Leukocyte adhesive function in Intensive Care patients with non-infectious and infectious systemic inflammation

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Research

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Author contribution: AL, JW, KD, JL, QC and PK designed the study and interpreted the data. ES, JM, JW and PK recruited patients and collected blood samples. AL, AJ, BP and QC performed the LAFA blood assays. AL, YRO and KE analysed the assay results. AL, QC, PK, JL, KD and JW drafted the initial manuscript with all authors editing and approving the final version.
Abstract

The lack of rapid and accurate diagnostic tools to distinguish infectious (known as sepsis) and non-infectious causes of inflammation in patients with systemic inflammation remains a significantly unmet clinic need, particularly in the intensive care unit (ICU). As a hallmark of inflammation, circulating leukocytes must be activated and undergo a cascade of interactions with blood vessel endothelium before transmigrating into surrounding tissues, a process called leukocyte recruitment. Given the divergent disease aetiologies, it was hypothesised that the ability of circulating leukocytes to interact with endothelial cells and cause inflammation may differ when responding to infectious and non-infectious inflammatory stimuli, providing potential markers to differentiate these two diseases. In the present study, a flow-based blood testing platform, named leukocyte adhesive function assay (LAFA), was used to mimic blood microcirculation in vitro so that the patient leukocyte ability to interact with multiple endothelial molecules, including P+E selectins, VCAM-1 and IL-8, can be studied. The leukocyte adhesive functions of multiple leukocyte subsets were quantitatively assessed using a range of cell kinetic parameters, including cell speed, straightness, dwell time etc. When analysed on P+E selectin substrate, a significantly lower value of cell straightness was observed in septic CD4 cells than non-infectious cells (0.78±0.04 vs 0.92±0.01, p < 0.01), suggesting a difference in CD4 cells ability to adhere to selectins in infectious and non-infectious patients. Additionally, an impaired ability to respond to IL-8 was observed in septic neutrophils compared to non-infectious cells, evidenced by a significantly reduced cell dwell time (91.7±14.0 vs 150.6±19.0 seconds, p < 0.05). Thus, our study promisingly showed the ability of LAFA to detect different adhesive function between leukocytes from ICU patients with infectious and non-infectious systemic inflammation. LAFA generated a number of novel markers that might distinguish infectious inflammation from non-infectious causes that warrant further study and novel opportunities for the rapid and accurate diagnosis of sepsis.
Introduction

Dysregulated host response, previously known as Systemic Inflammatory Response Syndrome (SIRS), is a complex inflammatory systemic response to non-infectious or infectious foreign insults. Non-infectious causes include major surgery, trauma, burns and severe tissue injury, whereas infectious causes (also known as sepsis) include bacterial, fungal or viral infection. All causes can result in similar clinical manifestations with the descriptors recently being redefined with the SEPSIS-3 criteria. Due to different aetiology, the treatments for non-infectious and infectious causes of systemic inflammation differ dramatically.

Antibiotic therapy and source control are the first line of treatment for sepsis, whilst antibiotic therapy is not indicated for non-infectious causes. For many patients it is extremely difficult to rapidly and accurately determine disease pathogenesis and the current practice is that patients will be given antibiotics when potential infection is suspected, frequently leading to overuse of antibiotics for patients with non-infectious causes. Antibiotics are continued until the treating clinician is satisfied that the cause of the systemic inflammation is not infection. This overuse of antibiotics in patients with non-infectious causes could result in the emergence of resistant pathogens, as well as other avoidable side effects. It is critical to develop new technology to aid the early differentiation between non-infectious and infectious inflammation. This will allow more targeted therapies to be applied earlier, avoiding unnecessary treatments and may improve survival rates.

