Significant immunomodulatory effects of *Pseudomonas aeruginosa* quorum-sensing signal molecules: possible link in human sepsis

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**ABSTRACT**

Pathogenic bacteria use quorum-sensing signal molecules to co-ordinate the expression of virulence genes. Animal-based studies have demonstrated the immunomodulatory effects of quorum-sensing signal molecules. In the present study, we have examined the impact of these molecules on normal human immune function in vitro and compared this with immune changes in patients with sepsis where quorum-sensing signal molecules were detected in the sera of patients. Quorum-sensing signal molecules inhibited normal dendritic cell and T-cell activation and proliferation, and down-regulated the expression of co-stimulatory molecules on dendritic cells; in MLDCRs (mixed lymphocyte dendritic cell reactions), secretion of IL (interleukin)-4 and IL-10 was enhanced, but TNF-α (tumour necrosis factor-α), IFN-γ (interferon-γ) and IL-6 was reduced. Quorum-sensing signal molecules induced apoptosis in dendritic cells and CD4+ cells, but not CD8+ cells. Dendritic cells from patients with sepsis were depleted and ex vivo showed defective expression of co-stimulatory molecules and dysfunctional stimulation of allogeneic T-lymphocytes. Enhanced apoptosis of dendritic cells and differential CD4+ Th1/Th2 (T-helper 1/2) cell apoptotic rate, and modified Th1/Th2 cell cytokine profiles in MLDCRs were also demonstrated in patients with sepsis. The pattern of immunological changes in patients with sepsis mirrors the effects of quorum-sensing signal molecules on responses of immune cells from normal individuals in vitro, suggesting that quorum-sensing signal molecules should be investigated further as a cause of immune dysfunction in sepsis.

**INTRODUCTION**

Bacteria inhabit the gastrointestinal tract and are essential for nutrition and the well-being of the host [1]. Bacteria in the gut secrete many intraluminal chemicals which, in some circumstances, may include QSSMs (quorum-sensing signal molecules). QSSMs are transmitters for bacteria to communicate with one another and they have a key role in the switching of processes such as virulence, biofilm formation, sporulation, mating and competence.

**Key words:** apoptosis, CD4+ T-cell, dendritic cell, quorum-sensing signal molecule, sepsis.

**Abbreviations:** 7-ADD, 7-amino-actinomycin D; AHL, N-acylhomoserine lactone; APC, allophycocyanin; DC, dendritic cell; HLA-DR-ECD, anti-HLA-DR Phycoerythrin-Texas Red®-x; IFN-γ, interferon-γ; IL, interleukin; Lin-FITC, lineage cocktail 1 labelled with FITC; LPS, lipopolysaccharide; MLDCR, mixed lymphocyte DC reaction; 3-oxo-C6-HSL, N-(3-oxohexanoyl)-l-homoserine lactone; 3-oxo-C12-HSL, N-(3-oxododecanoyl)-l-homoserine lactone; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; QSSM, quorum-sensing signal molecule; Th, T-helper; TNF-α, tumour necrosis factor-α.

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for DNA [2–4]. The complexity of QSSMs is determined by various mechanisms involving signal production, detection, relay and response [5]. Gram-negative bacterial pathogens that can colonize the gut produce autoinducers or the QSSMs AHLs (N-acylhomoserine lactones) [5,6]. Some of the best-described AHLs belong to the 4-14 carbon chain family of AHLs. 3-oxo-C6-HSL [N-(3-oxohexanoyl)-]homoserine lactone] and 3-oxo-C12-HSL [N-(3-oxododecanoyl)-]homoserine lactone] have been identified in pathogenic bacteria (e.g. Pseudomonas aeruginosa) [7,8]. AHLs can modulate immune function in animals, enhancing bacterial survival. In vitro studies have demonstrated that 3-oxo-C12-HSL can suppress secretion of TNF-α (tumour necrosis factor-α) and IL (interleukin)-12 by murine macrophages, and induce potent inflammation in the skin of mice [9,10]. Previous studies have shown that 3-oxo-C12-HSL has immunosuppressive effects on human PBMCs (peripheral blood mononuclear cells) [11,12]. This could result from direct effects on T-cells and/or DCs (dendritic cells), which are key antigen-presentation cells. DCs translate innate into adaptive immunity by integrating antigen processing and subsequent presentation to naive T-lymphocytes in secondary lymphoid compartments, using co-stimulatory molecules whose expression depends on DC activation and maturation [13]. A single mature activated DC is capable of stimulating 100–3000 T-cells [14].

