Published in final edited form as:

J Cell Physiol. 2018 April ; 233(4): 2681–2692. doi:10.1002/jcp.26159.

Development of the LYVE-1 gene with an acidic-amino-acid-rich (AAAR) domain in evolution is associated with acquisition of lymph nodes and efficient adaptive immunity

Shuan Shian Huang¹, Li Ya-Wen², Wu Jen-Leih², Johnson Frank E.³, and Jung San Huang⁴

¹Auxagen Inc., St. Louis, Missouri

²Graduate Institute of Life Sciences, National Defense Medical Center and Institute of Cellular and Organismic Biology, Academia Sinica, Taipei, Taiwan

³Department of Surgery, Saint Louis University Medical Center, St. Louis, Missouri

⁴Department of Biochemistry and Molecular Biology, Doisy Research Center, Saint Louis University School of Medicine, St. Louis, Missouri

Abstract

CRSBP-1 (mammalian LYVE-1) is a membrane glycoprotein highly expressed in lymphatic endothelial cells (LECs). It has multiple ligands, including hyaluronic acid (HA) and growth factors/cytokines (e.g., PDGF-BB and VEGF-A) containing CRS motifs (clusters of basic amino-acid residues). The ligand binding activities are mediated by Link module and acidic-amino-acid-rich (AAAR) domains, respectively. These CRSBP-1/LYVE-1 ligands have been shown to induce opening of lymphatic intercellular junctions in LEC monolayers and in lymphatic vessels in wild-type mice. We hypothesize that CRSBP-1/LYVE-1 ligands, particularly CRS-containing growth factors/cytokines, are secreted by immune and cancer cells for lymphatic entry during adaptive immune responses and lymphatic metastasis. We have looked into the origin of the Link module and AAAR domain of LYVE-1 in evolution and its association with the development of lymph nodes and efficient adaptive immunity. Lymph nodes represent the only major recent innovation of the adaptive immune systems in evolution particularly to mammals and bird. Here we demonstrate that the development of the LYVE-1 gene with the AAAR domain in evolution is associated with acquisition of lymph nodes and adaptive immunity. LYVE-1 from other species, which have no lymph nodes, lack the AAAR domain and efficient adaptive immunity. Synthetic CRSBP-1 ligands PDGF and VEGF peptides, which contain the CRS motifs of PDGF-BB and VEGF-A, respectively, specifically bind to CRSBP-1 but do not interact with either PDGFR or VEGFR2. These peptides function as adjuvants by enhancing adaptive immunity of pseudorabies virus

Correspondence: Jung S. Huang, PhD, Department of Biochemistry and Molecular Biology, Saint Louis University School of Medicine, Doisy Research Center, Room 413, 1100 S. Grand Blvd., St. Louis 63104, MO. huangjs@slu.edu.

AUTHORS' CONTRIBUTIONS

Y.W.L. and J.L.W. performed bioinformatics analyses of the LYVE-1 structure and function in evolution. S.S.H. performed bioinformatics analyses of adaptive immunity involving lymph nodes in evolution. F.E.J. critically reviewed the manuscript. J.S.H. determined the effects of synthetic PDGF and VEGF peptides on the efficacy of pseudorabies virus vaccine in pigs.

COMPETING FINANCIAL INTERESTS

We confirm that there are no competing financial interests associated with this work.

(PRV) vaccine in pigs. These results support the notion that LYVE-1 is involved in adaptive immunity in mammals.

Keywords

adaptive immunity; adjuvants; lymph nodes; LYVE-1 evolution

1 | INTRODUCTION

Cell-surface retention sequence (CRS) binding protein-1 (CRSBP-1) was identified in 1995 in simian sarcoma virus (SSV)-transformed cells and liver tissue by its ability to bind PDGF-BB, VEGF-A¹⁶⁵ and synthetic oligopeptides containing the CRS motifs (clusters of basic amino acid residues) of these growth factors (termed PDGF and VEGF peptides, respectively) (Boensch, Kuo, Connolly, Huang, & Huang, 1995). CRSBP-1 interacts with the autocrine v-sis oncogene product (PDGF-BB) and retains it on the cell surface in SSV-transformed cells (Boensch, Huang, Connolly, & Huang, 1999). All known members of the PDGF superfamily, including PDGF-AA, PDGF-BB, placental growth factor (PIGF) and VEGF-A/C/D, possess CRS motifs at the C-terminal part of the molecules and are putative physiological ligands of CRSBP-1 (Achen et al., 1998; Houck et al., 1991; Joukov et al., 1996; LaRochelle, May-Siroff, Robbins, & Aaronson, 1991; Ostman, Andersson, Betsholtz, Westermark, & Heldin, 1991; Pollock & Richardson, 1992). These CRS motifs are evolutionarily conserved in mammals. cDNA cloning, sequencing, and expression of bovine CRSBP-1 reveals that bovine CRSBP-1 cDNA encodes a 322-amino-acid-residue protein and that bovine CRSBP-1 expressed in transfected cells is a 120-kDa type I membrane disulfide-linked dimeric protein which has multiple ligand (hyaluronic acid [HA] and growth factors/cytokines/oligopeptides containing CRS motifs) binding activities (Huang et al., 2003). The deduced amino acid sequences of human and murine CRSBP-1 exhibit 61% and 56% identity with that of bovine CRSBP-1 and are identical to those of human and murine lymphatic vessel endothelial HA receptor-1 (LYVE-1). LYVE-1 was cloned using CD44 sequence homology cloning in 1999 (Banerji et al., 1999). Both CD44 and LYVE-1 (or CRSBP-1) are members of the Link protein superfamily that bind the large extracellular matrix glycosaminoglycan HA through their cell-surface Link module domains.