In order to differentiate sepsis from non-infectious inflammation, most work has focussed on the determination and characterisation of potential pathogens in patient blood and/or tissues. Currently, blood cultures or other clinically relevant positive microbiology is the gold standard for the identification of infection in ICU patients with systemic inflammation. A significant proportion of patients with infection will have negative cultures. The turn-around time for microbiological culture results is usually 12-72 hours. Earlier administration of antibiotics has been associated with improved survival and the “time critical” nature of both recognition and treatment of patients with sepsis is increasingly emphasised. Additionally, even though a number of molecular tools have been developed to characterise infectious pathogens, the lack of rapidity and sensitivity is a common drawback of these techniques. Biomarkers of host immune response, such as procalcitonin (PCT) and C-reactive protein (CRP), have been investigated to separate infectious and non-infectious
inflammation. Unfortunately, PCT tests lack the negative predictive power to withhold antibiotics in critically ill patients, suggesting the clinical applications of these biomarkers remain to be better defined. Thus, there is urgent clinical demand for new sepsis diagnostic tests, particularly that specifically reflect the patient immune activation during inflammation.

The recruitment of leukocytes from the circulation to the surrounding tissues is a hallmark of immune system activation during inflammation. To be recruited, circulating leukocytes must undergo a cascade of interactions with blood vessel endothelial cell (EC) surface and subsequently transmigrate into the tissue. Thus, leukocyte adhesive function is defined as the leukocyte ability to interact with and transmigrate through the ECs. Initially, the fast travelling leukocyte will tether and roll on the ECs, which requires the interaction between leukocytes expressing P-selectin glycoprotein ligand-1 (PSGL-1) and endothelial P or E selectins. The rolling leukocytes will then be activated by chemokines and reduce their speed. This allows the interaction between leukocyte α4β1 integrins with their endothelial ligands, such as vascular cell adhesion molecule-1 (VCAM-1), leading to leukocyte firm adhesion on endothelial surface. The adherent leukocytes then crawl along the endothelial surface until they find an optimal spot to leave the vasculature. Thus, the activity of leukocyte PSGL-1 and α4β1 integrin plays a central role in the regulation of leukocyte adhesive function, which will then determine the leukocytes potential to transmigrate and cause inflammation. Additionally, the leukocyte expressing chemokine receptors may facilitate the leukocyte recruitment process via chemotaxis.

Given the divergent causes of inflammation, it was hypothesised that leukocyte adhesive functions could be altered differently in non-infectious and infectious patients. In the present study, a newly developed blood test platform, the leukocyte adhesive function assay (LAFA), was used to identify such differences. By analysing blood samples from ICU patients with non-infectious or infectious systemic inflammation using LAFA, we aimed to compare the ability of specific leukocyte subsets to interact or respond to given endothelial adhesive substrates, including P+E selectin or VCAM-1 in the presence and absence of IL-8, and thereby the ability of LAFA to distinguish non-infectious and infectious patients may be evaluated.
**Methods and materials**

**Patient recruitment**

This study was approved by Metro South Human Research Ethics Committee, Brisbane Australia (Reference number: 17/QPATH/571). Patients were recruited for this study by screening patients who were admitted to the Intensive Care Unit (ICU), at Princess Alexandra Hospital, Brisbane Australia. Eligible patients were over 18-years of age with a newly identified systemic inflammatory response who could provide prior consent or had a surrogate who could provide prior consent. Patients who had pre-existing inflammatory conditions, such as multiple sclerosis, Crohn's disease, colitis, arthritis, lupus, etc. or were currently on anti-adhesion therapy, including Natalizumab, Vedolizumab or an anti-adhesion clinical trial were excluded from the study. All patients enrolled had any two or more of the following four criteria for systemic inflammation, regardless of the causes of inflammation:

1. Body temperature >38°C or <36°C
2. Heart rate >90 per minute
3. Respiratory rate >20 breaths per minute or PaCO₂ <32mmHg.
4. White blood cell count >12,000/mm³ or <4,000/mm³ or >10% bands.

The blood samples were collected within 48 hours after the first identification of the systemic inflammatory response. Whole blood was collected in a Heparin blood collection tubes (5ml) for LAFA assays and an EDTA tube (4ml) for full blood exam (FBE). A full blood cell exam was performed using a Mindray BC5000 Haematology Analyser according to manufacturer’s instructions.

**Sepsis Adjudication**

Two clinicians (JW / PK) provided a retrospective and independent assessment of all recruited patients. They were blinded to LAFA results but assessed patients’ medical records and laboratory results to determine either definite “infection”, definite “no infection” or “possible infection”. Concordance between the two assessors was required for a patient to be allocated to the definite infection or no infection group. Where the assessors differed (on 3 occasions) patients were categorised as having possible infection.