In sepsis, an alteration in the number and activity of gut microflora and, in particular, colonization by pathogenic Gram-negative bacteria may lead to production of QSSMs, thereby inducing disruption of gut homoeostasis [15]. This alteration of the gut biological–chemical environment could result in systemic entry of toxic substances, including QSSMs, and modulation of subsequent host inflammatory responses [16,17]. It has been postulated that high levels of QSSMs may be immunosuppressive and/or dysregulate key elements of cell-mediated immunity. Although animal studies suggest a selective effect on T-cell subsets [10], there is limited data from human in vitro studies and, to the best of our knowledge, none from human in vivo studies.

In the present study, we investigated the presence of QSSMs in vivo, the effects of QSSMs in vitro on DCs and T-cell subsets from normal individuals, and the host immune status in sepsis. Our findings offer a new and novel insight into the possible pathogenesis of severe sepsis in humans.

**Materials and Methods**

**Patients and controls**

The present study was carried out in the Division of Surgery, Queens Medical Centre, Nottingham, U.K. Seventeen healthy donors donated blood samples. Forty serum samples from patients with sepsis were used for the QSSM analysis. Patients were classified as being septic, having severe sepsis or septic with shock, according to the definitions outlined by the ACCP (American College of Chest Physicians)/SCCM (Society of Critical Care Medicine) [18]. Of the 40 serum samples studied, 17 were from patients with sepsis, 16 were from patients with severe sepsis and seven were from patients with septic shock. The study was approved by the Local Research Ethics Committees and all patients and volunteers gave written informed consent. The 3-oxo-C6-HSL and 3-oxo-C12-HSL used in the present study were synthesized by the Institute of Infection, Immunity and Inflammation, University of Nottingham, Nottingham, U.K.

**Cell preparation**

PBMCs were isolated from heparinized blood by density-gradient centrifugation over Histopaque 1077 (Sigma). DCs were generated from adherent monocytes in RPMI 1640 (Sigma–Aldrich), containing 500 i.u./ml GM-CSF (granulocyte/macrophage colony-stimulating factor; R&D Systems) and 500 i.u./ml IL-4 (R&D Systems), for 7 days. Circulating DCs were isolated from PBMCs using an established magnetic method (Miltenyi Biotec), and DC numbers were counted using light microscopy. T-cells were isolated from PBMCs using CD3+ magnetic microbeads (Miltenyi Biotec). Yields of > 93 % purity were obtained.

**MLDCR (mixed lymphocyte DC reaction) assay**

Isolated blood DCs were irradiated with 20 Grays in a cell irradiator (137Cs source) and used to stimulate allogeneic CD3+ T-cells from a single healthy volunteer, distributed at 5 × 10⁴ cells/well. The cells were cultured with QSSMs (10, 25, 50 and 100 μmol/l) for 6 days at 37°C in a humidified 5 % CO₂ incubator. Proliferation was assessed by the incorporation of [³H]thymidine. Either DCs or T-cells were incubated with 3-oxo-C12-HSL (10, 25, 50 and 100 μmol/l) for 24 h, washed twice and MLDCRs were set up.

