracellular cAMP concentration

Monocytes in RPMI were incubated with a range of ES/S or no stimulus for various intervals up to 2 min. The reaction was stopped by adding lysis buffer. Next, the cAMP content of these samples was measured using the cAMP Biotrak Enzymeimmunoassay System (Amersham Biosciences, Buckinghamshire, UK). The cAMP concentrations were calculated from these values and the mean cell volume of human monocytes[17].

Inhibition of cAMP

Monocytes were pre-incubated with the protein kinase A inhibitor adenosine-3',5'-cyclic monophosphorothioate,triethyl ammonium salt (Rp-cAMPS; 1 mM; BioLog Life Science Institute, Bremen, Germany) for 45 min, followed by 1 h incubation with ES/S or H_2O and then stimulated for 18-22 h with 100 ng of LPS/mL. Thereafter, the cytokine production was measured.

Statistical analysis

Differences between the values for cells incubated with ES/S and those for cells incubated with H_2O were analysed with a Wilcoxon test using Graphpad Prism version 4.02.

Results

Effect of secretions on cytokine and chemokine production by monocytes

The results revealed that secretions decreased the LPS-induced production of the pro-inflammatory cytokines TNF-α, IL-12p40 and MIF by monocytes in a dose-dependent manner without effecting IL-1β or IL-6 (Table 1). The production of the anti-inflammatory cytokine IL-10 was increased by secretions. Furthermore, secretions inhibited the LPS-induced production of the chemokine MIP-1β by monocytes, increased MCP-1 and IL-8, but had no effect on RANTES. Secretions did not affect the base-line levels of IL-1β, IL-6, IL-10, IL-12p40, TNF-α, RANTES or MIP-1β (data not shown). In contrast, 70 µg of secretions/mL increased the production of MCP-1 by naïve monocytes from 15 (3-53) to 1049 (425-9063) pg/mL and that of IL-8 from 578 (136-1436) to 3236 (1879-5934) pg/mL while decreasing the production of MIF from 72 (19-318) pg/mL to below the detection limit (10 pg/mL) when using 35 or 70 µg of secretions/mL (n = 6-8).

To determine whether the effective components are secreted or excreted by maggots, we compared the effects of S pools to ES pools from the same maggots on the cytokine and chemokine profile of monocytes. The results showed better effects of S than of ES when using equal protein concentrations (data not shown). However, the protein concentration was 30 ± 2% lower for S than ES pools meaning that we used the products of more maggots when testing the secretions. Therefore, we tested the differences in effects when using the volume of the S pools necessary for getting for example 35 µg and used the same volume for testing the ES pools (which was 50 µg). The results showed equal effects of S and ES indicating that the active component is secreted by maggots. Therefore, we combined the results for S and ES and refer to it as secretions.

Table 1 Effect of maggot secretions on the LPS-induced production of various cytokines and chemokines by monocytes.

	Control cells		Maggot secretions (µg/mL)			
			0.35	3.5	35	70
	Median (ng/mL)	Range (ng/mL)	(%)	(%)	(%)	(%)
IL-1β	0.5	0.2 -1.6	97 ± 4	81 ± 11	80 ± 10	91 ± 16
IL-6	25	13 - 40	89 ± 7	95 ± 4	92 ± 11	116 ± 15
IL-8	153	81 - 310	120 ± 7*	121 ± 9*	149 ± 15*	268 ± 63*
IL-10	0.4	0.05 - 2.1	108 ± 8	142 ± 11**	206 ± 35**	209 ± 35**
IL-12p40	0.3	0.1 - 5.4	98 ± 6	82 ± 6*	42 ± 7**	39 ± 10**
TNF-α	11	5 - 25	109 ± 14	84 ± 5*	29 ± 5*	19 ± 4*
MIF	0.08	0.04 - 0.2	85 ± 21	41 ± 14**	13 ± 5**	5 ± 3**
MCP-1	37	12 - 68	119 ± 8	134 ± 17	250 ± 33**	367 ± 66**
MIP-1β	20	1 - 155	107 ± 11	104 ± 20	23 ± 9**	17 ± 7*
RANTES	0.4	0.2 - 1.5	101 ± 3	109 ± 6	103 ± 10	74 ± 14

The results of the control cells, shown as the median value and the range, are set at 100%. The effect of secretions is expressed as a percentage relative to these values. The results are means ± SEM of 6-10 experiments. Values are significantly (*$p<0.05$ and **$p<0.005$) different from those for control monocytes stimulated with LPS.

