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Letter to the Editor

Transcellular migration of neutrophils is a quantitatively significant pathway across dermal microvascular endothelial cells

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Abstract: Neutrophil extravasation is central to inflammatory skin diseases like psoriasis and atopic dermatitis. *In vivo*, neutrophils have been shown to migrate through cell-to-cell junctions (paracellular pathway) or directly through the body of the endothelial cell (transcellular pathway). *In vitro*, however, neutrophil migration is a largely paracellular process where cells preferentially cross at tricellular corners devoid of tight junctions. To approximate the type of cells encountered by extravasating neutrophils *in vivo*, we developed a neutrophil-migration assay using primary human dermal microvascular endothelial cells. We show here that a large proportion of migrating neutrophils traverse a monolayer of microvascular endothelium using a purely transcellular pathway. In addition, we demonstrate that F-actin is rearranged similarly in neutrophils undergoing diapedesis along either route. This *in vitro* model closely simulates the physiological process of neutrophil extravasation *in vivo* and can be further utilized to evaluate the relative contribution of distinct migratory pathways to the pathophysiology of inflammatory skin disease.

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Background

Neutrophil extravasation is a critical component of both acute inflammation in the skin and chronic diseases like psoriasis (1). Although much is known about leucocyte adhesion to activated endothelium during inflammation, the route by which neutrophils extravasate during diapedesis remains unclear. Early serial EM studies involving leucocyte trafficking through inflamed rat mesenteric vessels *in vivo* found that the majority of extravasating neutrophils migrated through cell-to-cell junctions (paracellular pathway) while some were able to traverse the endothelium by passing directly through the body of the endothelial cell (transcellular pathway) (2,3). Using a similar type of analysis, a more recent report showed that neutrophils migrate through microvessels using a purely transcellular route in response to injection of the chemotactic peptide FMLP (4,5). Because of the technical difficulty of analysis, no consensus has been reached as to the predominant route of migration *in vivo*.

Questions addressed

Most *in vitro* reports have demonstrated that neutrophils cross the endothelium in a largely paracellular manner, migrating through tricellular corners at the junction of three endothelial cells (6,7). Recently, a study by Carman et al. demonstrated clear evidence of transcellular migration by a small proportion (5%) of migrating neutrophils (8). A subsequent study showed that the proportion of neutrophils migrating along a transcellular path can be increased under conditions of high TNF-α stimulation and ectopic expression of the adhesion molecule ICAM-1 in the endothelium (9). Each of these *in vitro* studies were conducted with human umbilical vein endothelial cells (HUVECs), a macrovascular cell type, despite evidence that leucocyte migration *in vivo* is mediated by microvascular endothelium (6–9). To more closely approximate the type of cells encountered by extravasating neutrophils *in vivo*, we developed a neutrophil-migration assay using primary human dermal microvascular endothelial cells (HDMVECs).

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Experimental design

To analyse neutrophil transmigration in HDMVECs, we adapted a protocol established for monitoring leucocyte transmigration across HUVECs (8,10) (see Supplementary Information, Methods). Briefly, HDMVECs grown to confluence on fibronectin-coated coverslips were stimulated with tumor necrosis factor (TNF)-α in low serum media. Blood was taken from healthy human donors and neutrophils were purified by double ficoll gradient centrifugation and found to be >97% pure by nuclear morphology. Neutrophils were added at a 2:1 ratio per endothelial cell and allowed to migrate for 8–10 min at 37°C. Cells on coverslips were then fixed and stained with anti-human CD45 (Chemicon, Billerica, MA, USA) to identify the neutrophils, phalloidin (Invitrogen, Carlsbad, CA, USA) to mark the body of the cell and VE-cadherin (R&D Systems, Minneapolis, MN, USA) to distinguish junctional regions of the endothelium.

Results

In adherent or postmigratory neutrophils, CD45 was evenly distributed (see Fig. S1a). Neutrophils in the process of diapedesis could be distinguished by their ring-like cluster of intense CD45 expression, which marked the point of penetration through the endothelium (see Fig. S1a). During paracellular migration, leucocytes have been shown to orient themselves perpendicular to the endothelium resulting in a redistribution of F-actin (11). We observed a similar redistribution of F-actin (phalloidin stained) in neutrophils migrating along a transcellular path. Furthermore, CD45 was found to cluster with F-actin in a similar pattern during both transcellular and paracellular migration suggesting that neutrophils orient themselves in a similar manner relative to the endothelium during diapedesis along either route (see Fig. S1 and Movies 1 and 2).

