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► To cite this version:

Pisake Boontham, Adrian R. Robins, Palanichamy Chandran, David Pritchard, Miguel Camara, et al.. Significant immunomodulatory effects of *Pseudomonas aeruginosa* quorum-sensing signal molecules: possible link in human sepsis. *Clinical Science*, 2008, 115 (11), pp.343-351. 10.1042/CS20080018. hal-00479423

HAL Id: hal-00479423

<https://hal.science/hal-00479423>

Submitted on 30 Apr 2010

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

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Key words: Dendritic Cells, CD4+ T Cell, Apoptosis, Human, Quorum-sensing signal molecule

Nonstandard abbreviations used: QSSM, quorum-sensing signal molecule; MLDCR, mixed lymphocyte dendritic cell reaction; AHL, acylhomoserine lactone; 3-oxo-C6-HSL, *N*-(3-oxohexanoyl)-L-homoserine lactone; 3-oxo-C12-HSL, *N*-(3-oxododecanoyl)-L-homoserine lactone

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ABSTRACT

Pathogenic bacteria use quorum-sensing signal molecules to coordinate the expression of virulence genes. Animal-based studies have demonstrated the immunomodulatory effects of quorum-sensing signal molecules. 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 patients' sera. Quorum-sensing signal molecules inhibited normal dendritic cell and T cell activation and proliferation, down-regulated expression of co-stimulatory molecules on dendritic cells; in mixed lymphocyte dendritic cell reactions (MLDCRs), secretion of IL-4 and IL-10 was enhanced, but TNF- α , IFN- 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 apoptotic rate, and modified Th₁/Th₂ cytokine profiles in mixed lymphocyte dendritic cell reactions 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 well-being of the host [1]. Bacteria in the gut secrete many intraluminal chemicals which, in some circumstances, may include quorum sensing signal molecules (QSSMs). 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 for DNA uptake [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, *N*-acylhomoserine lactones (AHLs) [5,6]. Some of the best-described AHLs belong to the 4-14 carbon chain family of AHLs. *N*-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C6-HSL) and *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) have been identified in pathogenic bacteria (eg. *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 by murine macrophages of tumour necrosis factor-alpha (TNF- α) and interleukin-12 (IL-12), and induce potent inflammation in the skin of mice [9,10]. Recent studies have shown that 3-oxo-C12-HSL has immunosuppressive effects on human peripheral blood mononuclear cells (PBMCs) [11,12]. This could result from direct effects on T cells and/or dendritic cells (DCs), which are key antigen presenting cells (APCs). DCs translate innate into adaptive immunity by integrating antigen (Ag) processing and subsequent presentation to naive T lymphocytes in secondary lymphoid compartments, using costimulatory molecules whose expression depends on DC activation and maturation [13]. A single mature activated DC is capable of stimulating 100 to 3,000 T cells [14].

In sepsis, alteration in 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 homeostasis [15]. This alteration of 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, there is limited data from human *in vitro* studies and, to the best of our knowledge, none from human *in vivo* studies.

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 man.

Stage 2(a) POST-PRINT

MATERIALS AND METHODS

The study was carried out in the Division of Surgery, Queens Medical Centre, Nottingham. Seventeen healthy donors donated blood samples. Forty serum samples from septic patients 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 American College of Chest Physicians (ACCP)/Society of Critical Care Medicine (SCCM) [18]. Of the forty serum samples studied, 17 were from patients with sepsis, 16 were from patients with severe sepsis and 7 were from patients with septic shock. The study was approved by the Local Research Ethics Committees. All patients and volunteers were required to give informed written consent. All 3-oxo-C6-HSL and 3-oxo-C12-HSL used in the study were synthesised by the Institute of Infection, Immunity and Inflammation, University of Nottingham.

Cell Preparation

PBMCs were isolated from heparinized blood by density gradient centrifugation over Histopaque 1077 (Sigma, UK). DCs were generated from adherent monocytes in tissue culture medium (TCM), containing 500 IU/ml of granulocyte-macrophage colony stimulating factor (GM-CSF) and 500 IU/ml of IL-4, for 7 days (ds). Circulating DCs were isolated from PBMCs using an established magnetic method (Miltenyi Biotec, Germany), and DC numbers were counted using light microscopy. T cells were isolated from PBMCs using CD3⁺ magnetic microbeads (Miltenyi Biotec, Germany). Yields of >93% purity were obtained.

Mixed Lymphocyte Dendritic Cell Reaction (MLDCR) 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 5x 10⁴ cells per well. The cells were cultured with QSSMs (10, 25, 50, 100 μMol) for 6 ds 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) for 24 hours (hrs), washed twice and MLDCRs set up.

DC Surface Markers

200 μ l heparinized blood or generated DCs were stimulated with 1 μ g/ml of LPS (Sigma-Aldrich, USA.), in the presence or absence of 100 μ M of 3-oxo-C12-HSL at 37 °C for 24 hrs. Cells were labeled using lineage cocktail 1 (Lin-FITC) (Becton Dickinson), anti-HLA-DR Phycoerythrin-Texas Red®-x (HLA-DR-ECD) (Beckman Coulter) and anti-CD86 allophycocyanin (CD86-APC) (Becton Dickinson) for 30 minutes (mins) at 6-12 °C. For whole blood analyses, RBCs were lysed using OptiLyse C (Beckman Coulter). Samples were washed and fixed with 2% paraformaldehyde before flow cytometric analysis (EPICS ALTRA, Beckman & Coulter, UK).