Effect of secretions on the sensitivity of monocytes to LPS and LTA

The results revealed that the production of TNF-α by monocytes was down-regulated significantly by 35 µg of secretions/mL for all concentrations of LPS (Figure 1A). The IL-12p40 production by monocytes was dose-dependently inhibited by secretions (Figure 1B) while the production of IL-10 by monocytes was enhanced (Figure 1C). In addition, secretions (35 µg/mL) reduced the production of TNF-α (Figure 2A) and IL-12p40 (Figure 2B) by monocytes in response to LTA dose-dependently, while enhancing IL-10 (Figure 2C).

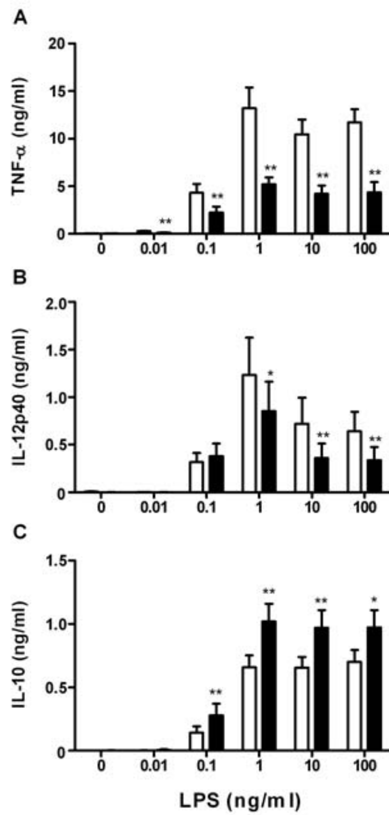

Figure 1 Effect of maggot secretions (35 µg/mL) on the production of TNF-α (A), IL-12p40 (B) and IL-10 (C) by monocytes challenged with a range of LPS. The results are means and SEM of 10-11 experiments. Values are significantly (*p<0.05 and **p< 0.005) different from those for control-incubated monocytes stimulated with LPS.

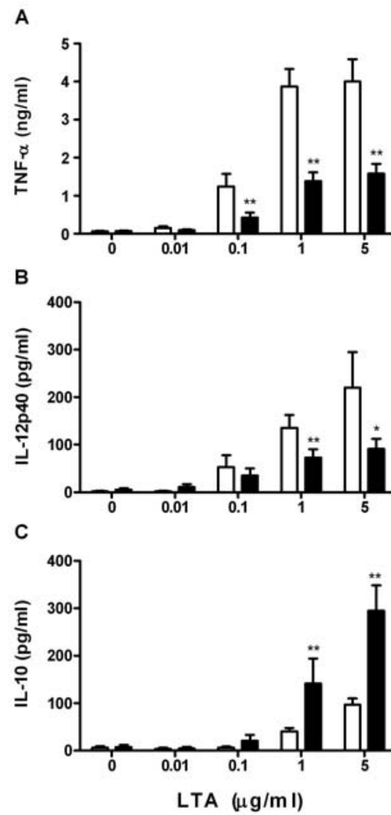

Figure 2 Effect of maggot secretions (35 µg/mL) on the production of TNF-α (A), IL-12p40 (B) and IL-10 (C) by monocytes challenged with a range of LTA. The results are means and SEM of 10 experiments. Values are significantly (*p<0.05 and **p< 0.005) different from those for control-incubated monocytes stimulated with LTA.

Effect of supernatants of secretions-treated monocytes on cell migration

Since incubation of monocytes with secretions resulted in an altered production of several chemokines, we investigated the effect of such monocyte culture supernatants on migration of monocytes. The results revealed that the chemotactic activity of monocytes towards supernatants of LPS-stimulated monocytes was abrogated when incubated in the presence of 35 µg of secretions/mL (Figure 3A). Secretions did not induce migration of naïve monocytes. Interestingly, secretions blocked migration of monocytes towards the chemotactic factor fMLP (Figure 3B). Furthermore, the chemotactic response of monocytes

towards combinations of supernatants and fMLP was decreased in the presence of secretions.