LYVE-1 has been found to be highly expressed in lymphatic endothelial cells (LECs). LYVE-1 is localized to the luminal and abluminal faces of lymphatic vessels (Banerji et al., 1999). Specific expression of LYVE-1 in LECs has made LYVE-1 a suitable lymphatic marker for studying lymphangiogenesis (Jackson, 2004). However, the finding of multiple ligand binding activities of CRSBP-1 (mammalian LYVE-1) prompted us to probe the potential role of CRSBP-1 in lymphatic function by generating CRSBP-1 null (CRSBP-1^{-/-}) mice (Huang et al., 2006). CRSBP-1^{-/-} mice are overtly normal and fertile but exhibit subtle and identifiable morphological and functional alterations of lymphatic capillary vessels in tissues, marked by the lack of typical irregularly-shaped lumens in tissues, and constitutively increased interstitial-lymphatic transport (as determined by tail dermal injection with high-molecular-weight FITC-dextran). The CRSBP-1 ligands PDGF-BB and HA enhance interstitial-lymphatic transport of injected FITC-dextran with the potency of PDGF-BB >>

HA in wild-type but not in CRSBP-1^{-/-} mice (Huang et al., 2006). CRSBP-1 ligands, including PDGF-BB, VEGF-A, PDGF, and VEGF peptides induce disruption of VE-cadherin-mediated intercellular adhesion and opening of intercellular junctions in LEC monolayers as determined by immunofluorescence microscopy and transwell permeability assay (Hou et al., 2011; Hou, Liu, Huang, & Huang, 2012). Both PDGF and VEGF peptides specifically interact with the AAAR domain of CRSBP-1. In addition, PDGF-BB, VEGF-A, VEGF-C, VEGF-D, and other cytokines (e.g., FGF-2, IGFBP-3, CCL1, CCL19, and CCL21) containing CRS motifs have been shown to be involved in the transport of immune and cancer cells from the interstitial space into lymphatic vessel lumens (termed lymphatic entry) (Alvarez, Vollmann, & von Andrian, 2008; Cao, 2005; Cao et al., 2012; Das et al., 2013; Huang et al., 2003; Johnson & Jackson, 2014; Lund, Medler, Leachman, & Coussens, 2016; Roberti et al., 2012; Stacker et al., 2001; Yan et al., 2016). Since lymphatic entry of antigen-presenting cells is known to be a rate-limiting step in adaptive immunity (Teoh, Johnson, Hanke, McMichael, & Jackson, 2009), we hypothesize that CRSBP-1, which controls lymphatic entry of cells, plays an important role in adaptive immunity. In this communication, we show evolutionary evidence to support an important role of LYVE-1 (or CRSBP-1) in efficient adaptive immunity which involves lymph nodes. We also demonstrate that synthetic PDGF and VEGF peptides function as adjuvants and enhance adaptive immunity of commercial pseudorabies vaccine in pigs.

2 | MATERIALS AND METHODS

2.1 | Materials

Pseudorabies virus (PRV) vaccine (PRV/Marker Gold*) was purchased from Intervet/Schering-Plough Animal Health (Millsboro, DE). Chemical agents including 2,2'-azinodi-(3-ethyl benzothiazoline 6-sulfonate) were purchased from Sigma (St. Louis, MO). Horseradish peroxidase and goat anti-pig IgG were purchased from Jackson ImmunoResearch Inc. (West Grove, PA). PDGF peptide (a 19-mer peptide containing the amino acid sequence of YVRVRRPPKGKHKRKFKHTH), VEGF peptide (a 25-mer peptide containing the amino acid sequence of KKSVRGKGKGQKRKRKKSRYKSWSV) were synthesized by C. S. Bio Co. (Menlo Park, CA).

2.2 | Alignment of amino acid sequences of LYVE-1 orthologs in vertebrates

Elephant shark (*C. milii*) LYVE-1 cDNA and protein sequences were obtained from the NCBI GenBank database (XM_007909338). Multiple alignments of the amino acid sequence of *C. milii* LYVE-1 with those of other 13 vertebrate orthologs—human (NM_006691), chimpanzee (XM_004050702), dog (XM_003639783), cow (NM_205815), rat (NM_001106286), mouse (NM_053247), chicken (NM_001199587), bird (XM_017824964), frog (XM_002942269), coelacanth (XM_005989706), whale shark (XP_020377923), salmon (BT048800), and zebrafish (NM_001309475) were performed using the NCBI multiple alignment program. The 14 amino acid sequences of LYVE-1 were analyzed for their relatedness in a phylogenetic tree. The phylogenetic tree was conducted in MEGA7 (Kumar, Stecher, & Tamura, 2016) using the Neighbor-Joining method (Saitou & Nei, 1987). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances

were computed using the Poisson correction method (Zuckerkindl & Pauling, 1965) and were in the units of the number of amino acid substitutions per site.

2.3 | Effects of PDGF and VEGF peptides on the immunity of PRV vaccine in pigs

Female pigs (Yorkshire strain, 10 weeks old, house inbred and specific pathogen-free) were studied. Pigs were housed in separate rooms at a commercial farm, Maioli, Taiwan. The study was performed according to Taiwan Enforcement Rules of Animal Protection. In the experiment, 24 pigs were divided into four groups (six pigs per group). Pigs were vaccinated intramuscularly with 1 ml of normal saline (control), PRV/Marker Gold* (Manufacturer: Intervet/Schering-Plough Animal Health) alone, PRV/Marker Gold* + 200 µg PDGF peptide (PDGF), and PRV/Marker Gold* + 200 µg VEGF peptide (VEGF). All pigs in each group were challenged with a local virulent strain isolate (termed TNL) of PRV virus at week 4 post vaccination. The challenge dose was 10^5 TCID₅₀ in a 2 ml volume. Each animal was challenged with 1 ml of inoculum per nostril over a 30 s period. The titers of antibody to PRV in the sera of pigs were estimated using standard procedures according to the protocol of the manufacturer. All pigs without vaccination (control) died after challenge with virulent PRV virus. By contrast, all pigs vaccinated with and without PDGF peptide or VEGF peptide survived after challenge with virulent PRV virus. However, VEGF and PDGF peptides enhanced PRV vaccine immunity in pigs by increasing serum titers of antibody to PRV by 9- and 4-fold compared to those of pigs vaccinated in the absence of either peptides.