T Cell Activation Markers

PBMCs were stimulated with 100 ng/ml of super-Ag (Staphylococcal enterotoxin-F) (Sigma, UK), in the presence or absence of 100 μ Mol of 3-oxo-C12-HSL for 24 hrs. Cells were then labelled with CD3-FITC and CD69-PE for 30 mins at 4 °C, washed and fixed with 2% 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, USA). Appropriate controls were used and 10, 25, 50 and 100 μ Mol of 3-oxo-C12-HSL and 24 hrs incubation. Antibodies (Abs) used for DCs and T cells: HLA-DR-APC and Lin-FITC for DCs, CD3+, CD4+ or CD8+ for T cells. The cells were analysed by flow cytometry within 2 hrs 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 (BD Pharmingen, UK) for T cells. (caspase 3-PE (BD Pharmingen, UK) and cytochrome C-PE (Santa Cruz Biotechnology, UK)).

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 were analysed using a cytokine bead array (Beckton & 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 μ l) and examined for the presence of AHLs by Thin Layer Chromatography (TLC) on RP-2 F254s reversed-phase TLC plates (Macherey & Nagel, Düren, Germany) treated with a methanol-water solvent system. Synthetic AHLs were run as standards. After chromatography, TLC plates were dried and overlaid with LB medium containing 0.75% agar seeded with the *Escherichia coli* AHL biosensor (pSB1075). This sensor was used to detect AHLs. Following incubation for 6 to 16 hrs at 30°C, AHLs were visualized as a pseudocolour image when viewed with a Luminograph LB980 (Berthold, Pforzheim, Germany) photon video camera.

Statistical Analysis

The Minitab version 13 software was used in analysing the data. The unpaired Student-t test was used to analyse the statistical differences between two independent samples. Where comparisons of more than two independent samples were required, analysis of variance (ANOVA) with post hoc Holm-Sidak test was used. For the non-parametric data, the Kruskal-Wallis was used, as well as the Mann-Whitney U test with Bonferroni corrections for the post-hoc analysis of multiple comparisons. Chi-square 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 μ Mol) did not affect cell viability (Figure 1D). 3-oxo-C12-HSL (10 to 100 μ Mol) 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 μ Mol 3-oxo-C12-HSL (Figure 1 B).

3-oxo-C12-HSL Inhibited Upregulation of CD86 on DCs Stimulated by LPS and Inhibited T Cell Activation Marker Expression

LPS upregulated the expression of CD86 on isolated DCs. 3-oxo-C12-HSL inhibited this LPS-induced expression of CD86 on isolated DCs (Figure 1 C). After stimulation with super-Ag, 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 1 C).

3-oxo-C12-HSL Induced Apoptosis in DCs and CD4+T Cells but not CD8+T Cells

After 24 hrs incubation with 3-oxo-C12-HSL, there was a significant dose-dependent increase in the number of apoptotic DCs (Figure 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 (Figure 2A and 2B).

3-oxo-C12-HSL Induced Increased Active Caspase-3 and Cytochrome-C Production in DCs and CD4+T Cells

Intracellular active caspase 3 and cytochrome C with the presence of 3-oxo-C12-HSLs were increased in DCs, CD4+T and CD8+T cells in a time-dependent manner. The optimal time for significant activation of cytochrome C was at 6 hrs, but for active caspase 3, this was both time and cell subsets dependent (Figure 3).

Th₁ 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 antiinflammatory), at 25, 50 and 100 μ Mol ($P < 0.01$ and $P < 0.001$, Kruskal-Wallis) (Table 1). Production of IL-6, TNF- α and IFN- γ (Th₁ proinflammatory) was decreased in supernatants from MLDCRs. Inhibition of TNF- α , IL-6 and IFN- γ occurred from 10 μ Mol to 100 μ Mol 3-oxo-C12-HSL ($P < 0.05$, $P < 0.01$ and $P < 0.001$, Kruskal-Wallis) (Table 1).

QSSMS IN 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 ($P < 0.05$, Chi-square 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 than healthy controls (** $P < 0.01$, ANOVA) (Figure 5 A). DCs were isolated from blood of patients with sepsis, and used to stimulate proliferation of allogeneic T cells. Responses of allogeneic T cells stimulated by DCs from patients with sepsis was low, compared with DCs from healthy controls (** $P < 0.001$, ANOVA). After patients received treatment (72 hrs after admission), the number and function of DCs increased and was reflected in enhanced T cell responses in the MLDCRs (Figure 5 B).

Inhibition of Expression of CD86 on DCs in Patients with Sepsis

Expression of CD86 on DCs isolated from blood of patients with sepsis was very low (2%). This was substantially lower when compared with healthy controls (50%) ($P < 0.001$, t-test) (Figure 5 C).

Enhanced Apoptosis in DCs and CD4+Th₁ 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, t-test). There was enhanced apoptosis in Th₁ (32%) (not statistically significant) and reduced apoptosis in Th₂ cells (42%), compared with controls (**P<0.01, 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, showed significantly increased production of IL-10, and significantly decreased production of IL-2, IL-6 and IFN- γ (*P<0.05, **P<0.01, t-test) (Figure 7).

DISCUSSION

Bacteria and their products have been postulated to be linked in the pathogenesis of sepsis, in particular, QSSMs [1]. Individual bacterial species can produce multiple distinct QSSMs; the best documented are those produced by *Pseudomonas aeruginosa*, and include C4-HSL 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 *Pseudomonas aeruginosa*. It is thought that these QSSMs are not able, normally, 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 *Pseudomonas aeruginosa* specifically has been shown to induce severe gut epithelial cell barrier damage. With gut barrier impairment, QSSMs produced *in situ* may readily enter into the circulation and, inhibit host immunity. Our 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 lack of a standardised technique.