**DC surface markers**

Heparinized blood (200 μl) or generated DCs were stimulated with 1 μg/ml LPS (lipopolysaccharide; Sigma–Aldrich), in the presence or absence of 100 μmol/l 3-oxo-C12-HSL at 37°C for 24 h. Cells were labelled using Lin-FITC (lineage cocktail 1 labelled with FITC; Becton Dickinson), HLA-DR-ECD (anti-HLA-DR Phycoerythrin-Texas Red®-x; Beckman Coulter) and CD86-APC (anti-CD86 allophycocyanin; Becton Dickinson) for 30 min at 6–12°C. For whole-blood analyses, red blood cells were lysed using OptiLyse C (Beckman Coulter). Samples were washed and fixed with 2 % (w/v) paraformaldehyde before flow-cytometric analysis (EPICS Altra; Beckman Coulter).
T-cell activation markers
PBMCs were stimulated with 100 ng/ml super-antigen (Staphylococcal enterotoxin F; Sigma), in the presence or absence of 100 μmol/l 3-oxo-C12-HSL for 24 h. Cells were then labelled with CD3-FITC (Becton Dickinson) and CD69-PE (CD69-phycocerythrin) for 30 min at 4 °C, washed and fixed with 2 % (w/v) paraformaldehyde before flow-cytometric analysis.

QSSMs and apoptosis in DCs and T-cells
DC and T-cell apoptosis was determined using Annexin V-PE/APC and 7-AAD (7-amino-actinomycin D; Pharmingen). Appropriate controls were used and 10, 25, 50 and 100 μmol/l 3-oxo-C12-HSL for an incubation of 24 h. Antibodies used for DCs and T-cells were: HLA-DR-APC and Lin-FITC for DCs, and anti-CD3ε, anti-CD4+ or anti-CD8+ for T-cells. The cells were analysed by flow cytometry within 2 h of preparation.

Apoptotic pathways, in particular caspase 3 and cytochrome c in DCs and CD4+ T-cells, were studied by flow cytometry using HLA-DR-ECD and Lin-FITC for DCs, and CD4-FITC and CD8-PECy7 (CD8-PE coupled to the cyanine dye Cy7; BD Pharmingen) for T-cells. Caspase 3-PE was from BD Pharmingen and cytochrome c-PE was from Santa Cruz Biotechnology.

Cytokine levels in supernatants of MLDCRs
Supernatants from multiwell-plate cultures of DCs and T-cells were collected on day 6 and frozen at −70 °C until further analysis. Cytokines IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ (interferon-γ) were analysed using a cytokine bead array (Becton Dickinson).

QSSMs in serum
Serum samples were analysed for the presence of AHLs as previously described [19]. Samples were extracted with acetonitrile (5 ml), filtered and evaporated to dryness under reduced pressure. The resulting extracts were dissolved in acetonitrile (50 μl) and examined for the presence of AHLs by TLC on RP-2 F254s reversed-phase TLC plates (Macherey & Nagel) treated with a methanol/water solvent system. Synthetic AHLs were run as standards. After chromatography, TLC plates were dried and overlaid with LB (Luria–Bertani) medium containing 0.75 % agar seeded with the Escherichia coli AHL biosensor (pSB1075). This sensor was used to detect AHLs. Following incubation for 6–16 h at 30 °C, AHLs were visualized as a pseudocolour image when viewed with a Luminograph LB980 photon video camera (Berthold).

Statistical analysis
The Minitab version 13 software was used for analysing the data. An unpaired Student’s t test was used to analyse the statistical differences between two independent samples. Where comparisons of more than two independent samples were required, ANOVA with a post-hoc Holm–Sidak test was used. For the non-parametric data, the Kruskal–Wallis test was used, as well as the Mann–Whitney U test with Bonferroni corrections for the post-hoc analysis of multiple comparisons. A χ2 test was used to test statistical significance between the presence of QSSMs in the serum, and mortality and blood cultures.

RESULTS
Modulation of DC and T-cell function in vitro by QSSMs
3-oxo-C12-HSL suppressed the MLDCR
3-oxo-C12-HSL (100 μmol/l) did not affect cell viability (Figure 1D). 3-oxo-C12-HSL (10–100 μmol/l) inhibited DC activation of allogeneic T-cells with both freshly isolated and in-vitro-generated and activated DCs (Figures 1A and 1B respectively). To establish whether DCs or T-cells were inhibited by the QSSMs, modified MLDCRs were carried out using in-vitro-generated DCs. Both in-vitro-generated activated DCs and allogeneic T-cells were significantly inhibited by 10, 25, 50 and 100 μmol/l 3-oxo-C12-HSL (Figure 1B).