2.4 | ELISA for PRV-specific antibody titers in the sera of pigs vaccinated with PRV vaccine in the absence and presence of PDGF peptide or VEGF peptide

ELISA assay was performed in ninety-six-well u-bottom microtiter plates (Nunc) coated with 5 µg/well of PRV as described (Ho, Hsiang, Hsiang, & Chang, 1998). After washing, 50 µl of goat anti-pig IgG conjugated with horseradish peroxidase, diluted to 1:3000 in 0.05% non-fat milk, was added to each well for 1 hr at 37°C. After washing, 50 µl/well of the substrate (0.5 mg/ml 2,2'-azinodi-[3-ethyl benzo-thiazoline 6-sulfonate], 0.03% H₂O₂, 50 mM sodium citrate buffer) was added, and the optical density (OD) at 490 nm was measured in an ELISA reader after incubation at 37°C in the dark for 15 min. The standard deviation (SD) from duplicate was below 10% of the mean.

2.5 | Statistical analysis

Student's *t*-test was used for determining the significance of a difference between two sample means. The values were presented as mean ± SD. *p* < 0.05 was considered significant. Comparisons between two groups were performed using the Mann-Whitney test.

3 | RESULTS

3.1 | CRSBP-1 (mammalian LYVE-1) is distinct from zebrafish LYVE-1 in cell-type expression and lymphatic function

Our finding of subtle but identifiable morphological and functional alterations in lymphatic vessels in CRSBP-1^{-/-} mice led us to hypothesize that CRSBP-1 (mammalian LYVE-1) regulates lymphatic function (Huang et al., 2006). However, this caused controversy because these phenotypes were not identified by two other groups in LYVE-1^{-/-} mice created by

them (Gale et al., 2007; Luong et al., 2009). We decided to address this by three approaches: 1) Our CRSBP-1^{-/-} mice were donated to Jackson Laboratory, Bar Harbor, ME (Stock # 06221.B6. 129S1-X1kdl<tmJhua>/J) in 2006 made available to investigators who are interested in investigating the role of CRSBP-1 in lymphatic function (Huang et al., 2006); 2) We determined the molecular mechanism by which CRSBP-1 ligands (including PDGF-BB, VEGF-A, oligopeptides containing CRS motifs of PDGF-BB and VEGF-A, and HA) induce opening of lymphatic intercellular junctions in LEC monolayers and in lymphatic vessels in wild-type mice (Chen, 2009; Hou et al., 2011, 2012; Huang et al., 2006). The results are consistent with the hypothesis (Hou et al., 2011, 2012; Huang et al., 2006); and 3) We identified and cloned the authentic zebrafish ortholog (zLyve-1) of LYVE-1 and examined the phenotype of lymphatic vessels in zLyve-1 antisense morpholino-knockdown zebrafish (Chen et al., 2013). The cloned zLyve-1 comprises the Link module but lacks the acidic-amino-acid-rich (AAAR) domain. zLyve-1 expressed in cultured cells exhibits HA binding activity but lacks ¹²⁵I-labeled CRS motif-containing peptide binding activity (Chen et al., 2013) seen in mammalian homologs (Huang et al., 2003). This is consistent with the notion that the Link module and AAAR domain in mammalian LYVE-1 are responsible for mediating HA and CRS motif (present in a group of growth factors/cytokines) binding, respectively. Authentic zLyve-1 is specifically expressed in the posterior cardinal vein (PCV) that gives rise to precursors of LECs which form the thoracic duct (TD). Zebrafish with zLyve-1 knockdown exhibits defects in TD formation (Chen et al., 2013). These results suggest that zLyve-1 is required for TD formation and that zLyve-1 is distinct from mammalian LYVE-1 in cell-type expression (PCV cells and LECs, respectively) and lymphatic function.

3.2 | Elephant shark (*C. milii*) LYVE-1 has a primordial AAAR domain and possesses a basal adaptive immunity

The structural and functional differences between the LYVE-1 homologs from mammals and zebrafish prompted us to look into the origin of the Link module and AAAR domain of LYVE-1 in evolution and its association with the development of lymph nodes and efficient adaptive immunity. We hypothesize that CRS motif-containing growth factor/cytokine binding activity of LYVE-1 in mammals plays an important role in efficient adaptive immunity involving lymph nodes. The major lineages of jawed vertebrates include cartilaginous fish (elephant shark, coelacanth, and whale shark), bony fish (e.g., zebrafish and salmon), amphibian, reptile, bird, and mammals (mouse, rat, dog, cow, chimpanzee, human). Cartilaginous fish are the earliest extant vertebrate group with major components of the adaptive immune system. Elephant shark (*C. milii*), a holocephalan cartilaginous fish, is the most ancient vertebrate known to possess a basal adaptive immunity based on immunoglobulins, T-cell receptors and major histocompatibility complex molecules. The *C. milii* adaptive immune system shares features like those seen in mammals. However, the immunogenome of *C. milii* exhibits the presence of cytotoxic natural and CD8⁺ T-cells but lacks the canonical CD4 co-receptor, transcription factor RORC, and several cytokines/cytokine receptors associated with helper and regulatory functions of CD4⁺ T-cells in mammals (Venkatesh, Lee, Ravi, & Maurya, et al., 2014), suggesting the presence a primordial type of helper function. The adaptive immunity of *C. milii* is characterized by the presence of a full-blown cytotoxic system and a primordial helper system that leads to a

T_H1-type response through IFN- γ , IL12, and TNF- α . These unusual types of CD8⁺ T-cells may be capable of recognizing exogenous antigens presented by MHC class II molecules for producing antibodies.