In this article, we document, *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 our MLDCRs, significant immunosuppressive effects were documented with 25, 50 and 100 μMol 3-oxo-C12-HSL on both *in vitro* generated (from CD14⁺ precursors) and freshly isolated blood DCs and T cells.

To delineate 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 upregulation of expression of the key costimulatory molecule CD86 by culture with LPS; this was reduced by coculture with 3-oxo-C12-HSL. Down-regulation of CD86 expression will result in diminished Ag presentation to naive T cells and generation of peptide specific cytotoxic T cells [13]. Furthermore, the activation of T cells (CD69 expression with super-Ag) 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 our MLDCR assays 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 proinflammatory cytokines (IFN- γ , TNF- α , IL-1, IL-6) and IL-8 by monocytes following activation [22]. IL-10 has a significant inhibitory effect on DC function, expression of costimulatory molecules and the ability to synthesize IL-12 [23]. An animal study has demonstrated that QSSMs inhibited Th₁ but stimulated the Th₂ pathway and activated T cells to produce proinflammatory 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 production of suppressive mediators, and increased apoptosis of immune cells [24,25]. Increased numbers of Th₂ cells with concurrent decreased numbers of Th₁ cells has previously been shown in patients with sepsis, compared with non-septic controls [24]. Hotchkiss *et al* (2001) demonstrated the depletion of B and CD4+T lymphocytes but not CD8+ or NK cells in patients with sepsis; apoptosis was documented to be the cause of the depletions [26]. The same group demonstrated depletion of DCs in the circulation during sepsis, which they attributed to increased apoptosis [26]. A recent

study showed Th₁ cells in patients with sepsis, undergoing apoptosis and enhanced IL-10 production [27]. The specific causes for these processes are unclear.

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

Also, our study showed significantly decreased expression of the important co-stimulatory molecule CD86 on *ex vivo* DCs from septic patients, 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. One study has shown decreased expression of HLA-DR and CD86 on macrophages in patients with sepsis, compared with controls [28]. Further, our 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 corroborates a previous study in critically ill patients, showing a shift to the Th₂ cytokine pathway [24].

Recently, 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 involved) [31, 32]. We demonstrated increased active caspase-3 and cytochrome-C in DCs and CD4⁺T cells, corroborating the apoptotic process.

In summary, we have documented, in humans, the selective suppressive effects of 3-oxo-C12-HSL *in vitro* on DC and CD4⁺ T cells, through induction of apoptosis, alteration in phenotypic profile, function and modulation of cytokine production (increased Th₂: IL-4, IL-10; reduced Th₁: TNF- α , IFN- γ , IL-6). Also, we have

demonstrated, in man, QSSMs in the blood in 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). 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.

Acknowledgement: P Boontham is a recipient of a Royal Thai Army grant, P Chandran a Lloyds TSB/ Royal College of Surgeons of Edinburgh (RCSEd) grant, RA Robins is supported by Cancer Research UK and A McKechnie is a recipient of the Maurice Wall Foundation/RCSEd grant. S Chuthapisith is supported by Siriraj Medical School, Thailand.

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FIGURE LEGENDS

Figure 1 (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 (** $P < 0.01$, 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 hrs, washed and used in the assays. At any one time, either T cells or DCs were so treated ($*P < 0.05$, ANOVA with post-hoc test). **(C)** The effect of 3-oxo-C12-HSL (100 μMol) on expression of costimulatory molecules (CD86) on freshly isolated and LPS stimulated blood DCs and expression of activation markers (CD69) induced by super-Ag (SAG) on blood T cells. Data shown is means \pm SEMs. ($*P < 0.05$; ** $P < 0.01$, ANOVA with post-hoc test) **(D)** A representative flow cytometric result identifying cell death after incubating PBMCs with 100 μMol 3-oxo-C12-HSL for 24 hrs. 3-oxo-C12-HSL did not affect cell viability. The percentages of dead cells (R1) were 4.17 and 4.78%, respectively, in the incubation of PBMCs in the absence and presence of 100 μMol 3-oxo-C12-HSL.

Figure 2 (A) A representative example of the apoptotic effect of 3-oxo-C12-HSL in generated DCs, CD4⁺ and CD8⁺ T cells. Right upper quadrant represents dead cells, right lower cells undergoing apoptosis and left lower viable cells. **(B)** The effect of 3-oxo-C12-HSL on apoptosis of DCs, DC4⁺ and CD8⁺ T cells; 5 different samples shown. Data shows median (line), mean (dot) values and interquartile ranges (box). ($*P < 0.05$; ** $P < 0.01$, ANOVA with post-hoc test)

Figure 3 (A) The effect of 3-oxo-C12-HSL on intracellular cytochrome C in DCs and T cell subsets. Five different donors were used to isolate DCs and T cell subsets. The significant increase of intracellular cytochrome C was document in at 6 hrs in CD4⁺ and CD8⁺ T cells, but not in DCs. ($*P < 0.05$, ANOVA with post-hoc test) **(B)** The 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 of intracellular active caspase 3 was documented at different time points. (* $P < 0.05$, ANOVA with post-hoc test)