3-oxo-C12-HSL inhibited the up-regulation of CD86 on DCs stimulated by LPS and inhibited T-cell activation marker expression
LPS up-regulated the expression of CD86 on isolated DCs. 3-oxo-C12-HSL inhibited this LPS-induced expression of CD86 on isolated DCs (Figure 1C). After stimulation with super-antigen, T-cells expressed high levels of CD69. When 3-oxo-C12-HSL was added concurrently, expression of CD69 on activated T-cells was reduced (Figure 1C).

3-oxo-C12-HSL induced apoptosis in DCs and CD4+ T-cells but not CD8+ T-cells
After incubation for 24 h with 3-oxo-C12-HSL, there was a significant dose-dependent increase in the number of apoptotic DCs (Figures 2A and 2B). Apoptosis in CD4+ T-cells was also increased but CD8+ T-cells showed no significant change in apoptosis after incubation with increasing concentrations of 3-oxo-C12-HSL (Figures 2A and 2B).

3-oxo-C12-HSL induced an increase in active caspase 3 and cytochrome c production in DCs and CD4+ T-cells
Intracellular active caspase 3 and cytochrome c in the presence of 3-oxo-C12-HSLs were increased in DCs, CD4+ T-cells and CD8+ T-cells in a time-dependent manner. The optimal time for significant activation of cytochrome c was at 6 h, but for active caspase 3 this was both time- and cell-subset-dependent (Figure 3).
Figure 1  Effect of 3-oxo-C12-HSL on T-cells and DCs

(A) Allogeneic T-cell stimulation assay with DCs and T-cells isolated from blood using DC/T-cell ratios of 1:20, and four different concentrations of 3-oxo-C12-HSLs (**P < 0.01 compared with no addition of 3-oxo-C12-HSL, measured using a Kruskal–Wallis test with post-hoc Mann–Whitney U tests with Bonferroni correction).

Five different donors were used to isolate DCs. (B) Allogeneic T-cell stimulation assays with in-vitro-generated DCs and isolated T-cells were each incubated with four different concentrations of 3-oxo-C12-HSL for 24 h, washed and used in the assays. At any one time, either T-cells or DCs were treated (∗P < 0.05 compared with no addition of 3-oxo-C12-HSL, measured using ANOVA with a post-hoc test).

(C) Effect of 3-oxo-C12-HSL (100 μmol/l) on the expression of co-stimulatory molecules (CD86) on freshly isolated and LPS-stimulated blood DCs, and the expression of activation markers (CD69) induced by super-antigen (sAg) on blood T-cells. Values are means ± S.E.M. (∗P < 0.05 and **P < 0.01 compared with controls, measured using ANOVA with a post-hoc test). (D) A representative flow cytometric result identifying cell death after incubating PBMCs with 100 μmol/l 3-oxo-C12-HSL for 24 h. 3-oxo-C12-HSL did not affect cell viability.

Th (T-helper) 1 and Th2 cytokine production by 3-oxo-C12-HSL

Supernatants removed from MLDCRs were analysed for cytokine profiles. 3-oxo-C12-HSL induced production of IL-4 and IL-10 (Th2 anti-inflammatory) at 25, 50 and 100 μmol/l (P < 0.01 and P < 0.001, measured using the Kruskal–Wallis test; Table 1). Production of IL-6, TNF-α and IFN-γ (Th1 pro-inflammatory) was decreased in supernatants from MLDCRs. Inhibition of TNF-α, IL-6 and IFN-γ occurred from 10 μmol/l to 100 μmol/l 3-oxo-C12-HSL (P < 0.05, P < 0.01 and P < 0.001, measured using the Kruskal–Wallis test; Table 1).

QSSMs in the blood of patients with sepsis

Activation of AHL biosensors was documented in the sera of 37.5 % of patients with sepsis, with an increased frequency (86 %) in patients with septic shock, suggesting the presence of AHL QSSMs in the positive samples (Figure 4 and Table 2). No activation of the AHL biosensors was detectable in the sera of 17 healthy volunteers. The presence of QSSMs in the serum of patients correlated with mortality rate (P < 0.05, measured using a χ² test; Table 3).