Whole-genome analysis of *C. milii* has provided insight into the origin of adaptive immunity. We identified the cDNA nucleotide and protein amino acid sequences of *C. milii* LYVE-1 in the gene bank (XM_007909338). This enabled us to analyze the evolutionary and structure-function relationships of this LYVE-1 and orthologs derived from tetrapods (human, chimpanzee, dog, cow, mouse, rat, chicken, bird, and frog), cartilaginous fish (whale shark and coelacanth), and bony fish (salmon and zebrafish). Coelacanth is closer to cartilaginous fish than to any other bony fishes but some of its features are unlike any other known living creature (Amemiya, Alföldi, Lee, & Fan, et al., 2013). The cDNA sequence of *C. milii* LYVE-1 reveals that it encodes a type I membrane glycoprotein comprising a Link module and an AAAR domain (Figure 1), both of which exhibit homology to their counterparts in all mammalian and avian LYVE-1 analyzed (Figure 2). The Link module of *C. milii* LYVE-1 contains two disulfide bonds in the Link module and one disulfide bond in the N-terminal and C-terminal extensions of the Link module. This structural feature is present in all tetrapod LYVE-1 analyzed and in the homologs from other cartilaginous fish (coelacanth and whale shark) but not in those homologs derived from bony fish (zebrafish and salmon) (Figure 3). LYVE-1 from bony fish does not possess the disulfide bond in the N-terminal and C-terminal extensions of the Link module. However, the different disulfide features in the Link modules seen in evolution do not appear to affect the HA binding affinity of LYVE-1 because both zebrafish and mammalian LYVE-1 expressed in transfected cells bind HA (10³ kDa) with a half-maximal binding concentration of ~30 nM as determined by HA binding activity assay (Chen et al., 2013; Huang et al., 2003). This suggests that the HA binding activity of LYVE-1 is important but should not play a pivotal role in acquisition of innovation such as efficient adaptive immunity involving lymph nodes during evolution. The AAAR domain is present in the homologs derived from *C. milii*, mammals, chicken, and bird but not in those from coelacanth, whale shark, and bony fish (zebrafish and salmon) (Figure 4). However, *C. milii* LYVE-1 lacks the half-cystine residue (equivalent to the Cys-201 in human LYVE-1) next to the AAAR domain as seen in all homologs derived from mammals, chicken, and bird (Figure 2). The Cys-201 residue is responsible for homodimerization of LYVE-1 during its biosynthesis in cells in these organisms (Huang et al.2003). It is conserved in LYVE-1 from mammals, chicken and bird, all of which have the AAAR domain. Because of its proximity to the AAAR domain (with two amino acid residue separation) (Figure 2), we hypothesize that the intermolecular disulfide bond involving Cys-201 plays a role in the growth factor/cytokine (CRS motif) binding activity (which is mediated by the AAAR domain) rather than in the HA binding activity which is mediated by the distant Link module. This hypothesis is supported by the findings that disulfide-linked homodimeric bovine CRSBP-1 and monomeric zebrafish LYVE-1 exhibit similar HA binding activity with a half-maximal binding concentration of ~30 nM in transfected cells (Chen et al., 2013; Huang et al., 2003). We also hypothesize that the AAAR domain, which lacks the equivalent human Cys-201 residue, in *C. milii* LYVE-1 is a primordial AAAR domain as compared to those in LYVE-1 homologs from mammals, chicken, and bird (Figures 2 and 3).

3.3 | Development of the LYVE-1 gene with an AAAR domain in evolution is associated with acquisition of lymph nodes and adaptive immunity

Basic lymphatic systems are known to be present in teleosts. Evolutionary trajectory of lymphoid tissues is seen in vertebrates (Boehm, Hess, & Swann, 2012). Primary lymphoid tissues, such as bone marrow and thymus, have specialized stromal microenvironments that generate the primary repertoire B and T lymphocytes. Secondary lymphoid tissues, such as spleen and lymph nodes, specialize in the coordination of immune responses by spatially organizing the interaction of lymphocytes with each other and/or with antigen-presenting cells (Boehm & Swann, 2014; Boehm et al., 2012). Lymphoid tissues such as bone marrow, thymus, and spleen, are evolutionarily more ancient than lymph nodes. Lymph nodes represent the only major recent innovation of the adaptive immune systems in evolution particularly to mammals and bird (Boehm & Swann, 2014; Boehm et al., 2012). The evolutionary emergence of lymph nodes occurred 100 million years (Ma) ago, as do bird and mammals, whose LYVE-1 genes possess the AAAR domain (Figure 4). This suggests that the LYVE-1 gene acquires the AAAR domain (which confers the growth factor/cytokine binding activity) at evolution stages like mammals and bird (Figures 2 and 4). The development of the LYVE-1 gene with an AAAR domain in evolution is associated with lymph node development (Figure 4). In mammals, lymph nodes are well established as key elements in the adaptive immune system (van de Pavert & Mebius, 2010). This supports an important role of LYVE-1 in adaptive immunity in mammals.

3.4 | Synthetic CRSBP-1 ligands, PDGF, and VEGF peptides, function as adjuvants to enhance immunity of live PRV vaccine

Lymphatic entry of antigen-presenting cells is known to be an important rate-limiting step in primary adaptive immunity to viruses and tumor cells (Teoh et al., 2009). Adaptive immunity, which involves lymph nodes, may also increase prognosis of certain diseases (Isik & Firat, 2017; Isik, Idiz, & Firat, 2016). Ideal effective adjuvants should augment immunity to viruses and tumor cells at this important rate-limiting step. However, none of all known adjuvants, including alum, acts at this rate-limiting step. We identified a novel mechanism involving CRSBP-1 (LYVE-1) which controls the transport of cells and fluid from tissues to lymphatic capillary vessels (Hou et al., 2011, 2012; Huang et al., 2006). It may be possible to harness this mechanism to improve the effectiveness of vaccines. Accumulating evidence indicates that, during immune response, antigen-loaded dendritic cells enter the lymphatic vessels by releasing molecules that induce disruption of VE-cadherin trans dimer-mediated intercellular adhesion ("button") between lymphatic lining cells. These molecules are groups of CRS motif-containing growth factors (e.g., the PDGF superfamily) (Achen et al., 1998; Boensch et al., 1995, 1999; Houck et al., 1991; Joukov et al., 1996; LaRochelle et al., 1991; Ostman et al., 1991; Pollock & Richardson, 1992;) and cytokines (e.g., IGFBP-3, CCL1, CCL19, and CCL21) (Alvarez et al., 2008; Cao, 2005; Cao et al., 2012; Das et al., 2013; Huang et al., 2003; Johnson & Jackson, 2014; Lund et al., 2016; Roberti et al., 2012; Stacker et al., 2001; Yan et al., 2016) which bind to CRSBP-1 (LYVE-1) localized to the surface of lymphatic vessels. This stimulates a response, and acts like a token to gain entry to the lymphatic vessel network. We designed specific CRSBP-1 ligands: synthetic PDGF peptide and VEGF peptide which contain the CRS motifs (YVRVRRPPKKGKHKRKFH and KKSVRGKGKGQKRKRKKSRYKSWSV) of PDGF-BB and VEGF-A, respectively. PDGF