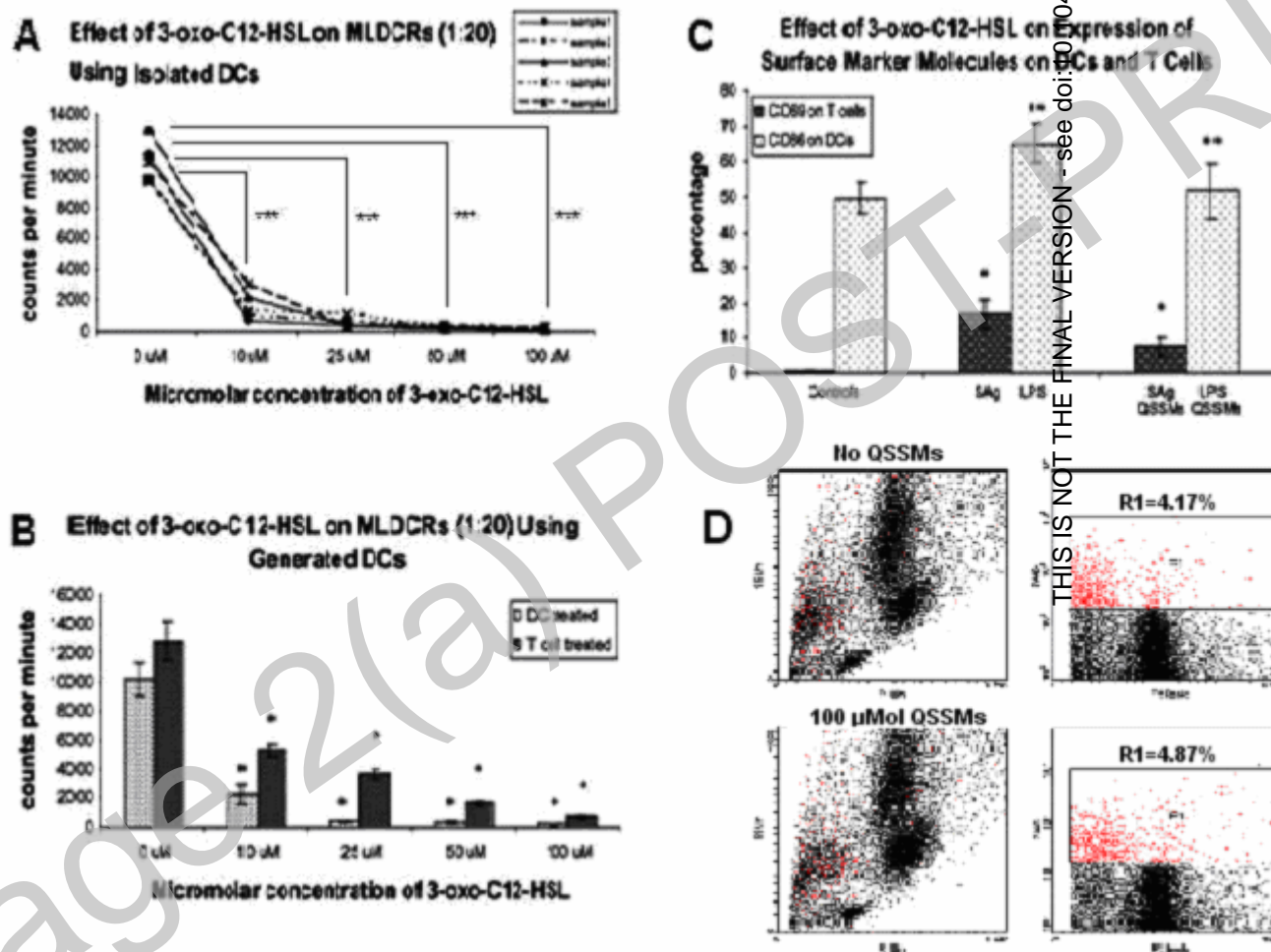
Figure 4 Detection of AHLs by TLC using the *E. coli* based AHL biosensor pSB1075. The presence of AHLs was visualised as a pseudocolour image when viewed with a Luminograph LB980 (Berthold, Pforzheim, Germany) photon video camera. Representative serum samples collected from patients with sepsis. C is the control image obtained using the synthetic AHL standards 3-oxo-C12-HSL (Oc12) and 3-oxo-C14-HSL (Oc14). One to 12 were serum samples from patients. In these two representative images, QSSMs were detected in the serum of patients 1, 6, 7 and 10 (arrow).

Figure 5 (A) Proliferation of controls and patients with sepsis on admission and after >72 hrs of treatment. P values shown are comparisons between controls and each time Number of blood DCs (cells/ml) in 20 ml blood from patients with sepsis. Data represents 10 specimens for controls and patients (on admission), and 6 for patients >72 hrs on therapy. P values shown are comparisons between controls and each time point in hospital. (** $P < 0.01$, NS: not significant, ANOVA with post-hoc test) **(B)** Allogeneic T cell stimulation assay of freshly isolated blood DCs (MLDCRs); data shown is the means of T cell point in hospital. (** $P < 0.01$, *** $P < 0.001$, ANOVA with post-hoc test) **(C)** The expression of CD86 on freshly isolated blood DCs from 10 patients with sepsis, compared with healthy controls. (*** $P < 0.001$, t-test)

Figure 6 Apoptosis of isolated blood DCs, CD4⁺ Th₁ and CD4⁺ Th₂ cells from patients with sepsis compared with controls; data show medians (line) and means (dot). Data represents 5 samples. (** $P < 0.01$, *** $P < 0.001$, t-test)

Figure 7 Decreased production of Th₁ regulatory proinflammatory 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. (* $P < 0.05$, ** $P < 0.01$, t-test)