Modulation of DC and T-cell function in patients with sepsis

Impaired DC function (MLDCR) from patients with sepsis DC numbers in blood were lower in patients with sepsis than healthy controls (P < 0.01; measured using an ANOVA) (Figure 5A). DCs were isolated from the blood of patients with sepsis and were used to stimulate...
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Figure 2
(A) A representative example of the apoptotic effect of 3-oxo-C12-HSL in generated DCs, and CD4+ and CD8+ T-cells. The right-upper quadrant represents dead cells, the right-lower quadrant represents cells undergoing apoptosis, and the left-lower quadrant represents viable cells. (B) The effect of 3-oxo-C12-HSL on apoptosis of DCs, and CD4+ and CD8+ T-cells; five different samples are shown. Values show the median (line) and mean (dot) values, and interquartile ranges (box). (*P < 0.05 and **P < 0.01 compared with no addition of 3-oxo-C12-HSL, using ANOVA with a post-hoc test).

Figure 3
(A) The effect of 3-oxo-C12-HSL on intracellular cytochrome c. Five different donors were used to isolate DCs and T-cell subsets. A significant increase in intracellular cytochrome c was seen at 6 h in CD4+ and CD8+ T-cells, but not in DCs. *P < 0.05 compared with the 0 h incubation time, measured using ANOVA with a post-hoc test. (B) Effect of 3-oxo-C12-HSL on intracellular active caspase 3 in DCs and T-cell subsets. Five different donors were used to isolate DCs and T-cell subsets. The significant increase in intracellular active caspase 3 was documented at different time points (*P < 0.05 compared with the 0 h incubation time, using ANOVA with a post-hoc test).

Inhibition of expression of CD86 on DCs in patients with sepsis
Expression of CD86 on DCs isolated from the blood of patients with sepsis was low compared with DCs from healthy controls (P < 0.001, measured using ANOVA). After patients received treatment (72 h after admission), the number and function of DCs increased and was reflected in enhanced T-cell responses in the MLDCRs (Figure 5B).

Enhanced apoptosis in DCs and CD4+ Th1 cells, but inhibition in CD4+ Th2 cells, in patients with sepsis
In patients with sepsis, blood DCs had a substantial number undergoing apoptosis (68%) compared with controls (15%) (P < 0.001, measured using a Student’s t test). There was enhanced apoptosis in Th1 cells (32%) (not statistically significant) and reduced apoptosis in Th2 cells (42%) compared with controls (P < 0.01, measured using a Student’s t test) (Figure 6).

Enhanced Th2 cytokine, but decreased Th1 cytokine, production in MLDCRs from patients with sepsis
Supernatants removed from MLDCRs using isolated blood DCs from patients with sepsis had a significantly increased production of IL-10, and a significantly decreased production of IL-2, IL-6 and IFN-γ (P < 0.05 and P < 0.01, measured using a Student’s t test) (Figure 7).

DISCUSSION
Bacteria and their products have been postulated to be linked to the pathogenesis of sepsis, in particular, QSSMs [1]. Individual bacterial species can produce multiple distinct QSSMs; the best documented are those produced by P. aeruginosa, and include C4-HSL.
Table 1  Levels of Th1 and Th2 cytokines in MLDCR supernatants at day 7