**Antibodies, chemical and reagents**

Human recombinant VCAM-1, P-selectin, E-selectin and IL-8 were all purchased from R&D Systems (Minneapolis, MN). Antibodies (Abs) against human leukocyte surface molecules,
Anti CD4-Alexa488, CD8-PE, CD14-Alexa488, CD15-APC and CD16-BV510, CD25-APC were all obtained from either BD Biosciences (San Diego, CA) or BioLegend (San Diego, CA).

Leukocyte adhesive function assay (LAFA)

LAFA utilises microfluidic technology to mimic blood circulation in vitro, allowing a direct assessment of leukocyte interaction with endothelial adhesive substrates (e.g. P+E selectins, VCAM-1 and IL-8). Specific leukocyte subsets are fluorescently labelled with different fluorophores, allowing the detection of multiple cell types concurrently. To perform LAFA, a Polymethyl methacrylate (PMMA)-bottom microfluidic chip (channel Width×Depth×Length: 1,000×200×18,000µm) from Microfluidic ChipShop (Jena, Germany) was employed. Recombinant human VCAM-1 protein (10µg/ml) with or without IL-8 (1µg/ml), or a combination of P-selectin (10µg/ml) and E-selectin (0.5µg/ml) was gently loaded into the channels and incubated overnight at 4°C. To identify specific leukocyte subsets, 100 µl whole blood was mixed with the two Ab cocktails for 5 minutes (min) at room temperature (RT) respectively: 1) anti-CD14-Alex488 (1:50), anti-CD15-APC (1:33.3) and Anti-CD16-BV421 (1:500); 2) anti-CD4-Alex488 (1:50 dilution), anti-CD8-PE (1:66.7) and anti-CD25-APC (1:500). These two sets of antibody cocktails allow the detection of following leukocyte subsets: CD14 (most likely monocytes), CD15+CD16+ (mainly neutrophils), CD4 (mainly CD4 lymphocytes), CD8 (mainly CD8 lymphocytes) and CD4+CD25+ (most likely CD4 regulatory cells) cells. The blood was then pulled into the microfluidic channels by a syringe pump at a shear stress of 1.5 dyne/cm².

Fluorescence microscopy time series were recorded on an InCell Analyser 2200 (GE Healthcare, Seattle, WA) with a 10X objective. All data acquisition was recorded at 1 frame per two second for 10 minutes, at the centre of the channel (approximately 9mm from the channel inlet). All experiments were performed in a 37ºC temperature controlled environment.

Cell tracking and data analysis

Cell tracking was accomplished using TrackMate from Fiji image analysis software. Cells were tracked automatically by detecting quality-filtered fluorescent spots in each frame and then linked with a maximum distance of 75µm and maximum gap size of 2. All tracks were subsequently checked manually and corrected for errors. Cell kinetic parameters, including cell density, speed, diffusion coefficient, straightness, dwell time and track length were determined for each interacting cell. Each parameter characterises cell migratory behaviours from one specific aspect. These cell kinetic parameters and their descriptions are list in Table 1.
Table 1 A list of cell kinetic parameters with their definition that were used to characterise migratory behaviours.