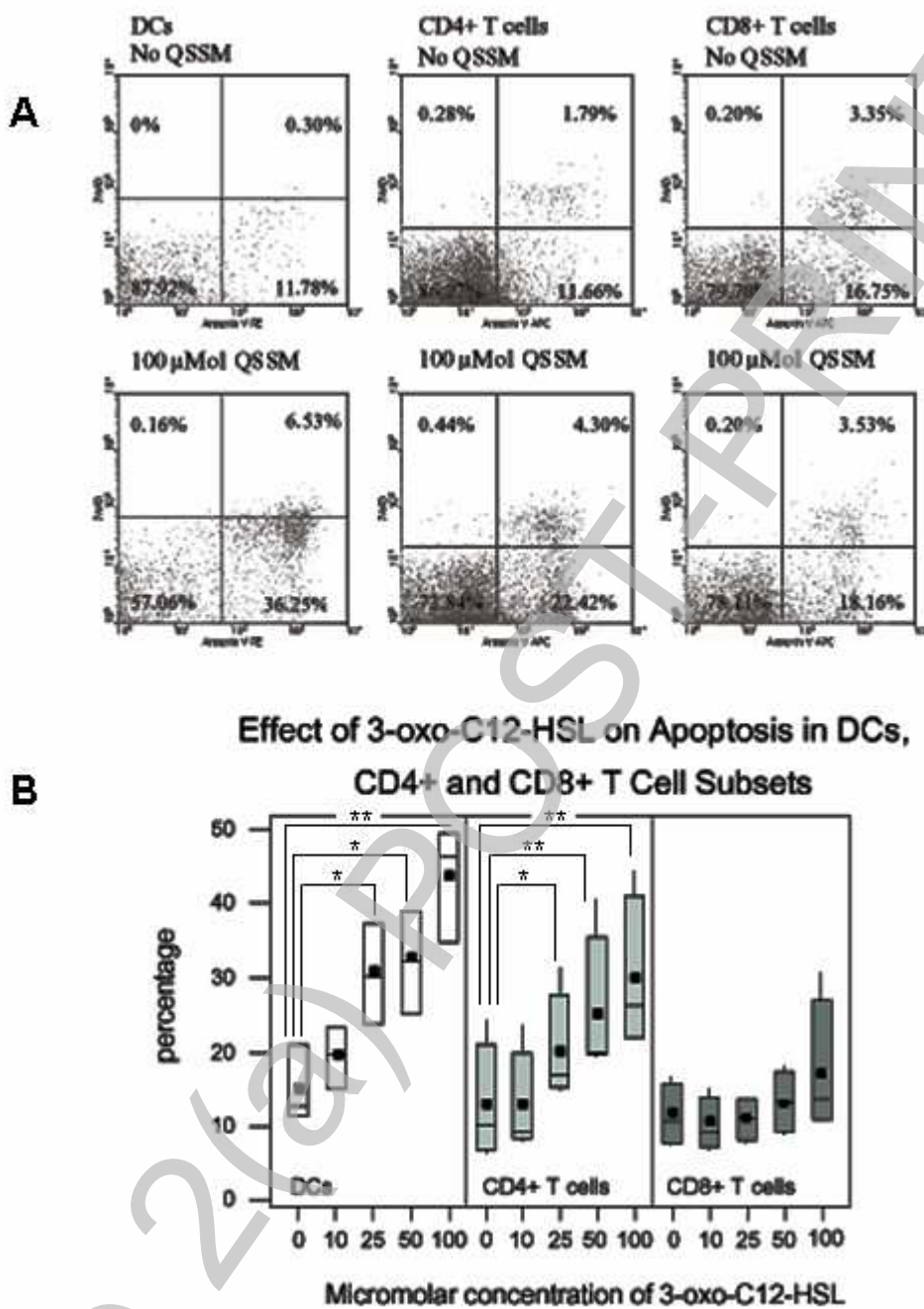
Figure 1



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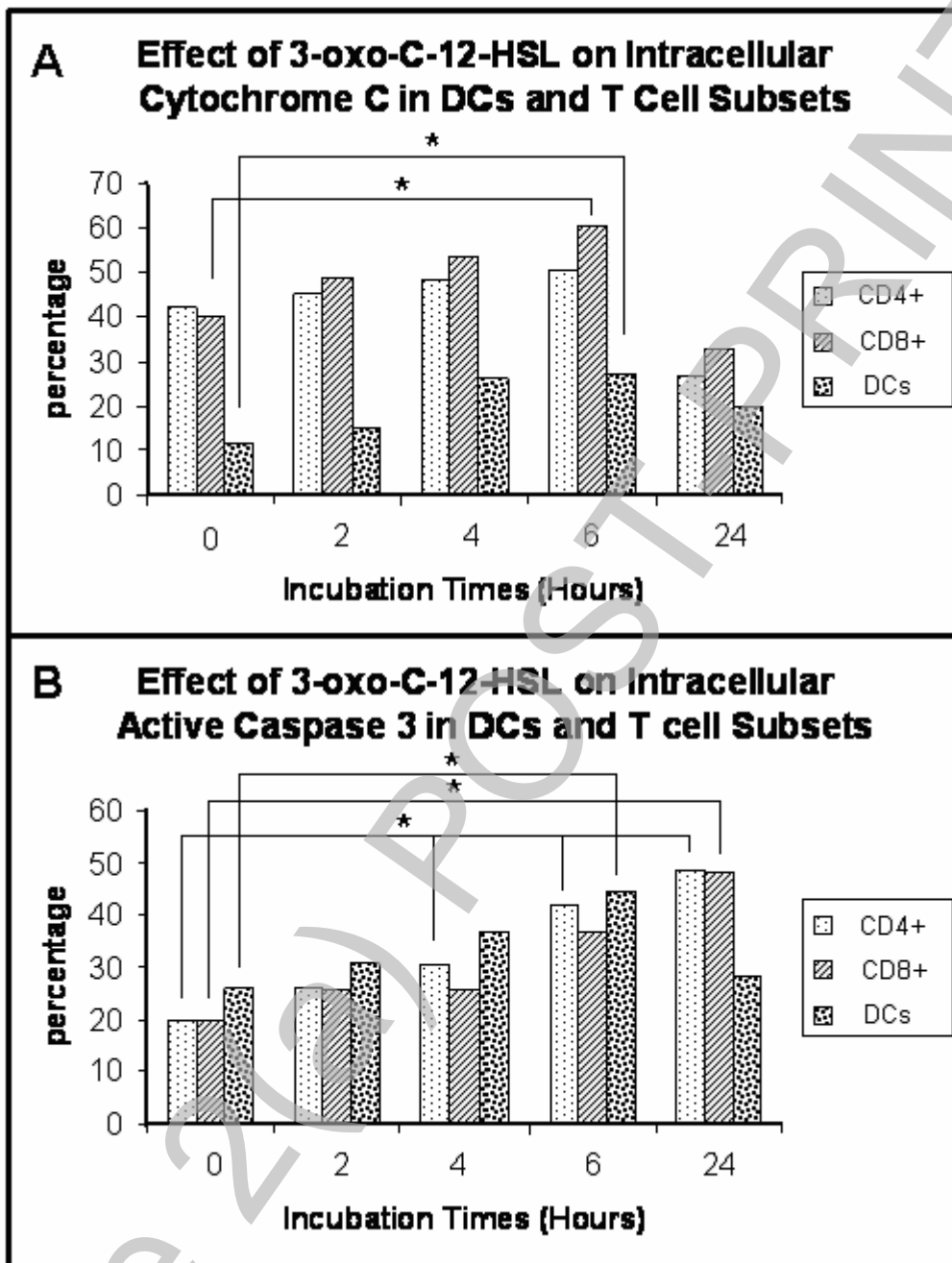