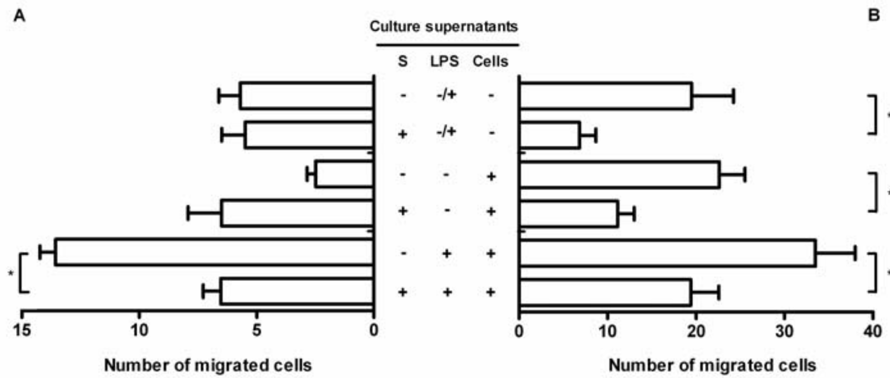

Figure 3 Effect of maggot secretions on the chemotactic activity of monocytes. Migration of monocytes in response to chemokines in cell-culture supernatants without (A) or with (B) 10 nM of fMLP was monitored using a Boyden microchemotaxis chamber. Results are means and SEM of six experiments. Each experiment was performed in quadruplicate. Values are significantly (*p<0.05 and **p<0.005) different from those for monocytes stimulated without secretions.

Effect of secretions on the phagocytosis and intracellular killing of S. aureus by monocytes

The results showed secretions (3.5 and 35 µg/mL) not to affect the phagocytosis and intracellular killing of *S. aureus* 42D by monocytes (Table 2). The antibacterial functions of monocytes incubated for 18 h with secretions were identical (data not shown).

Table 2 Effect of maggot secretions on the phagocytosis and intracellular killing of *Staphylococcus aureus* by monocytes.

Secretions (µg/mL)	Phagocytosis (%) at various intervals (min)		Intracellular killing (%) at various intervals (min)	
	30	60	30	60
0	12 ± 3	39 ± 4	38 ± 11	61 ± 7
3.5	21 ± 4	35 ± 4	44 ± 8	55 ± 10
35	19 ± 2	34 ± 3	38 ± 12	57 ± 10

Results are means ± SEM of 6 experiments.

Effect of secretions on cell surface receptors on monocytes

The results (Table 3) showed secretions (35 µg/mL) not to affect the expression of the pathogen-recognition receptors CD282, CD284 and CD14 or the Fcγ receptors CD16, CD32 and CD64, involved in the phagocytosis of opsonised bacteria, except for a reduction in the LPS-induced expression of CD32 (FcRII). Additionally, the expression of CD11b (together with CD18 complement receptor 3), involved in adhesion of monocytes to endothelial cells and phagocytosis of bacteria, was enhanced by secretions while complement receptor 1 (CD35) expression was not affected. CD54 expression was enhanced by secretions on naïve, but not LPS-stimulated, cells.

Table 3 Effect of maggot secretions on the expression of surface molecules on monocytes.

	No stimulus		LPS (100 ng/mL)	
	0	35 µg/mL	0	35 µg/mL
CD14	27 ± 3	26 ± 3	68 ± 12	60 ± 5
CD282	34 ± 2	34 ± 2	14 ± 2	13 ± 2
CD284	32 ± 2	29 ± 2	22 ± 5	18 ± 2
CD16	15 ± 4	12 ± 2	ND	ND
CD32	74 ±12	78 ± 18	273 ± 27	214 ± 19*
CD64	31 ± 14	35 ± 18	8 ± 1	8 ± 1
CD35	31 ± 4	28 ± 3	29 ± 3	28 ± 3
CD11b	243 ± 24	399 ± 52**	74 ± 14	122 ± 21**
CD54	386 ± 35	517 ± 54**	1365 ± 71	1293 ± 60

The results, expressed as the mean fluorescence intensity (MFI), are means ± SEM of 6-10 experiments. Values are significantly (*p<0.05 and **p<0.005) different from those for control-incubated monocytes. ND: not detectable.

Effect of secretions on the viability of monocytes

The results showed that secretions dose-dependently enhanced the percentage of viable monocytes (Table 4). Moreover, the LPS-induced increase in monocyte-survival was further enhanced by secretions.