<table>
<thead>
<tr>
<th>Cell kinetic parameters</th>
<th>Descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell density</td>
<td>The number of interacting cells detected as valid interactions (cells appear in more than 3 consecutive frames).</td>
</tr>
<tr>
<td>Normalised cell density</td>
<td>The correspondent cell counts were used to normalise the cell density results to reduce the effects from variable cell counts between individual blood donors.</td>
</tr>
<tr>
<td>R-factor</td>
<td>R-factor is calculated as (% of interacting cell type) / (% cell type in circulation).</td>
</tr>
<tr>
<td>Speed</td>
<td>The distant over time of a cell. The speed values of all detected cells were averaged for each blood sample. The lower the cell speed, the less the cell mobility, usually indicating stronger cell and substrate interaction.</td>
</tr>
<tr>
<td>Diffusion coefficient</td>
<td>The diffusion coefficient is calculated as mean square displacement divided by 4 times the time the cell travelled. A high diffusion coefficient value usually indicates a clear direction of cell movement. On the contrast, a low diffusion coefficient value suggests a non-directional cell movement, usually indicating strong cell and substrate binding.</td>
</tr>
<tr>
<td>Straightness</td>
<td>The ratio of displacement (the direct distance between two points) over track length. The straightness values of all detected cells were averaged for each blood sample. Low cell straightness value usually indicates high level of random cell migration which is independent from the blood flow, a consequence of strong cell and substrate interaction.</td>
</tr>
<tr>
<td>Dwell time</td>
<td>The total duration of a cell appearing in the video. The dwell time values of all detected cells were averaged for each blood sample. A high value of cell dwell time usually indicates a strong cell and substrate interaction.</td>
</tr>
<tr>
<td>Track length</td>
<td>The total length of a cell travelled. The track length values of all detected cells were averaged for each blood sample. The lower the cell track length, the less the cell mobility, usually indicating stronger cell and substrate interaction.</td>
</tr>
</tbody>
</table>

If a cell appeared in at least 3 consecutive frames, this interaction was defined as a valid interaction and this cell was included in the downstream data analysis, in which all cell kinetic parameters were then determined for this interacting cell. The values of each cell kinetic parameter from each valid cell were averaged within each leukocyte subset (e.g. CD4, CD8, CD15+CD16+ cells, etc) and presented as an averaged value for this subset.

Statistics
Data is reported as median (inter-quartile range) or mean (Standard deviation) as appropriate for its distribution. Appropriate parametric or non-parametric tests were used and a value of $p < 0.05$ was considered significant. The results for the infection, no infection and possible
infection groups are reported, with statistical comparisons made between the infection and no infection patients.
RESULTS

Demographics and stratification of ICU patients

A total of 28 patients were recruited to this study. Clinicians JW and PK reviewed the clinical records of each patient and independently adjudicated whether the patient had infection, no infection or were ‘indeterminate/possible infection’. This assessment was done independent of the LAFA results and the adjudication was not released until all LAFA assays were completed. As a result, 10 patients were deemed to have infection, 9 no infection and the remaining 9 possible infection. The baseline demographics for the ICU patients are presented in Table 2.

Table 2 Demographic description of Infection, No infection and Possible Infection (or unknown) ICU patients.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Infection (n=10)</th>
<th>No-Infection (n=9)</th>
<th>Possible Infection (n=9)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demographics</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>66 (58-72)</td>
<td>57 (29-57)</td>
<td>50 (45 – 77)</td>
<td>0.10</td>
</tr>
<tr>
<td>Gender (male)</td>
<td>4 (40%)</td>
<td>7 (78%)</td>
<td>5 (55%)</td>
<td></td>
</tr>
<tr>
<td>Admission characteristics</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medical</td>
<td>4</td>
<td>6</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Surgical</td>
<td>6</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Severity of Disease</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>APACHE 3 Score (median(IQR))</td>
<td>65 (44-89)</td>
<td>53 (41-71)</td>
<td>63 (54-75)</td>
<td>0.66</td>
</tr>
<tr>
<td>SOFA Score at inclusion (median(IQR))</td>
<td>9 (6-12)</td>
<td>4 (3-8)</td>
<td>5 (4-7)</td>
<td>0.08</td>
</tr>
<tr>
<td>Organ Support² and Outcome</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mechanical Ventilation (n)</td>
<td>7 (70%)</td>
<td>5 (56%)</td>
<td>5 (56%)</td>
<td></td>
</tr>
<tr>
<td>Vasopressor requirement (n)</td>
<td>9</td>
<td>3</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Renal Replacement Therapy (n)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ICU Mortality (n)</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>ICU LOS (days) (median(IQR))</td>
<td>3 (2-7.2)</td>
<td>3 (1-7)</td>
<td>4 (2.5 – 10)</td>
<td>0.58</td>
</tr>
</tbody>
</table>

² In the 24 hrs preceding Day 1

The clinical diagnosis and potential causes of systemic inflammation in each ICU patient are listed in Table 3.

Table 3 The clinical diagnosis and microbiology results for individual study patients.