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Figure 2



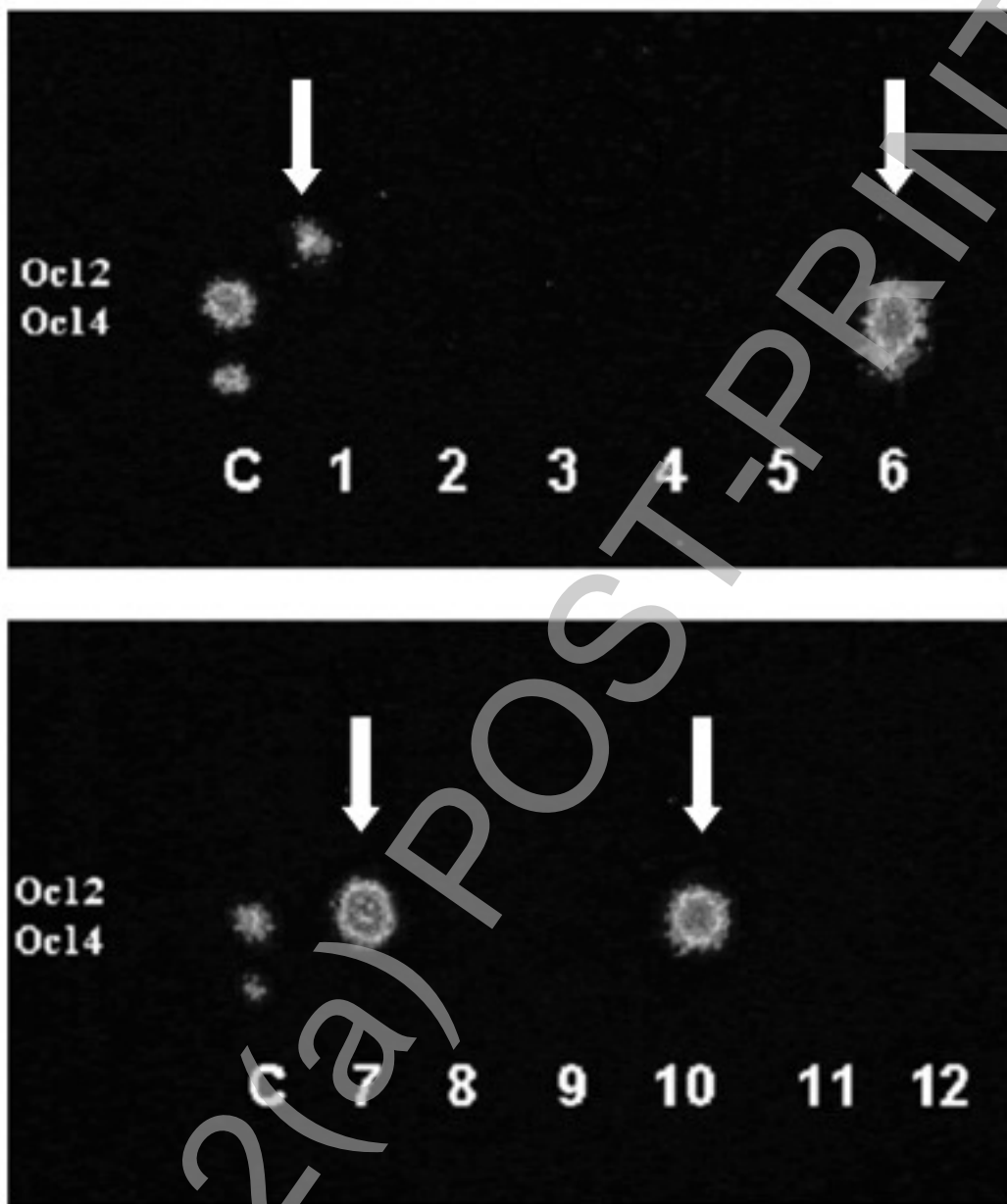
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Figure 3