<table>
<thead>
<tr>
<th>MLDCR cytokines</th>
<th>Controls 10 μmol/l</th>
<th>25 μmol/l</th>
<th>50 μmol/l</th>
<th>100 μmol/l</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4</td>
<td>21.7 ± 8.5</td>
<td>46.8 ± 27.2</td>
<td>98.2 ± 24.5</td>
<td>134.3 ± 31.9</td>
<td>207.7 ± 43.9</td>
</tr>
<tr>
<td>IL-10</td>
<td>3.6 ± 1.8</td>
<td>6.0 ± 2.2</td>
<td>38.1 ± 4.0</td>
<td>78.1 ± 8.7</td>
<td>104.7 ± 10.1</td>
</tr>
<tr>
<td>TNF-α</td>
<td>20.9 ± 3.5</td>
<td>11.0 ± 3.5</td>
<td>7.4 ± 0.6</td>
<td>5.3 ± 2.1</td>
<td>6.6 ± 1.0</td>
</tr>
<tr>
<td>IL-6</td>
<td>27.5 ± 6.4</td>
<td>13.4 ± 1.0</td>
<td>10.0 ± 1.0</td>
<td>9.3 ± 1.7</td>
<td>9.2 ± 1.0</td>
</tr>
<tr>
<td>IL-2</td>
<td>12.9 ± 2.0</td>
<td>5.4 ± 2.1</td>
<td>5.3 ± 1.2</td>
<td>4.3 ± 1.8</td>
<td>5.8 ± 1.6</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>696.0 ± 236.0</td>
<td>96.0 ± 32.0</td>
<td>95.0 ± 5.0</td>
<td>13.0 ± 4.0</td>
<td>12.0 ± 7.0</td>
</tr>
</tbody>
</table>

Table 2  Presence of QSSMs in the sera of patients with sepsis

All patients with severe sepsis, with or without shock, had blood cultures taken (n = 23), 17 of these (74 %) had positive cultures. Of the seven patients who had septic shock, six (85 %) had QSSMs detected in the serum and six (85 %) had positive blood cultures. ND, no blood culture was done.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Healthy controls (n = 17)</th>
<th>Patients with sepsis (n = 17)</th>
<th>Patients with severe sepsis (n = 16)</th>
<th>Patients with septic shock (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mortality (n)</td>
<td>0 (0 %)</td>
<td>1 (5.8 %)</td>
<td>6 (37.5 %)</td>
<td>4 (57 %)</td>
</tr>
<tr>
<td>Positive blood culture (n)</td>
<td>ND</td>
<td>ND</td>
<td>11 (68.7 %)</td>
<td>6 (85 %)</td>
</tr>
<tr>
<td>Detection of QSSMs in serum (n)</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
<td>9 (56 %)</td>
<td>6 (85 %)</td>
</tr>
</tbody>
</table>

Figure 4  Detection of AHLs by TLC using the E. coli-based AHL biosensor pSB1075

The presence of AHLs was visualized as a pseudocolour image when viewed with a Luminograph LB980 photon video camera. Representative serum samples collected from patients with sepsis are shown. C is the control image obtained using the synthetic AHL standards 3-oxo-C12-HSL (Oc12) and 3-oxo-C14-HSL (Oc14). Serum samples from the patients with sepsis are indicated as 1–12. In these two representative images, QSSMs were detected in the serum of patients 1, 6, 7 and 10 (indicated by the arrow).

Table 3  Mortality and blood culture status in patients in which QSSMs were or were not detected in their serum

Mortality was related with the presence of QSSMs in the serum.

<table>
<thead>
<tr>
<th>Detection of QSSMs in the serum</th>
<th>Mortality (n = 40)*</th>
<th>Blood culture (n = 22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>QSSMs not detected (n)</td>
<td>21 4 3 5</td>
<td>8 7 3 12</td>
</tr>
<tr>
<td>QSSMs detected (n)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*P < 0.05, using a χ² test.

(N-butyrylhomoserine lactone) and 3-oxo-C12-HSL. In animal studies, 3-oxo-C12-HSL was shown to be non-toxic to mammalian cells, and 3-oxo-C12-HSL was found to inhibit lymphocyte activation and proliferation [10].