<table>
<thead>
<tr>
<th>Study ID</th>
<th>ICU Diagnosis</th>
<th>Microbiology</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>No</th>
<th>Infection</th>
<th>Description</th>
<th>Microbiology</th>
</tr>
</thead>
<tbody>
<tr>
<td>NON-01</td>
<td>Trauma. Fall. Surgery for unstable T8 fracture</td>
<td>Scant Staphylococcus Aureus in ETA; considered colonisation</td>
<td></td>
</tr>
<tr>
<td>NON-02</td>
<td>Myocardial infarction with out of hospital cardiac arrest</td>
<td>Nil positive microbiology</td>
<td></td>
</tr>
<tr>
<td>NON-03</td>
<td>Exacerbation of asthma</td>
<td>Nil positive microbiology</td>
<td></td>
</tr>
<tr>
<td>NON-04</td>
<td>Decompressive craniectomy for malignant middle cerebral artery stroke</td>
<td>Nil positive microbiology</td>
<td></td>
</tr>
<tr>
<td>NON-05</td>
<td>Subarachnoid haemorrhage</td>
<td>Haemophilus and Klebsiella grown from ETA considered colonisation</td>
<td></td>
</tr>
<tr>
<td>NON-06</td>
<td>Trauma. Evacuation of subdural haematoma</td>
<td>Haemophilus in ETA day 3</td>
<td></td>
</tr>
<tr>
<td>NON-07</td>
<td>Reduced level of consciousness due to cerebral metastatic disease</td>
<td>Klebsiella in ETA</td>
<td></td>
</tr>
<tr>
<td>NON-08</td>
<td>Pulmonary oedema following coronary artery bypass grafts</td>
<td>Nil positive microbiology</td>
<td></td>
</tr>
<tr>
<td>NON-09</td>
<td>Lobectomy for carcinoid lung tumour</td>
<td>Nil positive microbiology</td>
<td></td>
</tr>
<tr>
<td>INF-01</td>
<td>Below knee amputation, Gangrenous foot</td>
<td>Pseudomonas and mixed enterics from tissue</td>
<td></td>
</tr>
<tr>
<td>INF-02</td>
<td>Urosepsis</td>
<td>E. Coli in blood (and urine)</td>
<td></td>
</tr>
<tr>
<td>INF-03</td>
<td>Perforated viscus with faecal peritonitis</td>
<td>Perforated viscus with faecal peritonitis</td>
<td></td>
</tr>
<tr>
<td>INF-04</td>
<td>Multitrauma. Fall. Spine, thorax, abdominal and pelvic injuries</td>
<td>Serratia in blood. Serratia and Pseudomonas in ETA. Enterococcus Faecalis in urine</td>
<td></td>
</tr>
<tr>
<td>INF-05</td>
<td>Necrotising fasciitis</td>
<td>Group G Streptococci and Proteus from tissue</td>
<td></td>
</tr>
<tr>
<td>INF-06</td>
<td>Septic arthritis</td>
<td>MRSA in blood and joint aspirate</td>
<td></td>
</tr>
<tr>
<td>INF-07</td>
<td>Fourniers gangrene</td>
<td>Streptococcus Constellatus and Proteus from tissue</td>
<td></td>
</tr>
<tr>
<td>INF-08</td>
<td>Urosepsis</td>
<td>Providencia in blood and urine</td>
<td></td>
</tr>
<tr>
<td>INF-09</td>
<td>Intraoperative aspiration. Metatarsal head osteotomies for osteomyelitis</td>
<td>Candida in urine</td>
<td></td>
</tr>
<tr>
<td>INF-10</td>
<td>Ischaemic bowel. Perforated colon</td>
<td>E. Coli in peritoneum</td>
<td></td>
</tr>
<tr>
<td>Possible Infection</td>
<td>UN-01</td>
<td>Febrile neutropenia post autologous stem cell transplant</td>
<td>Nil positive microbiology</td>
</tr>
<tr>
<td>--------------------</td>
<td>-------</td>
<td>--------------------------------------------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>UN-02</td>
<td></td>
<td>Exacerbation of asthma</td>
<td>Initial cultures negative. Serratia from ETA day 6. Coagulase negative Staphylococci from blood day 5</td>
</tr>
<tr>
<td>UN-03</td>
<td></td>
<td>Drug overdose with aspiration pneumonitis</td>
<td>Coagulase negative Staphylococci in blood; considered contaminant. Enterobacter Cloacae in ETA</td>
</tr>
<tr>
<td>UN-04</td>
<td></td>
<td>Hanging with aspiration pneumonitis</td>
<td>Coagulase negative Staphylococci in blood; considered contaminant significant aspiration with mixed flora in ETA</td>
</tr>
<tr>
<td>UN-05</td>
<td></td>
<td>Small bowel resection, colectomy, nephrectomy</td>
<td>Candida in urine</td>
</tr>
<tr>
<td>UN-06</td>
<td></td>
<td>Coronary artery bypass grafts following myocardial infarction</td>
<td>Nil positive microbiology</td>
</tr>
<tr>
<td>UN-07</td>
<td></td>
<td>Distal pancreatectomy and splenectomy</td>
<td>Nil positive microbiology</td>
</tr>
<tr>
<td>UN-08</td>
<td></td>
<td>Fall, Traumatic brain injury</td>
<td>Pseudomonas in ETA</td>
</tr>
<tr>
<td>UN-09</td>
<td></td>
<td>Embolisation of bleeding mycotic hepatic artery pseudoaneurysm</td>
<td>Nil positive microbiology</td>
</tr>
</tbody>
</table>