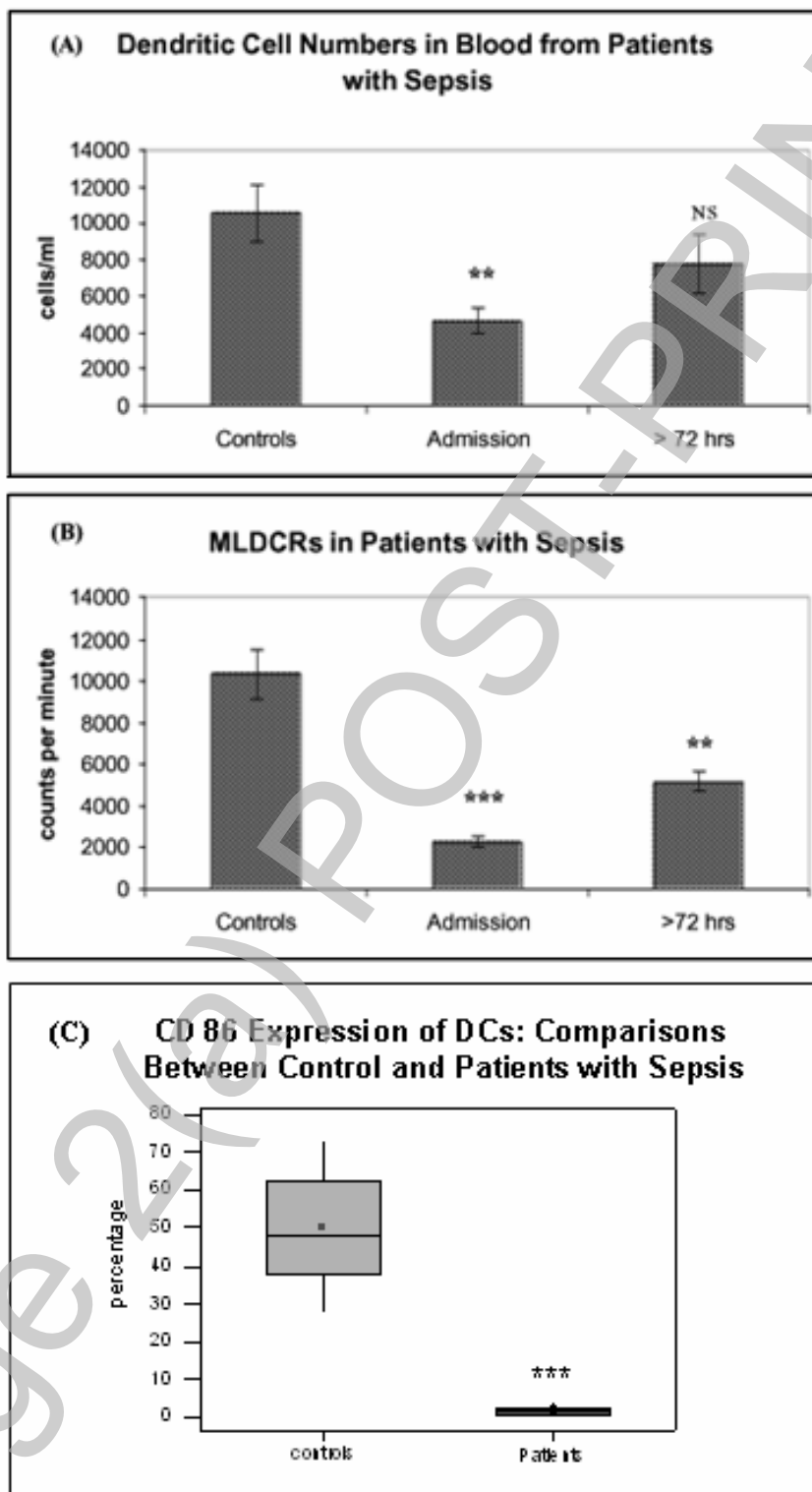
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Figure 4



