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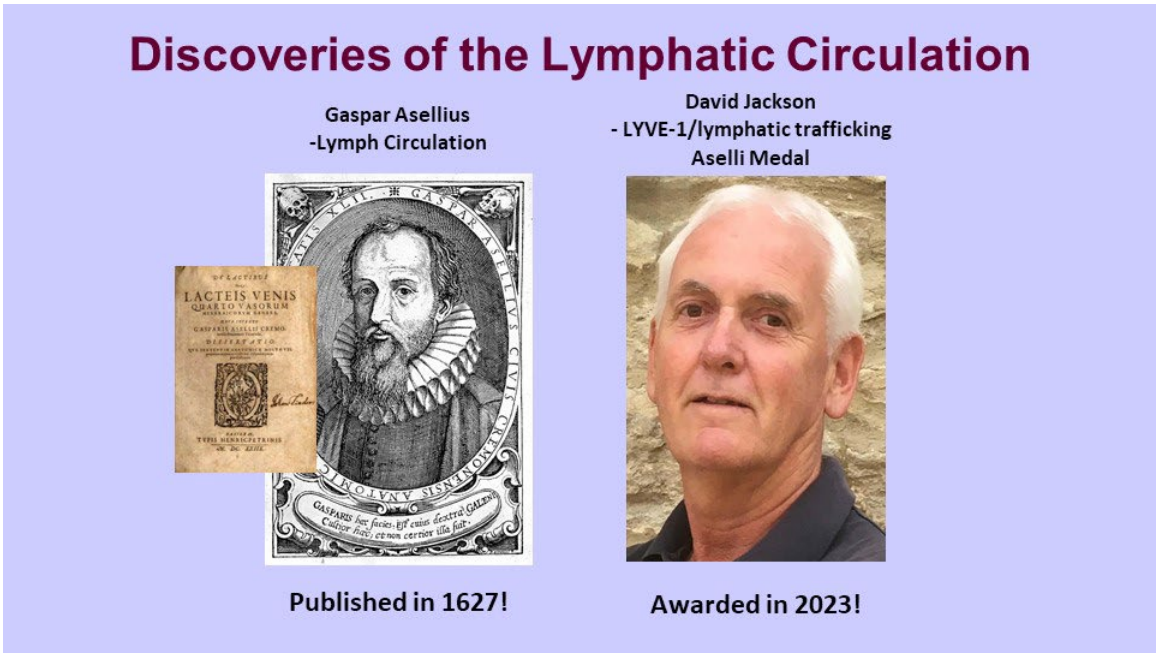
Special Feature

ASELLI AWARD WINNER 2023: THE LIFE AND TIMES OF A LYMPHOMANIAC

David G. Jackson, PhD

University of Oxford, MRC Translational Immune Discovery Unit, MRC Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford, United Kingdom



ABSTRACT

Winning a scientific prize is always a welcome honor and receiving the Aselli Award in 2023 was a particularly pleasant surprise. The following text provides a brief summary of the research carried out by my group in Oxford that led to the discovery of the lymphatic endothelial marker LYVE-1 and its emerging role as a pivotal receptor controlling the entry of immune cells and metastatic tumor cells to lymphatic capillaries in peripheral tissues. As a basic scientist and relatively late comer to the field of lymphology, my hope is that a closer partnership of basic and clinical research will help explain the intricate workings of the lymphatics and improve the picture for patients suffering from much neglected lymphatic disorders.

Keywords: Gasparo Aselli award, hyaluronan, LYVE-1

I was greatly honored to receive the Gasparo Aselli award at the International Society of Lymphology 2023 Genoa World Congress, for my work on immune and tumor cell trafficking via the lymphatics and sincerely thank Corradino and Corrado Campisi and Marlys Witte, the organizers, for nominating me.

As is so often the case in science, my introduction to the particular field of lymphatic biology was serendipitous. Having graduated with a B.A. (Mod.) and PhD in biochemistry from Trinity College Dublin, I moved to Oxford in 1987 and switched focus to leucocyte cell surface receptors and what was then the latest technology for their isolation by cDNA expression cloning. At Oxford, I established my own research group as an MRC Senior Fellow, and this led me to pursue research into CD44, a receptor for the extracellular matrix glycosaminoglycan, hyaluronan (HA), a huge linear copolymer of N-acetyl glucosamine and glucuronic acid whose many functions include that of a tissue 'filler' and adhesive ligand for leucocyte migration. This was followed by the cloning of a new CD44 homologue, aided by the biopharmaceutical company Human Genome Sciences, part founded by the Nobel prizewinner Craig Venter. It was not until we expressed the cDNA and generated antibodies for tissue immunostaining that we discovered the novel receptor which we subsequently named LYVE-1 (Lymphatic Vessel Endothelial receptor - 1). was a component of lymphatic vessels (1), an unexpected identification confirmed by a hematologist colleague we called upon one afternoon to look down our microscope. Ironically, we were not particularly enthused by such news at the time.