and VEGF peptides do not interact with either PDGF β -type receptor (PDGF β R) or VEGF receptor-2 (VEGFR2) (Boensch et al., 1995, 1999; Chen, 2009; Hou et al., 2011, 2012; Huang et al., 2006) and stimulate interstitial-lymphatic transport in wild-type mice but not in CRSBP-1^{-/-} mice, as determined by FITC-dextran tail dermal injection (in the absence of increased interstitial fluid pressure) (Huang et al., 2006) and λ -carrageenan-induced edema (in the presence of increased interstitial fluid pressure) assays (Hou et al., 2011). These oligopeptides mimic their parent growth factors and are capable of disrupting (unbuttoning) the VE-cadherin-mediated adhesion (“button”) at lymphatic intercellular junctions (Hou et al., 2011, 2012), resulting in wide opening of the junctions and allowing antigen-presenting cells to enter lymphatic vessels. We hypothesize that the synthetic specific CRSBP-1 ligands (PDGF and VEGF peptides) we designed should function as adjuvants and potentiate efficacy of commercially available vaccines which may contain alum as the adjuvant. This is because our peptides and alum augment immune responses by distinct mechanisms. To test this hypothesis, we determined the effects of PDGF and VEGF peptides on the efficacy of a commercially available PRV vaccine (PRV/Marker Gold*; manufacturer: Intervet/Schering-Plough Animal Health) in pigs. PRV/Marker Gold* vaccine is a genetically designed, live virus vaccine for the immunization of healthy pigs to prevent the disease caused by PRV (Aujeszky’s disease) (Klupp, Hengartner, Mettenleiter, & Enquist, 2004). In the experiment 24, 10-week-old pigs were divided into four groups (six pigs per group). Pigs were vaccinated intramuscularly with 1 ml of PRV/Marker Gold* alone, PRV/Marker Gold* alone $\pm$ 200 μ g PDGF peptide (PDGF) or VEGF peptide (VEGF) or normal saline (control). All pigs in each group were challenged with a virulent strain (termed TNL) of PRV virus (Ho et al., 1998) at day 28 (week 4) post vaccination. The challenge dosage was 10⁵ TCID₅₀ (50% tissue culture infective dose) in a 2 ml volume. The titers of the antibody to PRV virus in the sera of pigs were estimated using standard procedures according to the protocol of the manufacturer. As shown in Figure 5, pigs vaccinated with the vaccine containing PDGF peptide or VEGF peptide significantly increased the serum antibody titer by 4- and 9-fold, respectively, at day 14 (week 2) post vaccination and two weeks after challenge with wild-type virulent virus. It is important to note that the abilities (B > A) of PDGF peptide (A) and VEGF peptide (B) to potentiate immunity of PRV vaccine correlate with those of these two peptides to induce opening of lymphatic intercellular junctions (Hou et al., 2012; Huang et al., 2006). This support the notion that these peptides enhance adaptive immunity of the vaccine by attenuating lymphatic barrier during lymphatic entry of immune cells. Interestingly, both peptides potentiate the vaccine immunity even after challenge with virulent PRV. This indicates that the effects of these peptides are still in the memory of immune cells at least for 8 weeks after vaccination. Both peptides also protect the animal from the mortality caused by challenge with virulent PRV (Figure 5).

4 | DISCUSSION

Clustered basic and acidic amino acid residues in the Link module and AAAR domain, respectively, of mammalian LYVE-1 are presumably responsible for electrostatically interacting with target ligands acidic HA and basic CRS motifs in growth factor/cytokine molecules, respectively. This raises an important question: which interactions of LYVE-1 with HA or growth factors/cytokines containing CRS motifs play an important role in

inducing opening of lymphatic capillary intercellular junctions, resulting in lymphatic entry of immune cells? Several lines of evidence suggest that growth factors/cytokines containing CRS motifs (e.g., the PDGF superfamily, IGFBP-3, CCL19, CCL21), but not native HA or other forms of HA, are likely involved in lymphatic entry of antigen-presenting cells and naive T lymphocytes under physiological conditions. These include: 1) Native HA (molecular weight 10^3 kDa) has a random coil structure in solution (Weigel & Baggenstoss, 2017). Some HA receptors (e.g., LYVE-1, CD44, and HARE) initiate signaling responses upon binding HA in the 10–300 kDa range, but not larger 10^3 kDa HA (Weigel & Baggenstoss, 2017). The apparent K_d s of native HA and growth factors/cytokines for binding to mammalian LYVE-1 in cells are estimated to be ~ 30 nM and 0.5–0.7 nM, respectively, as determined by HA binding assay and I^{125} -labeled PDGF peptide or I^{125} -labeled VEGF peptide binding assay, respectively (Boensch et al., 1995; Chen et al., 2013; Huang et al., 2003) (Table 1). The interstitial fluid concentrations of HA in normal humans and lymphedema patients are ~ 2 nM and ~ 22 nM, respectively (Liu & Zhang, 1998), whereas the functional concentrations of PDGF-BB and VEGF-A in tissues are in the ranges of ~ 1 nM (Boensch et al., 1995). This suggests that growth factors/cytokines containing CRS motifs are likely more functional than native HA in inducing opening of lymphatic intercellular junctions under physiological conditions; 2) VEGF-A and VEGF peptide (an oligopeptide containing the CRS motif of VEGF-A) induce opening of lymphatic intercellular junctions more effectively than HA, given the optimal effective concentrations and magnitudes, as determined by in vitro and in vivo assays (Hou et al., 2011, 2012; Huang et al., 2006); and 3) Lymph nodes represent the only major recent innovation of the adaptive immune systems in evolution particularly to mammals and bird (Boehm & Swann, 2014; Boehm et al., 2012). Here we demonstrate that the development of LYVE-1 gene with an AAAR domain in evolution is associated with acquisition of lymph nodes and efficient adaptive immunity. This suggests that both lymph nodes and the AAAR domain of CRSBP-1, which confers its binding activity of growth factors/cytokines containing CRS motifs, are required to maintain efficient adaptive immune systems. These support the notion that the growth factor/cytokine-binding activity, rather than the apparent HA-binding activity mediated by the Link module, of mammalian LYVE-1 plays an important role in the development of efficient adaptive immunity.