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Figure 5



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Figure 6

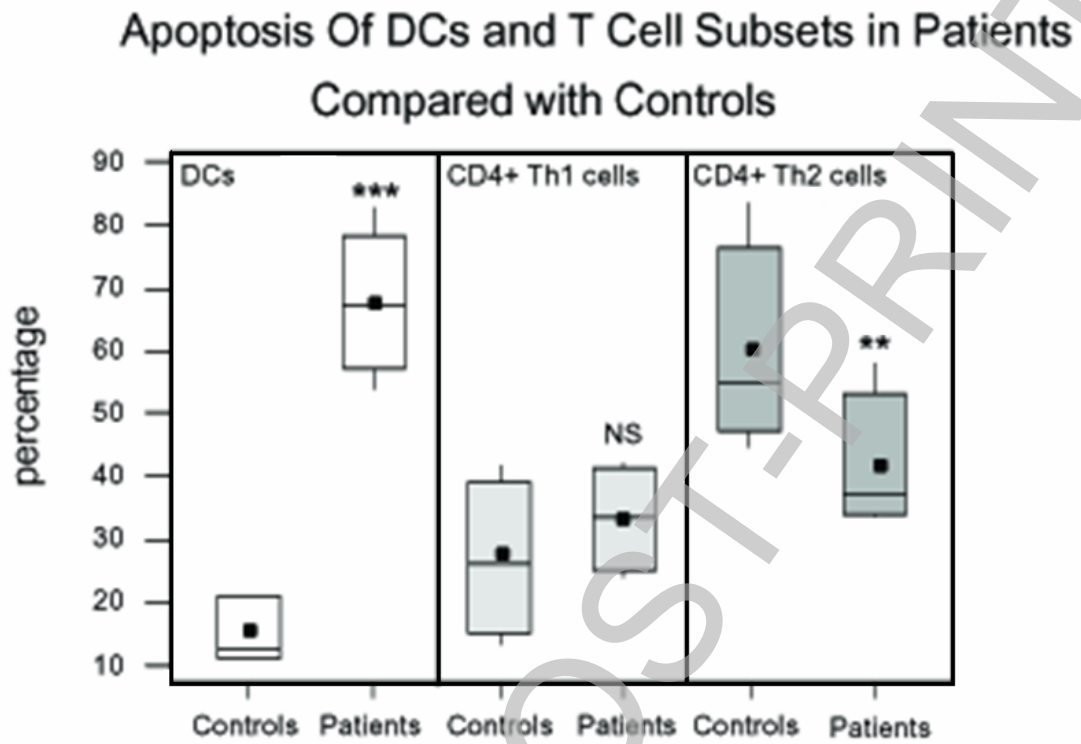
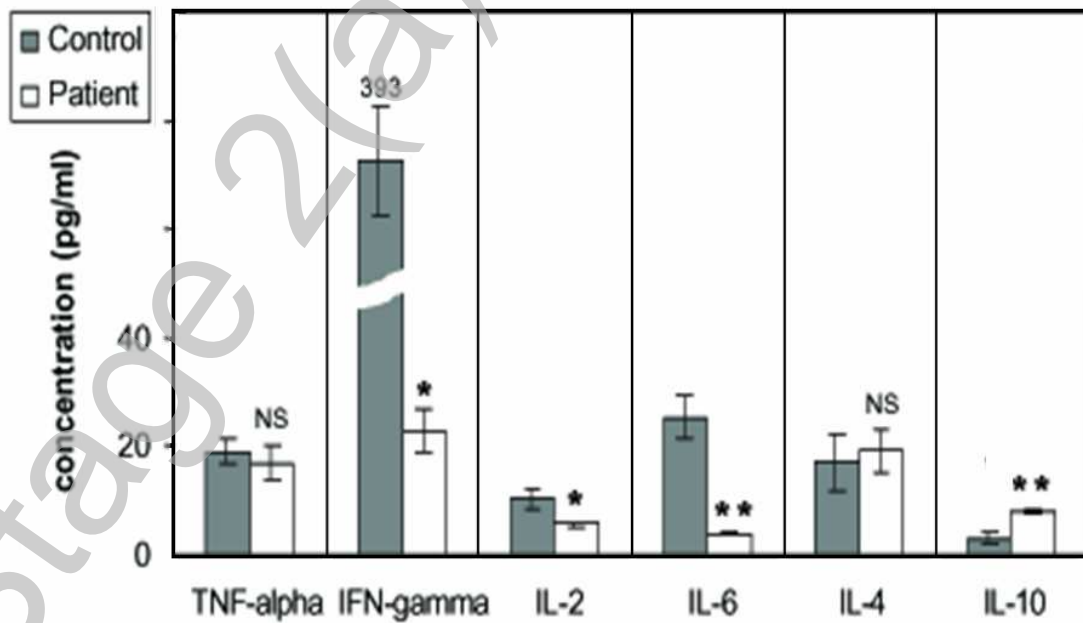


Figure 7



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TABLE

Table 1: Levels of Th₁ and Th₂ cytokines in MLDCR supernatants at day 7. (n=5; Kruskal-Wallis analysis)

MLDCR Cytokines	Controls	3-oxo-C12-HSL				P value
		10 µM	25 µM	50 µM	100 µM	
IL-4	21.7 ± 8.5	46.8±27.2	98.2±24.5	134.3±31.9	207.7± 43.9	<0.01
IL-10	3.6 ±1.8	6.0±2.2	38.1±4.0	78.1±8.7	104.7±10.1	<0.001
TNF-α	20.9±3.5	11.0±3.5	7.4 ± 0.6	5.3±2.1	6.6±1.0	<0.05
IL-6	27.5±6.4	13.4±1.0	10.0 ±1.0	9.3±1.7	9.2±1.0	<0.01
IL-2	12.9±2.0	5.4±2.1	5.3 ±1.2	4.3±1.8	5.8±1.6	=0.08
IFN-γ	696 ± 236	96.0±32.0	95± 5	13.0±4.0	12.0±7	<0.001

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), seventeen 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.

	Number of patients	Mortality (%)	Positive blood cultures (%)	Detection of QSSMs in serum (%)
Healthy controls	17	0 (0%)	ND	0 (0%)
Patients with sepsis	17	1 (5.8%)	ND	0 (0%)
Patients with severe sepsis	16	6 (37.5%)	11 (68.7%)	9 (56%)
Patients with septic shock	7	4 (57%)	6 (85%)	6 (85%)

ND: no blood culture was done

Table 3: Mortality and blood culture status in patients who had and had not QSSMs detected in their serum. Mortality was related with the presence of QSSMs in the serum (* P<0.05, Chi-square test).

Detection of QSSMs in the serum	Mortality (n=40)*	
	Survived	Died
QSSMs not detected	21	4
QSSMs detected	8	7
	Blood culture (n=23)	
	Negative	Positive
QSSMs not detected	3	5
QSSMs detected	3	12