To determine the percentage of migrating neutrophils traversing the endothelium along each route, active migration events were identified as transcellular (Fig. 1a left), or paracellular (Fig. 1a right), based on the presence or absence of co-localization with the junctional marker VE-cadherin, respectively. When transmigration was halted, the majority of neutrophils were found to follow a paracellular route. However, more than 25% were observed to follow a purely transcellular path passing directly through individual HDMVECs independent of junctional regions of the endothelial monolayer (Fig. 1b). In addition, we found that a similar proportion of neutrophils migrated in a transcellular manner under either 100 or 10 ng/ml concentrations of TNF-α stimulation, suggesting that the route of migration is not significantly altered by decreasing dosage of cytokine (see Fig. S2).

We and others have described protrusive structures extending from lymphocytes in the process of diapedesis (12–14). A recent report by Carman et al. demonstrated that lymphocyte cellular protrusions or 'podosomes' probe the endothelium in search of regions that are permissive for transcellular diapedesis. Ultrastructurally, we observed that neutrophils formed similar structures to penetrate the endothelium in our transmigration model (see Fig. S3a). Moreover, dermal microvascular endothelial cells were found to extend reciprocal processes towards the migrating neutrophil (see Fig. S3b). It remains unclear whether these endothelial extensions function to tether the neutrophil to the endothelium or if they play a role in the process of diapedesis.

Conclusions

This report demonstrates that a large proportion of migrating neutrophils traverse microvascular endothelium using a purely transcellular pathway. It is plausible that the large percentage of transcellular neutrophil diapedesis observed in HDMVECs but not in HUVECs is due to the different properties of microvascular (HDMVEC) and macrovascular (HUVEC) endothelium. Microarray comparison of gene expression profiles in HUVECs and human microvascular endothelial cells (HMEC-1) following TNF-α-stimulation demonstrated that less than half of the genes analysed were found to be regulated in a similar manner in both cell types (15). Moreover, TNF-α-stimulated HUVECs and microvascular endothelial cells showed clear differences in their regulation of genes critical to leucocyte transmigration, including the adhesion molecule ICAM-1 and neutrophil chemoattractant IL-8 (15).

Dynamic interactions between factors on the neutrophil and EC are central to diapedesis. Stimulation of the (proteinase-activated receptor) PAR_2 on the neutrophil surface was demonstrated to reduce total transendothelial migration through microvascular endothelial cells *in vitro* (16). Blockage of the endothelial junctional molecule VE-caherin accelerates neutrophil entry into inflamed mouse peritoneum *in vivo* and increases paracellular permeability *in vitro* (17–19). As of yet, attempts to specifically block the paracellular path and skew migration along a purely transcellular route have been unsuccessful (13).

Recently, ICAM-1 in concert with caveolin-1, the protein responsible for induction of vesicles known as caveolae in the endothelium, has been shown to facilitate transcellular migration of T lymphoblasts through HUVECs (10). However, the relative contribution of these factors to the path of neutrophil diapedesis and their involvement in the route of leucocyte transmigration *in vivo* remain unanswered. As EC differentiation is highly dependent on the unique *in vivo* microenvironment, the proportion of leucocytes migrating along each route may additionally be affected by