ETA = Endotracheal Aspirate, E. coli = Escherichia Coli, MRSA = Methicillin-resistant *Staphylococcus aureus*

*Migratory behaviours of leukocytes from patients with infectious and non-infectious inflammation.*

Leucocyte behaviour across the study groups is shown in Figure 1A to 1H, using a number of cell kinetic parameters. When analysed on P+E selectin substrates, the straightness of interacting CD4 cells in infectious patients was significantly lower than non-infectious cells (0.78±0.04 vs 0.92±0.01, \(p < 0.01\); Figure 1F), indicating a stronger CD4 cell binding to selectins in patients with an infectious cause. This notion was further supported by the observation that the dwell time of infectious CD4 cells was significantly longer than non-infectious cells (48.3±6.5 vs 29.3±3.6 seconds, \(p < 0.05\); Figure 1G). In addition, significantly lower numbers of interacting CD4 (55.0±19.1 vs 155.8±45.3, \(p < 0.05\)) and CD8 (53.4±13.7 vs 112.0±28.5, \(p < 0.05\)) cells were seen in patients with an infectious compared to non-
infectious cause of their systemic inflammation (Figure 1A). To reduce the effects from variable cell counts between individual blood donors (Supplementary Figure 1), the correspondent cell counts were also used to normalise the cell density results \(^{20}\). After the normalisation, no difference in the number of CD4 and CD8 interacting cells were observed (Figure 1B).

The blood samples were then analysed by LAFA on VCAM-1 substrate, a ligand of leukocyte α4β1 integrin. VCAM-1 supports firm leukocyte adhesion on the blood vessel wall, and this LAFA assay allows the assessment of leukocyte α4β1 integrin activity. As shown in Figure 2H, the track length of interacting CD14 cells from patients with infectious inflammation was significantly shorter than non-infectious CD14 cells (58.6±9.4µm vs 85.7±9.4µm, \(p < 0.05\)), suggesting a reduced CD14 cell mobility in patients with infection. Additionally, a significantly lower CD15+CD16+ (neutrophils) cell density (29.0±12.4 vs 188.1±81.1, \(p < 0.05\)) was detected in patients with infection than non-infectious patients (Figure 2A), while no such decrease was detected when the cell density was normalised by neutrophil counts (Figure 2B). The R-factor of CD15+CD16+ cells is significantly lower in infectious patients than non-infectious patients (0.28±0.07 vs 0.49±0.07, \(p < 0.05\)), suggesting a lower cell propensity in infectious patients to be recruited on VCAM-1 substrate (Figure 2C). The track length of CD15+CD16+ cells from patients with infections was also significantly lower than non-infectious cells (43.5±3.1 µm vs 60.0±3.4 µm, \(p < 0.01\)), suggesting a reduced cell mobility in infectious CD15+CD16+ cells on VCAM-1 substrate (Figure 2H).