QSSMs are produced in the gut by various transient intestinal bacteria such as *P. aeruginosa*. It is thought that these QSSMs are not normally able to pass through the gut barrier into the circulation. However, in critically ill patients (severe sepsis and trauma), intestinal mucosa and gut defences are damaged resulting in enhanced gut permeability. Impairment of gut permeability has been documented in human sepsis and pancreatitis, thus increasing the likelihood of translocation of enteric bacteria and various other possible mediators of the pathogenesis of sepsis and shock into the systemic circulation [20,21]. Sepsis associated with *P. aeruginosa* specifically has been shown to induce severe gut epithelial cell barrier damage. With gut barrier impairment, QSSMs produced in situ may readily
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Figure 5  Impaired DC function from patients with sepsis

(A) Proliferation of DCs from controls and patients with sepsis on admission and after > 72 h of treatment. P values shown are comparisons between controls and each time point. The number of blood DCs (cells/ml) is in 20 ml of blood from patients with sepsis. Values represent ten specimens for controls and patients (on admission), and six for patients > 72 h on therapy. P values shown are comparisons between controls and each time point in hospital (∗∗∗P < 0.001 and NS, not significant compared with controls, measured using ANOVA with a post-hoc test). (B) Allogeneic T-cell stimulation assay of freshly isolated blood DCs (MLDCRs); values shown are the means of T-cell counts from the different time points in hospital (∗∗∗P < 0.001 compared with controls, using ANOVA with a post-hoc test). (C) The expression of CD86 on freshly isolated blood DCs from ten patients with sepsis, compared with healthy controls (∗∗∗P < 0.001, measured using Student’s t test).

Figure 6  Apoptosis of isolated blood DCs, CD4+ Th1 cells and CD4+ Th2 cells from patients with sepsis compared with controls

The medians are indicated by the line and the means are indicated by the dot. Values represent five samples. (∗∗P < 0.01, ∗∗∗P < 0.001 and NS, not significant, measured using Student’s t test).

Figure 7  Decreased production of Th1 regulatory pro-inflammatory cytokines (IL-2, IL-6 and IFN-γ), but enhanced production of IL-10, in supernatant MLDCRs using isolated blood DCs from patients with sepsis compared with healthy controls

(*P < 0.05, **P < 0.01 and NS, not significant, measured using Student’s t test).

enter into the circulation and inhibit host immunity. The present study has documented the presence of a molecule with the same TLC migration as 3-oxo-C12-HSL in the blood of patients with severe sepsis (56%) and/or clinical shock (86 %) using AHL biosensor systems. QSSMs were not detected in the blood from 17 healthy volunteers. However, the levels of the QSSMs have not been quantified due to the lack of a standardized technique. In the present study we show, in vitro, the substantial immunosuppressive effects of QSSMs on human DCs and various T-cell subsets. A preliminary study demonstrated that 3-oxo-C12-HSL, but not 3-oxo-C6-HSL, significantly inhibited human PBMC responses to concanavalin A [11]. In the MLDCRs in the present study, significant immunosuppressive effects were documented with 25, 50 and 100 μmol/l 3-oxo-C12-HSL on both in-vitro-generated (from CD14+ precursors) and freshly isolated blood DCs and T-cells.

To explain more precisely the possible inhibitory mechanisms of 3-oxo-C12-HSL, their effects on the expression of key phenotypic makers on DCs and T-cells were studied. Blood DCs had an up-regulation of the expression of the key co-stimulatory molecule CD86 by culture with LPS; this was reduced by co-culture with 3-oxo-C12-HSL. Down-regulation of CD86 expression will result in diminished antigen presentation to naïve T-cells and generation of peptide-specific cytotoxic T-cells [13]. Furthermore, the activation of T-cells (CD69 expression with super-antigen) was also significantly suppressed by 3-oxo-C12-HSL. These findings offer
a possible explanation for the inhibitory effects of 3-oxo-C12-HSL in the MLDCRs. However, the precise molecular mechanisms responsible for these biological effects are not well established.

Supernatants from the MLDCR assays in the present study showed that 3-oxo-C12-HSL induced the secretion of IL-4 and IL-10 but suppressed the production of IL-2, IL-6, TNF-α, and IFN-γ. IL-10 is a potent modulator of monocyte/macrophage function and can suppress the production of pro-inflammatory cytokines (IFN-γ, TNFα, IL-1 and IL-6) and IL-8 by monocytes following activation [22]. IL-10 has a significant inhibitory effect on DC function, expression of co-stimulatory molecules and the ability to synthesize IL-12 [23]. An animal study has demonstrated that QSSMs inhibited the Th1, but stimulated the Th2, pathway and activated T-cells to produce pro-inflammatory cytokines when injected into the skin of animals [9]. Furthermore, an earlier study had documented that QSSMs were able to inhibit TNF-α and IL-12 production from macrophages and also promoted IgE production by IL-4-stimulated PBMCs [10].