Lymphotropic group A streptococci have been shown to spread to draining lymph nodes by capsular HA-mediated docking to the Link module of mammalian LYVE-1 (CRSBP-1) on lymphatic vessels (Lynskey et al., 2015). Dendritic cells and activated T lymphocytes have been recently shown to enter lymphatic vessels by cell-surface HA-mediated docking to the Link module of LYVE-1 on the basolateral surface of lymphatic vessels (Johnson et al., 2017). However, the roles of HA and/ or the Link module of CRSBP-1 in adaptive immunity is currently not clear. The possibility of the involvement of endogenous CRSBP-1 ligands (e.g., CCL21) in inducing opening of lymphatic intercellular junctions after docking to LYVE-1, resulting in lymphatic entry of dendritic cells, cannot be excluded. The ability of HA to induce opening of lymphatic intercellular junctions is much weaker than those of CRSBP-1 ligands (growth factors/cytokines) to induce such opening of lymphatic intercellular junctions (Hou et al., 2011; Huang et al., 2003). HA-mediated docking to the Link module/subsequent lymphatic entry should be much less efficient than the direct

lymphatic entry mediated by the AAAR domain because the binding affinity of HA to the Link module is much lower than those of growth factors/cytokines containing CRS motifs to the AAAR domain ($K_d \sim 30$ nM vs $K_d \sim 1$ nM). The interstitial concentration of HA in normal humans is 2 nM (Liu & Zhang, 1998). In addition, naive T lymphocytes do not express HA at the cell surface (Johnson et al., 2017). The HA-mediated docking mechanism should not be capable of delivering naive T lymphocytes to lymph nodes. The roles of the AAAR domain and Link module of mammalian LYVE-1 (CRSBP-1) in lymphatic trafficking and metastasis are summarized in Table 1.

It is possible that HA can be converted to low-molecular-weight (LMW) hyaluronic acid (LMW-HA) by fragmentation to become more effective to induce opening of lymphatic intercellular junctions. For example, very small HA (e.g., <10 kDa) has a rod-like structure (Weigel & Baggenstoss, 2017). This possibility is supported by a recent finding that LMW-HA (<10 kDa) but not high-molecular-weight HA (HMW-HA) ($1-2 \times 10^3$ kDa), promotes melanoma cell lymphatic metastases which can be blocked by the presence of an anti-LYVE-1 antibody (Du et al., 2016). This suggests that LMW-HA, but not HMW-HA, is efficient in inducing opening of lymphatic intercellular junctions, allowing lymphatic metastasis of melanoma cells. In addition, HMW-HA may function as a negative regulator for the opening of lymphatic intercellular junction induced by growth factors/cytokines containing CRS motifs. We previously showed that combination of PDGF-BB and HA abolishes the activity of either one in inducing opening of lymphatic intercellular junctions, as determined by tail dermal injection with high-molecular-weight FITC-dextran (Huang et al., 2006). HA does not appear to affect PDGF-BB binding to CRSBP-1 (Huang et al., 2006). We hypothesize that high interstitial concentrations of HA may contribute to the phenotype of lymphedema by blocking the potential attenuating effects of endogenous growth factors/cytokines containing CRS motifs such as PDGF-BB, VEGF-A, IGFBP-3, CCL1, CCL19, CCL21 (Alvarez et al., 2008; Das et al., 2013; Hou et al., 2011; Huang et al., 2003, 2006; Johnson and Jackson, 2014; Lund et al., 2016; Roberti et al., 2012; Yan et al., 2016).

CRSBP-1 ligands containing CRS motifs (including VEGF-A, PDGF-BB, VEGF, and PDGF peptides), which target the AAAR domain of CRSBP-1, have been shown to induce disruption of VE-cadherin-mediated lymphatic intercellular adhesion and opening of lymphatic intercellular junctions in LEC monolayers and in lymphatic vessels (Chen, 2009; Hou et al., 2011, 2012). This occurs by interaction with CRSBP-1 in the CRSBP-1-PDGF β R- β -catenin complex in the plasma membrane of target lymphatic capillaries, resulting in tyrosine phosphorylation of the complex, dissociation of β -catenin and p120-catenin from VE-cadherin (which trans-dimerizes between cells), and subsequent internalization of VE-cadherin, leading to disruption of VE-cadherin-mediated intercellular adhesion (VE-cadherin intercellular transdimerization), opening of lymphatic intercellular junctions and increased interstitial-lymphatic transport (lymphatic entry) of immune cells (which are present in the interstitial space near lymphatic vessels and presumed to secrete CRSBP-1 ligands) in animals (Hou et al., 2011, 2012). We hypothesize that other CRS-containing cytokines such as CCL19 and CCL21 also utilize the same mechanism involving membrane CRSBP-1-PDGF β R complexes (Hou et al., 2011) to facilitate homing of T lymphocytes and antigen-presenting dendritic cells (DCs) to the lymph nodes. CCL19 and

CCL21 function as chemokines to attract monocytes, NK cells, and immature B cells and dendritic cells to lymphatic vessels by interacting with the cell-surface chemokine receptor (CCR7) (Förster, Davalos-Misslitz, & Rot, 2008). They also promote lymphatic entry of these cells (Gunn et al., 1999; Robbiani et al., 2000), by inducing opening lymphatic intercellular junctions through interaction with CRSBP-1 in the CRSBP-1-PDGFβR-β complex on the plasma membrane of target LECs (in lymphatic vessels) via their C-terminal CRS motifs (⁸⁵RLQRTSAKMKRR⁹⁶ and ¹¹¹KTQKKQKQSKGCKR¹²⁴, respectively).

