JAM-C and reverse transendothelial migration of neutrophils under conditions of sepsis

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Abbreviations

HUVECs = human umbilical vein endothelial cells
JAM-C = junctional adhesion molecule C
LPS = lipopolysaccharide
LBT4 = leukotriene B4
mAb = monoclonal antibody
PepG = peptidoglycan
ROS = reactive oxygen species
rTEM = reverse transendothelial migration
TEM = transendothelial migration

Background

Sepsis is a dysregulated inflammatory response to infection and remains a major cause of mortality in the UK with around 37,000 deaths each year.¹

Neutrophils are the first leucocytes to arrive at sites of infection or tissue damage and exit the blood circulation via a tightly controlled process called transendothelial migration (TEM).² On arrival, neutrophils release cytokines and chemokines for the recruitment of additional leucocytes however, prolonged neutrophil activation can result in damage to the host.³ In sepsis, both neutrophil elastase and leukotriene B4 (LTB4) are reported to be upregulated.⁴

Recent studies suggest that neutrophils can undergo reverse TEM (rTEM) thereby returning to the circulation. Junctional adhesion molecule C (JAM-C) was reported to be a key regulator of rTEM such that pharmacological blocking of JAM-C increased rTEM.⁵, ⁶ Both LTB4 and elastase may have roles in rTEM, and work in mice suggests that neutrophil elastase regulates JAM-C expression.⁷ Interestingly, reactive oxygen species (ROS) are also reported to affect JAM-C expression and oxidative stress is a hallmark of sepsis.⁵

Remote organ injury commonly occurs in patients with sepsis and is associated with neutrophil infiltration. The role of rTEM in sepsis remains unknown but may explain why neutrophils are found in remote tissues where they can cause subsequent damage.

This in vitro laboratory pilot study aimed to investigate rTEM under conditions mimicking sepsis and determine the effects of JAM-C, elastase and LTB4 inhibition. Additionally, the effect of melatonin, a potent anti-oxidant, was also investigated.
Methods

Human umbilical vein endothelial cells (HUVECs) were cultured with lipopolysaccharide (LPS) and peptidoglycan (PepG) for 24 hours to mimic sepsis. Endothelial cell monolayers were co-treated with a JAM-C monoclonal antibody (mAb), elastase inhibitor, an LTB4 receptor antagonist or various doses of melatonin. The LTB4 receptor antagonist was added either as a pre-treatment before the neutrophils or as a post-treatment at the same time as the neutrophils. All other treatments were added before and after the neutrophils to provide treatment throughout.

Following approval by the University of Aberdeen College of Life Sciences and Medicine Ethics Review Board and written informed consent, whole blood was collected from healthy volunteers of both sexes and neutrophils were isolated by density centrifugation. Neutrophils were fluorescently labelled before being added to endothelial cell monolayers. After 1h, neutrophils which migrated remained between the endothelial cells while neutrophils which did not migrate were removed by thorough washing. After a further 24 hours, reverse migrated neutrophils returning to the apical surface were removed, lysed and quantified by measuring fluorescence (Figure 1). The proportions of neutrophils at each stage in the experiment could be calculated by measuring fluorescence (Figure 2).

High resolution light microscopy was performed to examine the neutrophils and endothelial cells in co-culture. Immunofluorescence was performed to determine JAM-C expression in the presence and absence of the JAM-C mAb.

Figure 1: Summary of in vitro model. The cross-section of a well is outlined with endothelial cells cultured on bottom. Green stars indicate fluorescently labelled neutrophils. Following 1 hour incubation, a proportion of neutrophils undergo TEM. Non-migrated neutrophils are removed then medium replaced. After 24 hours reverse migrated neutrophils return to the apical endothelial cell surface from which they are collected and quantified by measuring fluorescence. Abbreviations: TEM, transendothelial migration; rTEM, reverse TEM.
Results

Initial cell viability acid phosphatase assays were performed (data not shown) using a range of doses of each treatment. Doses were selected based on manufacturer recommendations and the current literature. When cells were treated with the JAM-C mAb (0.2μg/ml and 2μg/ml) no effect on cell viability was observed compared to vehicle controls (P ≥ 0.05). Likewise, cell viability was similar compared to vehicle controls when cells were treated with the LTB4 receptor antagonist (10nM, 100nM and 500nM). However, a dose-dependent effect was observed when cells were treated with the elastase inhibitor and cell viability was significantly lower (P < 0.01) at the 1mM dose. At the 0.01mM and 0.1mM dose, cell viability was no different than without the elastase inhibitor (P ≥ 0.05). Melatonin was not studied since previous work by the University of Aberdeen Anaesthesia and Intensive Care Research Group reported no effect of melatonin on cell viability at doses between 0.1μM and 500μM.8

Following analysis of preliminary reverse migration experiments using the same range of doses of each treatment as cell viability assays (data not shown), a final model was established. In the final model, each experiment used neutrophils isolated from a different volunteer (n=14) (Table 1).

High resolution light microscopy suggested neutrophils were adherent to the outer membrane of HUVECs, especially at cell junctions (Figure 3).

Figure 2: Cross-section of a well illustrating neutrophil fractions. Fraction 0 (F0) was quantified by measuring fluorescence of the neutrophils originally added. All other fractions were calculated as a proportion of F0. Fraction 1 (F1) denotes the proportion of neutrophils removed following the wash step while Fraction 2 (F2) denotes the proportion of reverse migrated neutrophils. Fraction 3 (F3) is the proportion of neutrophils remaining between the endothelial cells at the completion of the experiment. The proportion of neutrophils undergoing TEM after 1 hour = F0 – F1. Note in experimental conditions the endothelial cells are directly cultured on the bottom of the well. Abbreviations: TEM, transendothelial migration.
The proportion of neutrophils at each stage of the experiment for cells treated with LPS and PepG only is outlined in Figure 3. The proportion of neutrophils migrating within the first hour ranged from 50% to 85% with a median value of 67%. The proportion of neutrophils which reverse migrated ranged from 16% to 62% with a median of 46% (Figure 4).