Table 4 Effect of maggot secretions on the viability of monocytes

LPS (100 ng/mL)	Secretions (µg/mL)	Survival (%)
-	0	52 ± 6
-	0.35	54 ± 7
-	3.5	58 ± 5*
-	35	74 ± 4*
-	70	80 ± 2*
+	0	69 ± 4* ⌉ **
+	35	80 ± 3* ⌋

The results, expressed as the percentage viable cells, are means ± SEM of 6-10 experiments. Values are significantly (*p<0.05 and **p<0.005) different from those for control-incubated monocytes.

Effect of secretions on the intracellular cAMP concentration

Analysis of the results revealed the peak cAMP concentration to be reached 15 s after the addition of secretions (35 µg/mL) and to return gradually to basal values at 120 s (data not shown). Based on these results, the 15 s interval was chosen to determine the dose-effect relation for secretions. The results revealed 3.5 µg of secretions/mL to significantly enhance the cAMP concentrations with a maximum increase up to 1.9-fold over basal level after exposure to 70 µg of secretions/mL (Table 5). In agreement, Rp-cAMPS (1 mM) significantly attenuated the inhibitory effect of secretions (35 µg/mL) on the LPS-stimulated production of TNF-α from 71 ± 5% to 41 ± 12% (n = 9; p<0.005) and of IL-12p40 from 71 ± 6% to 32 ± 14% (p<0.005), whereas it blocked (p<0.05) the increase in IL-10 production by LPS-stimulated monocytes completely.

Table 5 Effect of maggot secretions on the cAMP concentration in monocytes

Secretions (µg/mL)	cAMP (µM)
0	0.91 ± 0.07
0.35	0.97 ± 0.08
3.5	1.09 ± 0.10*
35	1.33 ± 0.13*
70	1.70 ± 0.17*

Results are means ± SEM of 10 experiments. Values are significantly (*p<0.05) different from those for control-incubated monocytes.

Discussion

The main conclusion from the present study is that maggot secretions suppress the pro-inflammatory responses of monocytes without affecting their antimicrobial activities. This conclusion is based on the following observations. First, secretions reduced the production of the pro-inflammatory cytokines IL-12p40, TNF-α and MIF by LPS-stimulated monocytes whereas the production of anti-inflammatory cytokine IL-10 was enhanced. Addition of secretions to monocytes that had already been exposed to LPS resulted in similar effects on the cytokine profile, indicating that secretions can interfere with an ongoing inflammatory reaction (data not shown). The anti-inflammatory actions of secretions are not limited to modulation of the TLR-4 pathway as secretions exerted similar effects on cells stimulated with a TLR-2 ligand. Furthermore, secretions inhibited the LPS-induced production of TNF-α and IL-12p40 by cells in whole blood (unpublished observations). However, the production of the anti-inflammatory cytokine IL-10 by blood cells was not affected by secretions suggesting that the secretions-induced increase in IL-10 production by purified monocytes may be counteracted by cellular/molecular components of whole blood. The suggestion that maggots produce IL-10[18] was withdrawn by the authors (personal communication with dr K.Y. Mumcuoglu, Dept of Parasitology, Hebrew University-Hadassah Medical School, Jerusalem, Israel). Second, secretions decreased the chemotactic response of monocytes towards fMLP as well as to the chemotactic factors in supernatants of (LPS-stimulated) monocyte cultures. These results are in agreement with our earlier finding that ES reduced the migration of human neutrophils towards fMLP[10]. The secretions induced production of chemotactic factor MCP-1 and decreased production of migration inhibitor MIF by monocytes did not increase migration indicating that secretions inhibited migration independent of the levels of these chemokines; participation of MIP-1β inhibition cannot be excluded. The effect of secretions-induced increased levels of IL-8 and CD11b are not tested within our experimental set up. The increased expression of CD54 and CD11b on naïve monocytes is unlikely to influence chemotaxis as monocytes are triggered when migrating into a wound. Third, secretions did not affect the phagocytosis and intracellular killing of *S. aureus* by freshly isolated monocytes and by 18 h cultured monocytes. This is in agreement with our earlier findings that ES had no effect on the phagocytosis and intracellular killing of *Candida albicans* by neutrophils[10]. Additionally, maggots aid in the removal of bacteria from wounds by ingesting bacteria together with liquefied necrotic tissue and subsequently killing them in their digestive tract[19,20]. An important implication of the above observations that secretions interfered in a similar fashion with activation of both the TLR-2 and TLR-4 pathways is that the reported differences in effects of maggots on survival of gram-positive and gram-negative bacteria[21] are likely the result of antibacterial activity[1] and not of differential modulation of immune cell responses.