Through our naivete in the field, we were completely unaware that there was an urgent need for sensitive and unambiguous markers for lymphatics – particularly among those interested in the mechanisms underlying embryonic and adult lymphangiogenesis and its links with cancer metastasis. At a time when oncologists across the world were fixating on the prognostic significance of sentinel lymph node biopsies and the virtues of lymph node resection, other researchers also required new and more reliable reagents, not only to distinguish lymphatic vessels from blood vessels, but also to establish quantitative correlations between intra- and peritumoral lymphatic vessel density (LVD), lymph node involvement and systemic dissemination. Although additional markers such as VEGFR3 and 5' nucleotidase were becoming available, they had their various shortcomings (2,3). We happened to arrive at just the right time and with just the right marker, LYVE-1, for such studies. I can still remember the enthusiastic welcome I received when I attended my first Gordon conference on lymphatics in Ventura California, as a complete novice, and the number of key players that wished to collaborate, using our highly sensitive LYVE-1 antibodies and expertise in immunohistochemistry. The resulting alliances formed the basis for a plethora of seminal publications on the regulation and functional significance of lymphangiogenesis in murine models over the following years, both in the context of normal development and cancer metastasis (4-9) (*Fig 1*). Combined with sub-sequent analyses of archival human cancer tissues, the results from such studies appeared to largely confirm the notion that both VEGF-C/D-induced tumor lymphangiogenesis and an increase in peritumoral and/or intratumoral lymphatic vessel density were associated with poor disease prognosis, with the lymphatics acting as conduits for systemic cancer spread (10-15). While those studies remain largely valid to this day, the issue of precisely how tumors invade the lymphatics remains unresolved. Indeed, the relevance of lymphatic invasion and nodal metastasis for systemic dissemination of cancers is now increasingly questioned, as detailed further below.

Alongside these tumor-based studies, our motivation as basic scientists have been to determine the normal function of LYVE-1 in the lymphatics and particularly its roles in immunity and inflammation. The large degree of sequence homology with other known HA binding proteins, coupled with our initial findings using the recombinant receptor, had already underlined the identity of LYVE-1 as a *bona fide* hyaluronan receptor (1). However, as is common in scientific research, our quest

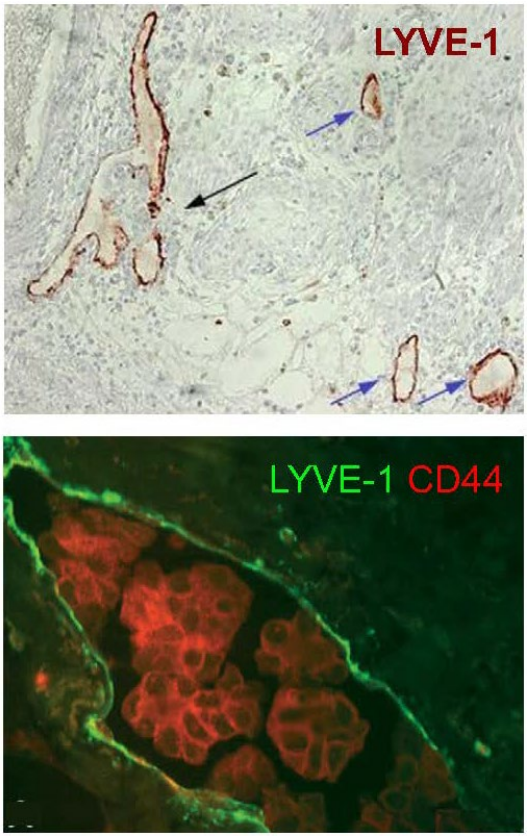


Fig. 1 Examples of LYVE-1 immunostained lymphatics in human cancers. Top panel: LYVE-1 immunoperoxidase stained intratumoral lymphatics (arrows) within invasive testicular cancer. Lower panel: Individual LYVE-1 immunofluorescent-stained lymphatic (green) containing CD44+ metastatic tumour cells (red) in lobular breast cancer (31,32).