Lymphatic metastasis is an important mechanism in the spread of human cancer. It contributes to mortality from solid tumors. Cancer cells enter the lymphatic capillaries by active movement, are carried in the lymphatic fluid to lymph nodes and then metastasize to other tissues. How cancer cells migrate from the interstitial space into the lymphatic vessel lumen, which have collapsed irregular shapes (closed configuration) in the resting state (in the absence of increased interstitial fluid pressure), is poorly understood. Two lines of evidence support the notion that the mechanisms involving CRSBP-1 by which immune cells enter lymphatic capillary vessels during adaptive immune responses are utilized by cancer cells to enter lymphatic vessels during metastasis. These include: 1) The interstitial-lymphatic transport (at the initial lymphatics) of immune and cancer cells has been shown to require physical contact between these cells and lymphatic vessels (Baluk et al., 2007). These cells are also known to produce CRSBP-1 ligands, such as members of the PDGF superfamily and other CRS-containing cytokines (e.g., IGFBP-3, CCL1, CCL19, and CCL21). The expression levels of PDGF-BB in cancer cells are correlated with their tendency to produce lymphatic metastasis (Cao, 2005; Cao et al., 2004). VEGF-C produced by cancer cells promotes cancer cell invasion by increasing lymphatic metastasis (Karaman & Detmar, 2014); and 2) We recently demonstrated that paclitaxel (Taxol), a microtubule-stabilizing agent, is a CRSBP-1 downstream inhibitor and is capable of inhibiting CRSBP-1 ligand-induced contraction of LECs and opening of lymphatic intercellular junctions in LEC monolayers (Hou et al., 2011, 2012). Paclitaxel is known to block lymphatic metastasis in lung cancer in mice (Liu, Meisner, Kwong, Wu, & Johnston, 2009). We reason that the mechanism involving CRSBP-1 by which immune cells enter lymphatic vessels may be hijacked by cancer cells in evolution for lymphatic entry during lymphatic metastasis (Table 1). This possibility is supported by the findings that CRSBP-1 antagonists (e.g., suramin) (Boensch et al., 1995, 1999) and CRSBP-1 downstream inhibitors including PDGFβR kinase inhibitors (which block signaling mediated by CRSBP-1-PDGFβR heterocomplexes stimulated by CRSBP-1 ligands) (Hou et al., 2011) and paclitaxel (Hou et al., 2012) have been shown to prevent and attenuate cancer metastasis in animal models (Kuwai et al., 2008; Liu et al., 2009; Singla, Bondareva, & Jirik, 2014).

ACKNOWLEDGMENTS

We thank Yao-Hong Wang, PhD, Yuanpei University, Hsinchu, Taiwan, for performing pig experiments. We also thank Robert M. Senior, MD, Professor, Washington University School of Medicine, St. Louis, Missouri, USA, for critically reviewing the manuscript. This work was supported by NIH grants HL 095261 and AR052578 to Auxagen (S.S.H.). This work was also supported by the President's Research Fund, Saint Louis University (J.S.H.).