The second conclusion pertains to the mechanisms by which secretions exert their effects on monocytes. Our results showed that the effects of secretions on the cytokine/chemokine profiles of LPS- and LTA-stimulated monocytes were not caused by an altered sensitivity to these stimuli. In agreement, secretions had no effect on the expression of surface molecules involved in the recognition of the bacterial products by (LPS-stimulated) monocytes, suggesting that secretions exert their effects either downstream of these receptors or on other, yet identified (intracellular) binding partners and targets. Based on our earlier finding that ES increased the intracellular cAMP levels in neutrophils[10], we presumed a similar mechanism by monocytes and indeed found that the cAMP concentrations were enhanced dose- and time-dependently by secretions. Pre-treating monocytes with Rp-cAMPS, an inhibitor of cAMP-dependent PKA-activation, attenuated the effects of secretions on LPS-stimulated cytokine production indicating that maggots exert their effects on monocytes through a cAMP-dependent mechanism. In agreement, others reported that activation of cAMP pathways is associated with reduced production of pro-inflammatory cytokines including TNF-α, IL-12 and MIP-1β, without affecting IL-1β production, while enhancing the production of IL-10[22,23]. Furthermore, cAMP-elevation is connected to decreased migration[24,25] whereas phagocytosis by freshly isolated monocytes remains unaffected[26]. However, elevation of cAMP is also associated with a moderate reduction in phagocytosis by incubated/stimulated monocytes and macrophages[26,27] which seems to be in contrast with our data. This discrepancy can be explained by our observation that secretions enhanced the viability of monocytes; although the total phagocytosis of bacteria remained the same, the amount of phagocytosis per viable monocyte decreased. As secretions did not decrease the expression of FcR, CR1 or CD11b (part of CR3), the reduction in phagocytosis per cell may be explained by interference of signalling pathways down-stream of receptor activation[27]. Of note, cAMP is known to inhibit apoptosis in several cell types[28-30].

What could be the clinical relevance of the present findings? Although pro-inflammatory responses are essential for healing of acute wounds, they can be detrimental in chronic wounds where inflammation persists. Some histological data exists that parts of chronic wounds seem to be stuck in different phases of healing with loss of synchronicity that leads to rapid healing[12]. Some part ready for epidermal resurfacing and fibroblast proliferation could be damaged by another part that is still in the inflammatory phase[31]. It has been reported that chronic leg ulcers are associated with elevated expression of pro-inflammatory cytokines, like TNF-α and MIF, compared to acute wounds[32-34]. These cytokines enhance the production and release of a large variety of other pro-inflammatory cytokines[35,36] as well as the synthesis of several matrix metalloproteinases and serine proteases[14,36,37]. When produced in excess these pro-inflammatory responses may cause deleterious extracellular matrix destruction[38-40], and growth factor and protease inhibitor inactivation[37,41-43] and are responsible for the failure of wound healing. In addition, TNF-α activates phagocytes to

produce reactive oxygen intermediates[44,45] which can be toxic to cells like endothelial cells, fibroblasts and leucocytes and may further promote tissue proteolysis by potentiating the effects of several proteinases while inactivating proteinase inhibitors[46,47]. Together, pro-inflammatory responses may be responsible for maintenance of chronic wounds. Furthermore, TNF-α is also related to the formation of ulcers by enhancing the production of plasminogen activator inhibitor-1[48,49] which can lead to impaired lysis of pericapillary fibrin cuffs[50,51]. Importantly, although the mechanisms underlying the immunomodulatory effects of secretions on monocytes are not fully elucidated, the findings from the present *in vitro* study show that maggot secretions potently inhibit the pro-inflammatory activities of monocytes. Secretions decrease migration of cells to the wound and reduce the amount of pro-inflammatory cytokines of the cells located in the wound while their overall antibacterial activities are unaltered. Consequently, the release of other pro-inflammatory cytokines, reactive oxygen intermediates and proteases will diminish bringing tissue destruction to a halt and may result in an environment beneficial for healing.

The exiting beneficial effect of maggots in diabetic foot ulcers and other chronic wounds found in clinical studies[6-9] could well be explained by the phenomena described in this study. Besides direct antibacterial features of maggots observed in other studies[19,20], and our earlier observations that ES can inhibit the formation of and brake down bacterial biofilms[1], we found that the maggots seem to preserve the important anti-bacterial function of human leucocytes while protecting the fragile regenerating woundbed against inflammation and tissue destruction by the same inflammatory cells.

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