for physiological function proved not to be anything but straightforward. Given the abundance of HA as a fundamental component of the tissue extracellular matrix and its accelerated turnover in inflammation and injury, we initially assumed that LYVE-1 had some involvement in the process, specifically HA uptake. This seemed all the more likely in light of earlier literature, which revealed the surprising observation that HA is transported from the tissues via lymph for degradation in local draining LNs. To test the hypothesis, we cultured lymphatic endothelial cells (LECs) isolated from surgically resected human and mouse skin, and looked for their ability to endocytose purified, fluorescently-labeled HA. To our surprise, we found that LYVE-1 contributed only minimally to HA uptake and was even partly downregulated during inflammation (16). Indeed, to add to our consternation we could neither detect binding of purified fluorescent HA nor find any obvious association of endogenous HA with lymphatics when we carried out dual staining for LYVE-1 in tissue sections of either skin or any of the other organs we examined.

Notably, while chasing this enigma, we discovered that LECs add a particular type of glycosylation (sialylation) to LYVE-1 that partially masks its receptor function (17), but as it turned out this was not the main reason for the lack of HA binding in our studies. After much brainstorming and appropriate experimentation using crosslinking mAbs, we finally made the discovery that LYVE-1 needs to be clustered at the LEC cell surface to bind HA, as its relatively low affinity for the sugar ligand means that many copies of the receptor must come together to mutually tether an individual HA chain (18,19). Consistent with such reasoning and with the help of colleagues at the Hammersmith in London, we then discovered that virulent strains of group A streptococci (*Streptococcus pyogenes*) that make a characteristically dense, mucus-like HA surface coat, can also bind to LECs via LYVE-1 (20), and furthermore, that they likely exploit the interaction to invade lymphatics for systemic infection (21) in the pathogenesis of tonsilitis as well as more serious streptococcal infections that can lead to necrotising fasciitis or rheumatic fever.

The realization that the organization of HA itself is critical for binding was a turning point in our thinking about LYVE-1 physiological function and led us to make the key discovery that migrating immune cells also utilize a surface HA coat to adhere to LECs and given the localization of LYVE-1 within the distinctive buttoned junctions of initial lymphatics (22), that it acted as a vessel entry receptor (23). Specifically, we showed in mice that the migration of antigen-presenting