Furthermore, the numbers of interacting CD4 (60.0±11.9 vs 146.5±45.6, \(p < 0.05\)) and CD8 (30.0±6.9 vs 58.8±12.4, \(p < 0.05\)) cells on VCAM-1 substrate were significantly reduced in patients deemed to have infection compared to non-infectious patients (Figure 2A). The R-factor of infectious lymphocytes was significantly higher than non-infectious lymphocytes (17.3±3.1 vs 5.4±1.2, \(p < 0.01\)) (Figure 2C).

All blood samples were also analysed on VCAM-1+IL-8 substrates. As shown in Figure 3C and 3D, the dwell time (91.7±14.0 vs 150.6±19.0 seconds, \(p < 0.05\)) and track length (45.1±7.8 vs 62.7±5.8 vs µm, \(p < 0.05\)) of CD15+CD16+ neutrophils were significantly lower in infectious patients than non-infectious patients, indicating a decreased mobility of infectious CD15+CD16+ cells and an impaired cell ability to respond to IL-8.
Discussion

This study explored leukocyte adhesive function assay (LAFA) as a new tool to distinguish patients with infectious systemic inflammation from those affected by non-infectious causes. LAFA assesses the functions of several important leukocyte expressing membrane proteins, including PSGL-1 (selectin receptor), α4β1 integrin (VCAM-1 receptor) and CXCR-1 (IL-8 receptor). Compared with non-infectious patients, an enhanced ability of CD4 cells from infectious patients to adhere to P+E selectin was detected. Additionally, an impaired response to IL-8 was observed in neutrophils from infectious patients. These findings may offer a new understanding on disease pathogenesis and possible new biomarkers for sepsis diagnosis.

Compared with cells from non-infectious patients, an enhanced PSGL-1 activity was seen in CD4 cells from patients with infection, evidenced by a reduced cell straightness and increased dwell time (Figure 1F and 1G). The pathological role of PSGL-1 in animal models of sepsis was demonstrated by previous studies, in which the blockage of PSGL-1 resulted in less lung damage and better animal survival rate \(^{23,24}\). However, the role of PSGL-1 in ICU septic patients remains less well understood. Thus, our results provide direct evidence for a potential involvement of CD4 PSGL-1 in disease pathogenesis in sepsis patients. Given its ability to distinguish the likelihood of infection in patients with unknown cause of inflammation, the divergent activity of PSGL-1 between non-infectious and infectious CD4 cells may also be useful markers for the diagnosis of sepsis.

Our study extends the results of previous work that described an enhanced neutrophil recruitment by VCAM-1 in patients with infection, but not in patients with non-infectious systemic inflammation \(^{21}\). In our study, interacting leukocytes were recorded during the entire period of flow experiments utilising recently developed LAFA technology to enable a more complete assessment of leukocyte recruitment and cell migratory behaviours. In contrast to the previous findings, a slightly decreased number of interacting neutrophils (CD15+CD16+ cells) on VCAM-1 substrate was detected in infectious patients when compared to non-infectious patients (Figure 2A and 2B). This difference could be due to the divergent approaches used to detect the interacting cells between the two studies. Our study also noted that the neutrophil mobility was lower in infectious patients than non-infectious patients (Figure 2H), suggesting that the reduced neutrophil mobility in neutrophils from infectious patients on VCAM-1 substrate may serve as a new marker to distinguish infectious patients from non-infectious patients.
IL-8 is a chemokine that is shown to guide the migration of leukocytes (mainly neutrophils) by forming a concentration gradient, a process known as chemotaxis. CXCR1, a receptor for IL-8, may be expressed on neutrophil cell membranes and plays an important role in the regulation of leukocyte functions and migratory behaviours. Our study analysed blood samples by LAFA using VCAM-1 plus IL-8 as substrates, allowing the assessment of leukocyte CXCR1 activities.