Reverse migration was compared between each treatment by calculating the percentage change relative to reverse migration in cells treated with LPS and PepG alone (Figure 4). When cells were treated with the JAM-C mAb, a statistically significantly higher proportion of neutrophils reverse migrated compared to cells treated with LPS/PepG alone (P=0.006). However, the proportion of neutrophils which reverse migrated was similar for cells treated with the elastase inhibitor, LTB4 receptor antagonist and melatonin compared to cells treated with LPS/PepG alone (Figure 5).

Although quantitative analysis of immunofluorescence was not possible, the addition of the JAM-C mAb appears to result in less JAM-C expression compared to cells treated with LPS and PepG alone (Figure 6).

Table 1: Final *in vitro* model volunteer characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Volunteers</th>
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<tbody>
<tr>
<td></td>
<td>n=14</td>
</tr>
<tr>
<td>Sex (male:female)</td>
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<tr>
<td>Age (median [range])</td>
<td>27 [21-62]</td>
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</tbody>
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Figure 3: High-resolution light microscopy of HUVECs treated with LPS and PepG in co-culture with neutrophils. Green outline: HUVEC outer membrane. Orange outline: neutrophil (= 12 to 17 µm). White arrow: neutrophil bound to the junction between HUVECs. No staining. Magnification 40x.
Figure 4: Reverse migration fractions LPS + PepG + vehicle control. Fractions are labelled on the x-axis. Each experiment used neutrophils isolated from a different volunteer (n = 14). Box and whisker plots show median, interquartile and full range of data. Fractions were calculated as a proportion of the total neutrophils initially added to each well (F0). No statistical analysis.

Figure 5: Reverse transendothelial migration of human neutrophils treated with LPS/PepG plus either anti-JAM-C mAb ([clone 208206], R&D Systems, Abingdon, UK; 2μg/ml), the elastase inhibitor (Sigma, Dorset, UK; 1mM), the LTB4 receptor antagonist ([LY223982], Cayman Chemical, Michigan, USA; 100nM) added either before or after addition of neutrophils, or various doses of melatonin (Sigma, Dorset, UK). Results are expressed as % of rTEM in the presence of LPS/PepG alone (for LPS/PepG only the % change is based on the baseline median value). Box and whisker plots show median, interquartile and full range. Data compared using Friedman/Wilcoxon test for paired data (n = 14). The final concentrations of LPS and PepG were 2.5mg/ml and 25mg/ml respectively.
Discussion

In this study, reverse transendothelial cell migration of human neutrophils from healthy volunteers under conditions mimicking sepsis was studied. In addition the effects of inhibiting JAM-C, elastase or LTB4, and the effect of melatonin were investigated. We found that around two thirds of neutrophils migrated into the endothelial cell layer and of these around 70% reverse migrated back. We also found that inhibition of JAM-C using a blocking mAb increased reverse migration but inhibition of elastase or LTB4 had no effect. Treatment with melatonin also did not affect reverse migration.

We found that under conditions mimicking sepsis, the proportion of neutrophils migrating within the first hour (median 67%) was consistent with previous static transmigration models where the percentage was reported to be around 60% when neutrophils were treated with TNF-α.⁹

We also found that a statistically significantly higher proportion of neutrophils reverse migrated from the endothelial monolayer in HUVECs treated with a JAM-C mAb under conditions of sepsis compared to LPS and PepG alone.
This finding is supported by immunofluorescent imaging of JAM-C expression. Altogether, these findings concur with previous work investigating JAM-C inhibition in ischaemia-reperfusion injury in a mouse model which, although differs in aetiology compared to sepsis, both result in oxidative stress and remote organ injury.\textsuperscript{5}

Although it had been suggested that elastase and/or LTB4 have a role in regulation of JAM-C and therefore reverse migration we found no difference when cells were co-treated with antagonists of these mediators along with LPS/PepG.

Melatonin is well-known for its role in sleep regulation but it is also a potent antioxidant, with marked anti-inflammatory effects.\textsuperscript{8,10} We hypothesised that given the role of oxidative stress in sepsis and ischaemia reperfusion, and the importance of oxidative stress in terms of neutrophil and endothelial cell activation melatonin may have some effects on migration of neutrophils. However, we found no effect of melatonin in our study.

Although, there was variable responses of each treatment between individual subjects, expression of results as percentage change from baseline values with LPS/PepG alone failed to demonstrate any effects of treatments other than JAM-C inhibition. This may represent inadequate power of the study. In this pilot study we recruited 14 volunteers in the time available.

**Conclusion**

In this study, a model and methods for investigating reverse migration of neutrophils under conditions mimicking sepsis was successfully established. We found that neutrophils did undergo reverse migration and that inhibition of JAM-C increased this, under conditions mimicking sepsis. This confirmed the role of JAM-C in the process of reverse migration.

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I was awarded a First Class degree and the results will be submitted for presentation at the Anaesthetic Research Society meeting in York in November 2015.
References


8. Lowes D, Almawash A, Webster N, Reid V, Galley H. Melatonin and structurally similar compounds have differing effects on inflammation and mitochondrial function in endothelial cells under conditions mimicking sepsis. British Journal of Anaesthesia. 2011;107(2):193-201.