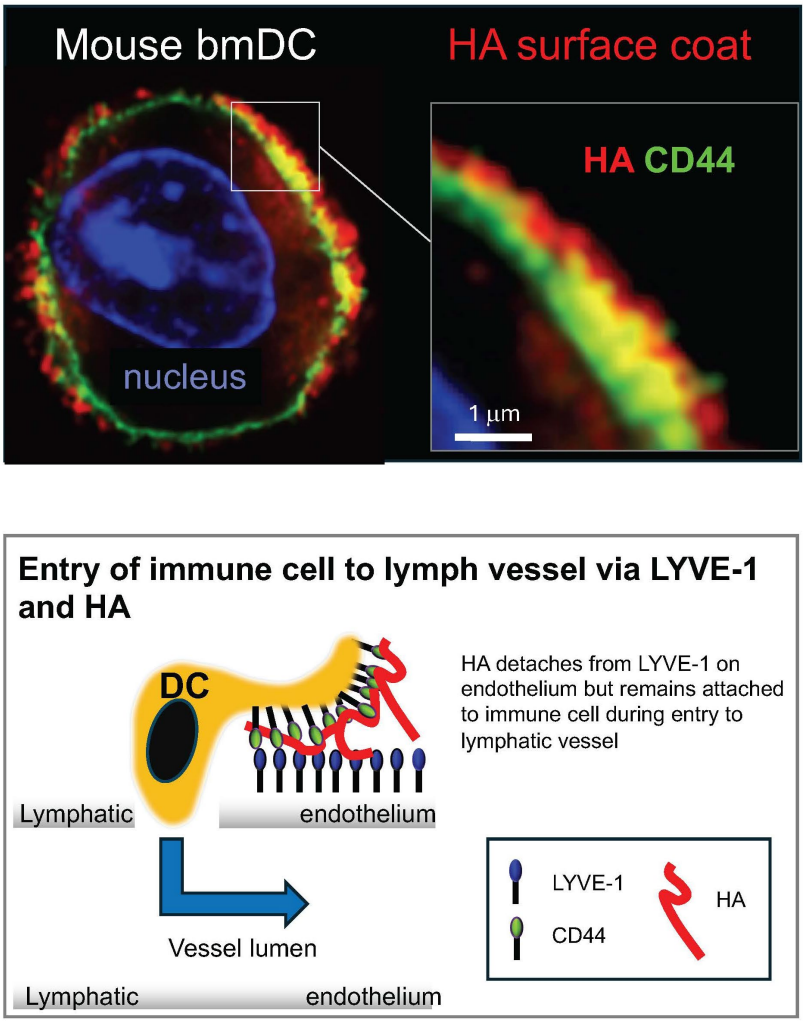


Fig. 2 How immune cells use their HA surface coat to enter lymphatic vessels via LYVE-1. Top panel shows a high magnification fluorescence microscopy image of the HA coat on the surface of a murine bone marrow derived DC (bmDC) and a zoomed-in portion showing association with its anchoring partner CD44. Lower panel shows a cartoon image of the proposed LYVE-1 mediated vessel entry mechanism for a migrating DC en route to a draining LN.

dendritic cells from inflamed skin to draining LNs was largely dependent on LYVE-1, and that its genetic deletion caused the migrating cells to logjam at the basolateral surface of initial capillaries rather than transit to the lumen, as did disruption of the DC HA coat by either enzymatic digestion or deletion of its anchoring receptor CD44 (24,25). These findings were soon extended to other immune cell populations, with the demonstration that monocyte/macrophages (but not T cells or neutrophils) also synthesize an HA surface coat and employ LYVE-1 adhesion to exit tissues via afferent lymphatics following their repair and remodeling of injured tissue and removal of dead cells and debris during the resolution of inflammation. Accordingly, in a mouse model of myocardial infarction, genetic deletion of LYVE-1 disrupted the clearance of inflammatory monocyte/macrophages from

the infarcted myocardium and exacerbated cardiac fibrosis (26). With recent insights gained from our LYVE-1 crystal structures, we now envisage migrating immune cells use their HA coat to crawl along the vessel surface and transmigrate via a transient and reversible sliding interaction with LYVE-1 that enables them retain the coat for further engagement inside the vessel lumen and in downstream lymph node sinuses (*Fig. 2*).

Drawing parallels from our studies of immune cells, it seemed likely and even obvious to us that tumor cells might equally exploit the LYVE-1 HA axis to facilitate lymphatic invasion and perhaps systemic metastasis. Like others, we observed that many if not all breast tumor cell lines synthesize a surface HA coat. Indeed, when we transplanted experimental murine mammary tumors such as 4T1 orthotopically in mice, we could disrupt their capacity to metastasize to local draining LNs by prior administration of LYVE-1 receptor blocking mAbs. As already mentioned, the notion that nodal metastasis can serve as a bridgehead for systemic spread and distant organ involvement is now disputed. Although key studies have reported that some tumors can spread systemically by direct invasion of cortical HEVs in the first affected LNs (27, 28), more recent genetic analyses indicate the majority of (but not all) organ metastases derive from the primary tumor rather than its lymph node metastases (29), implying that nodal involvement is more an indicator of progression than a route for systemic dissemination. Nevertheless, as it is increasingly apparent that lymph node invasion exerts considerable suppression of host antitumor immunity (30), the targeting of receptors such as LYVE-1 that aid lymphatic invasion may well prove effective as an adjunct to e.g., checkpoint inhibitor therapy in metastatic cancer even if not for direct anti-metastatic therapy itself.

There are still many more questions to be answered regarding cell migration via the lymphatics and the roles played by LYVE-1. These include a possible involvement in intraluminal crawling, the regulation of lymphangiogenesis, and the exocytic release of chemokines such as CCL21 from lymphatic endothelium for immune cell transmigration. Are there genetic variants of LYVE-1 that are associated with lymphatic or other diseases ? What is the physiological significance of LYVE-1 expression in tissues other than lymphatic endothelium, most notably discrete populations of yolk sac derived macrophages ? Beyond these issues, my ongoing studies will explore the therapeutic potential of LYVE-1 antibody blockade as a means of controlling alloimmune rejection following tissue transplantation.

While much of my research career has focused on LYVE-1, it is clear that no single receptor functions in isolation of others, and future work will have to unravel how LYVE-1 integrates with key receptors such as integrins, selectins, JAMs, cadherins and others to facilitate lymphatic entry during health and disease.

CONFLICT OF INTEREST AND DISCLOSURE

The author declares no competing financial interests exist.

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David G. Jackson, PhD
University of Oxford
Emeritus Professor of Human Immunology
MRC Translational Immune Discovery Unit
MRC Weatherall Institute of Molecular Medicine
John Radcliffe Hospital
Headington
Oxford OX3 9DS UK
Email: David.jackson@imm.ox.ac.uk