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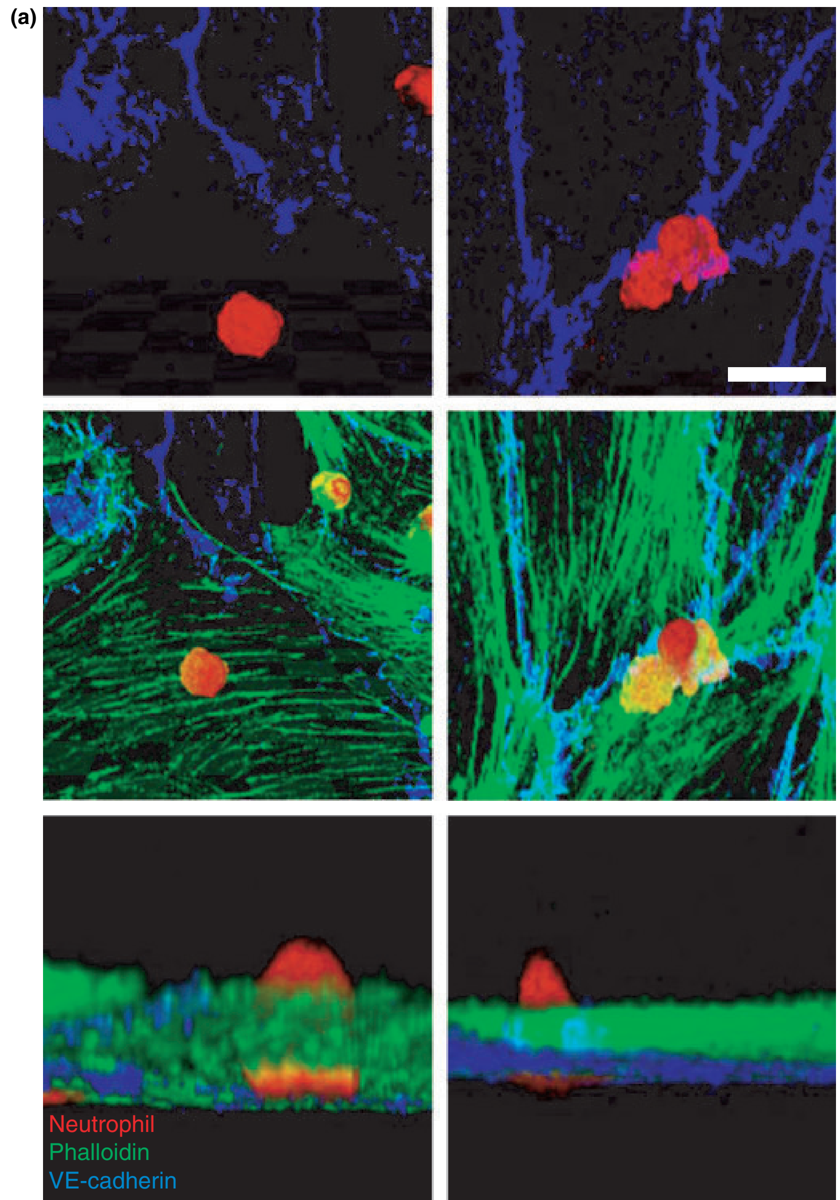

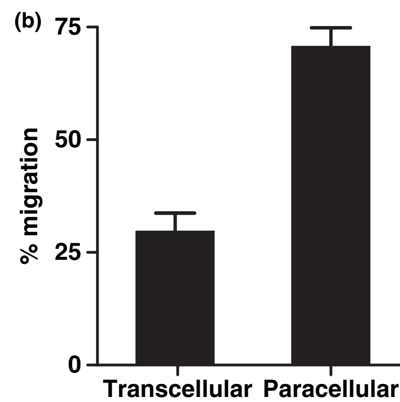

Figure 1. A significant fraction of neutrophils traverse dermal microvascular endothelium along a purely transcellular route. (a) Top views of merged confocal Z stacks showing a single neutrophil migrating through a region in the centre of the cell devoid of VE-cadherin stained cell-to-cell junctions (left) and another neutrophil migrating through a junctional region of the endothelium (right) CD45 (red), VE-cadherin (blue) (top panels). Pictures of the above transmigrating neutrophils stained with CD45 (red), VE-cadherin (blue) and phalloidin (green) (middle panels). Side views of the transmigrating neutrophils. Portions of the migrating cells can be found above and below the phalloidin stained (green) endothelial plane (bottom panels). Scale bar 20 um. (c) Graph calculating the percent total transcellular and paracellular migration through HDMVECs.

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organ system, vascular bed or tissue type (20,21). Moreover, the effect of concentration and inflammatory stimulus on the route of diapedesis is unclear.

The use of dermal microvascular cells in these assays represents a useful *in vitro* surrogate for the *in vivo* process of neutrophil transmigration and will enable further investigation into the molecular control of the transcellular route of diapedesis. Additional study is needed to evaluate the relative contribution of distinct migratory pathways to the pathophysiology of inflammatory skin disease.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Neutrophils adopt a similar orientation relative to the endothelial monolayer during transcellular and paracellular migration.

Figure S2. A similar proportion of neutrophils migrate along a transcellular route under different conditions of TNF-α stimulation.

Figure S3. Protrusive structures extend from both neutrophils and dermal endothelial cells preceding diapedesis.

Movie S1. CD45 clusters with F-actin during neutrophil transcellular diapedesis.

Movie S2. CD45 clusters with F-actin during neutrophil paracellular diapedesis.

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