Severely disrupted immune function has been reported in patients with sepsis, including decreased number and suppressed activity of circulating immune cells, dysregulation of the production of suppressive mediators and increased apoptosis of immune cells [24,25]. Increased numbers of Th2 cells with a concurrent decrease in the numbers of Th1 cells has previously been shown in patients with sepsis, compared with non-septic controls [24]. Hotchkiss et al. [26] demonstrated the depletion of B- and CD4+ T-lymphocytes, but not CD8+ or NK (natural killer) cells in patients with sepsis; apoptosis was documented to be the cause of the depletion [26]. The same group demonstrated the depletion of DCs in the circulation during sepsis, which they attributed to increased apoptosis [26]. A previous study has shown Th1 cells in patients with sepsis undergoing apoptosis and enhanced IL-10 production [27]. The specific causes for these processes are unclear.

The present study of patients with sepsis demonstrated severely impaired functions of DCs and T-cells, and confirmed the findings of a previous study showing reduced numbers of DCs in patients with sepsis [26]. We found a substantially enhanced apoptosis of DCs (68 %) in patients with severe sepsis. In the present study, DC numbers and functions improved following appropriate treatment.

In addition, the present study showed significantly decreased expression of the important co-stimulatory molecule CD86 on ex vivo DCs from patients with sepsis compared with controls. This provides a possible explanation for the suppressed MLDCR responses and stimulation of CD8+ T-cells of the ex vivo DCs in patients with sepsis. A previous study has shown a decreased expression of HLA-DR and CD86 on macrophages in patients with sepsis compared with controls [28]. Furthermore, the present study demonstrated the enhanced production of Th2 cytokines (IL-10), but decreased production of Th1 cytokines (IL-2, IL-6 and IFN-γ) in MLDCRs from patients with sepsis. This is in agreement with a previous study in critically ill patients showing a shift to the Th2 cytokine pathway [24].

Previously, an animal study has demonstrated apoptosis in macrophages and neutrophils from mice treated with 3-oxo-C12-HSL [29]. In our in vitro study, we found that 3-oxo-C12-HSL enhanced apoptosis of human immune cells, in particular, DCs and CD4+, but not CD8+, T-cells. These results may suggest a possible role of QSSMs in patients with severe sepsis; blood lymphocytes from such ill patients have been documented undergoing apoptosis [30]. The differential level of apoptosis documented in the T-cell subtypes studied may be due to variable rates of apoptosis (possibly related to different apoptotic pathways being involved) [31,32]. We demonstrated increased active caspase 3 and cytochrome c in DCs and CD4+ T-cells, corroborating the apoptotic process.

In summary, in the present study we have documented in humans the selective suppressive effects of 3-oxo-C12-HSL in vitro on DCs and CD4+ T-cells, through the induction of apoptosis, and the alteration in phenotypic profile, function and modulation of cytokine production (increased Th2 cytokines IL-4 and IL-10; reduced Th1 cytokines TNF-α, IFN-γ and IL-6). In addition, we have demonstrated, in humans, QSSMs in the blood of patients with severe sepsis. We have confirmed previously documented alterations in immune status in patients with sepsis (reduced DC numbers, increased apoptosis of CD4+ T-cells, and increased Th2 and decreased Th1 cytokine profiles) [25,26]. Immune changes in vivo in patients were comparable with 3-oxo-C12-HSL-induced changes in vitro. Our results offer a possible, but not necessarily the only, explanation for the deregulation and dysfunction of host defences in patients with sepsis. However, sepsis is a complex mechanism which involves many immunobiological pathways. Further studies are required to define more precisely the role of QSSMs in sepsis.

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