An impaired response to IL-8 induced chemotaxis in neutrophils from patients with infection has been previously reported. Consistently, in the present study, a reduced ability to respond to IL-8 was detected in infectious neutrophils, compared with non-infectious cells (Figure 3C and 3D). These results suggest that the neutrophil IL-8 receptors (e.g. CXCR1) may play different roles in regulation of inflammation in non-infectious and infectious patients, which may allow it to serve as a useful marker to distinguish these two diseases.

The “Possible Infection (or Unknown)” group provides several unique insights. Firstly, it is likely this group contains both non-infectious and infectious patients, which may explain in part why it is indistinguishable from either Non-infectious or Infectious group in majority of cell parameters when analysed on all adhesive substrate (Figure 1, 2 and 3). Additionally, patient UN-02 was determined to be “possible infection” by both ICU specialists even though no evidence of infectious pathogen was found (Table 3). Given a low CD4 straightness value (0.595) was detected in this patient, and our notion that CD4 straightness may serve as a biomarker for sepsis, this result would support the cause of inflammation in patient UN-02 is more likely to be infectious than non-infectious.

Despite the small cohort size, this study has provided promising data showing the ability of LAFA to detect different leukocyte adhesive functions in sepsis and non-infectious systemic inflammation, providing potential diagnostic biomarkers for sepsis. Larger clinic studies would be required to further validate these findings and the clinical utility of LAFA technology. While currently LAFA involves specialised laboratory staff and equipment, it is possible with further development the assay time can be reduced to less than 20 minutes and the results turn around may be under 1 hour. Additional automation is also possible which could mean LAFA may be suitable as a point-of-care test for sepsis diagnosis.
Figure legends

Figure 1 Divergent leukocyte migratory behaviours between non-infectious and infectious ICU patients determined by LAFA using P+E selectins as substrates. Blood samples were collected from ICU patients and then analysed by LAFA on P+E selectin substrates. Based on the assessments by two independent ICU specialists, all patients were then divided into No Infection (n=9), Infection (n=10) and Possible Infection (n=9) groups. The cell density (A), normalised cell density (B), R-factor (C), cell speed (D), diffusion coefficient (E), straightness (F), dwell time (G) and track length (H) of CD14, CD15+CD16+, CD4, CD8 and CD4+CD25+ interacting cells were determined. *, p < 0.05; **, p < 0.01 compared to No Infection ICU patients.

Figure 2 Divergent leukocyte migratory behaviours between non-infectious and infectious ICU patients determined by LAFA using VCAM-1 as substrate. Blood samples were collected from ICU patients and then analysed by LAFA on VCAM-1 substrate. Based on the assessments by two independent ICU specialists, all ICU patients were then divided into No Infection (n=9), Infection (n=10) and Possible Infection (n=9) groups. The cell density (A), normalised cell density (B), R-factor (C), cell speed (D), diffusion coefficient (E), straightness (F), dwell time (G) and track length (H) of CD14, CD15+CD16+, CD4, CD8 and CD4+CD25+ interacting cells were determined. *, p < 0.05; **, p < 0.01 compared to No Infection ICU patients.

Figure 3 Divergent leukocyte migratory behaviours between non-infectious and infectious ICU patients determined by LAFA using VCAM-1+IL-8 as substrates. Blood samples were collected from ICU patients and then analysed by LAFA on VCAM-1+IL-8 substrates. Based on the assessments by two independent ICU specialists, all ICU patients were then divided into No Infection (n=9), Infection (n=10) and Possible Infection (n=9) groups. The cell speed (A), straightness (B), dwell time (C) and track length (D) were then determined. *, p < 0.05 compared to No Infection ICU patients.

Supplementary Figure 1 Full blood cell counts. Blood samples were collected from ICU patients (n=28) and the full blood cell counts were determined by a haematology analyser. Based on the assessments by two independent ICU specialists, all ICU patients were then divided into No Infection (n=9), Infection (n=10) and Possible Infection (n=9) groups. *, p < 0.05 compared to no infection ICU patients.
Figure 3

A. Speed

B. Straightness

C. Dwell time

D. Track length
Supplementary Figure 1

Blood cell counts

- Total Leukocyte
- Neutrophils
- Lymphocytes
- Monocytes

Cell Counts (10^6 cells/ml)

- No Infection
- Infection
- Possible Infection

*
Acknowledgement/Declaration

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