Funding information

NIH, Grant numbers: HL 095261, AR052578; President's Research Fund

REFERENCES

- Achen MG, Jeltsch M, Kukk E, Makinen T, Vitali A, Wilks AF, ... Stacker SA (1998). Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). *Proceedings of the National Academy of Sciences of the United States of America*, 95, 548–553. [PubMed: 9435229]
- Alvarez D, Vollmann EH, & von Andrian UH (2008). Mechanisms and consequences of dendritic cell migration. *Immunity*, 29, 325–342. [PubMed: 18799141]
- Amemiya CT, Alföldi J, Lee AP, Fan S, Philippe H, Maccallum I, ... Lindblad-Toh K (2013). The African coelacanth genome provides insights into tetrapod evolution. *Nature*, 496, 311–316. [PubMed: 23598338]
- Baluk P, Fuxe J, Hashizume H, Romano T, Lashnits E, Butz S, ... McDonald DM (2007). Functionally specialized junctions between endothelial cells of lymphatic vessels. *The Journal of Experimental Medicine*, 204, 2349–2362. [PubMed: 17846148]
- Banerji S, Ni J, Wang SX, Clasper S, Su J, Tammi R, ... Jackson DG (1999). LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. *The Journal of Cell Biology*, 144, 789–801. [PubMed: 10037799]
- Banerji S, Hide BR, James JR, Noble ME, & Jackson DG (2010). Distinctive properties of the hyaluronan-binding domain in the lymphatic endothelial receptor Lyve-1 and their implications for receptor function. *The Journal of Biological Chemistry*, 285, 10724–10735. [PubMed: 19887450]
- Boehm T, & Swann JB (2014). Origin and evolution of adaptive immunity. *Annual Review of Animal Biosciences*, 2, 259–283. [PubMed: 25384143]
- Boehm T, Hess I, & Swann JB (2012). Evolution of lymphoid tissues. *Trends in Immunology*, 33, 315–321. [PubMed: 22483556]
- Boensch C, Kuo MD, Connolly DT, Huang SS, & Huang JS (1995). Identification, purification, and characterization of cell-surface retention sequence-binding proteins from human SK-Hep cells and bovine liver plasma membranes. *The Journal of Biological Chemistry*, 270, 1807–1816. [PubMed: 7829517]
- Boensch C, Huang SS, Connolly DT, & Huang JS (1999). Cell surface retention sequence binding protein-1 interacts with the v-sis gene product and platelet-derived growth factor β -type receptor in simian sarcoma virus-transformed cells. *The Journal of Biological Chemistry*, 274, 10582–10589. [PubMed: 10187853]
- Cao R, Björndahl MA, Religa P, Clasper S, Garvin S, Galter D, ... Cao Y (2004). PDGF-BB induces intratumoral lymphangiogenesis and promotes lymphatic metastasis. *Cancer Cell*, 6, 333–345. [PubMed: 15488757]
- Cao R, Ji H, Feng N, Zhang Y, Yang X, Andersson P, ... Cao Y (2012). Collaborative interplay between FGF-2 and VEGF-C promotes lymphangiogenesis and metastasis. *Proceedings of the National Academy of Sciences of the United States of America*, 109, 15894–15899. [PubMed: 22967508]
- Cao Y (2005). Direct role of PDGF-BB in lymphangiogenesis and lymphatic metastasis. *Cell Cycle*, 4, 228–230. [PubMed: 15655347]
- Chen W-H, Tseng W-F, Lin G-H, Schreiner A, Chen H-R, Voigt MM, ... Huang JS (2013). The LYVE-1 ortholog is required for thoracic duct formation in zebrafish. *Cellbio*, 2, 228–247.
- Chen H-R (2009). Role of CRSBP-1 in regulation of cell migration in lymphatic endothelial cells and its orthologue in zebrafish Master thesis (Thesis Advisor: Huang Jung S., PhD), Institute of Systems Biology and Bioinformatics, National Central University, Zhongli, Taiwan.
- Das S, Sarrou E, Podgrabinska S, Cassella M, Mungamuri SK, Feirt N, ... Skobe M (2013). Tumor cell entry into the lymph node is controlled by CCL1 chemokine expressed by lymph node lymphatic sinuses. *The Journal of Experimental Medicine*, 210, 1509–1528. [PubMed: 23878309]
- Du Y, Cao M, Liu Y, He Y, Yang C, Wu M, ... Gao F (2016). Low-molecular-weight hyaluronan (LMW-HA) accelerates lymph node metastasis of melanoma cells by inducing disruption of lymphatic intercellular adhesion. *Oncoimmunology*, 5, 1232235.
- Förster R, Davalos-Misslitz AC, & Rot A (2008). CCR7 and its ligands: Balancing immunity and tolerance. *Nature Reviews. Immunology*, 8, 362–371.

- Gale NW, Prevo R, Espinosa J, Ferguson DJ, Dominguez MG, Yancopoulos GD, ... Jackson DG (2007). Normal lymphatic development and function in mice deficient for the lymphatic hyaluronan receptor LYVE-1. *Molecular and Cellular Biology*, 27, 595–604. [PubMed: 17101772]
- Gunn MD, Kyuwa S, Tam C, Kakiuchi T, Matsuzawa A, Williams LT, & Nakano H (1999). Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization. *The Journal of Experimental Medicine*, 189, 451–460. [PubMed: 9927507]
- Hirose Y, Saijou E, Sugano Y, Takeshita F, Nishimura S, Nonaka H, ... Miyajima A (2012). Inhibition of Stabilin-2 elevates circulating hyaluronic acid levels and prevents tumor metastasis. *Proceedings of the National Academy of Sciences of the United States of America*, 109, 4263–4268. [PubMed: 22371575]
- Ho T-Y, Hsiang C-Y, Hsiang C-H, & Chang T-J (1998). DNA vaccination induces a long-term antibody response and protective immunity against pseudorabies virus in mice. *Archives of Virology*, 143, 115–125. [PubMed: 9505970]
- Hou W-H, I-H L, Tsai CC, Johnson FE, Huang SS, & Huang JS (2011). CRSBP-1/LYVE-1 ligands induce disruption of lymphatic intercellular adhesion by inducing tyrosine phosphorylation and internalization of VE-cadherin. *Journal of Cell Science*, 124, 1231–1244. [PubMed: 21444752]
- Hou WH, Liu IH, Huang SS, & Huang JS (2012). CRSBP-1/LYVE-1 ligands stimulate contraction of the CRSBP-1-associated ER network in lymphatic endothelial cells. *FEBS Letters*, 586, 1480–1487. [PubMed: 22673514]
- Houck KA, Ferrara N, Winer J, Cachianes G, Li B, & Leung DW (1991). The vascular endothelial growth factor family: Identification of a fourth molecular species and characterization of alternative splicing of RNA. *Molecular Endocrinology*, 5, 1806–1814. [PubMed: 1791831]
- Huang SS, Tang FM, Huang YH, Liu I-H, Hsu SC, Chen ST, ... Huang JS (2003). Cloning, expression, characterization, and role in autocrine cell growth of cell surface retention sequence binding protein-1. *The Journal of Biological Chemistry*, 278, 43855–43869. [PubMed: 12912978]
- Huang SS, Liu I-H, Smith T, Shah MR, Johnson FE, & Huang JS (2006). CRSBP-1/LYVE-1 null mice exhibit identifiable morphological and functional alterations of lymphatic capillary vessels. *FEBS Letters*, 580, 6259–6268. [PubMed: 17070806]
- Isik A, & Firat D (2017). Bilateral intra-areolar polythelia. *The Breast Journal*, 7. <https://doi.org/10.1111/tbj.12838>.
- Isik A, Idiz O, & Firat D (2016). Novel approaches in pilonidal sinus treatment. *Prague Medical Report*, 117, 145–152.
- Jackson DG (2004). Biology of the lymphatic marker LYVE-1 and applications in research into lymphatic trafficking and lymphangiogenesis. *APMIS*, 112, 526–538. [PubMed: 15563314]
- Johnson LA, & Jackson DG (2014). Control of dendritic cell trafficking in lymphatics by chemokines. *Angiogenesis*, 17, 335–345. [PubMed: 24232855]
- Johnson LA, Banerji S, Lawrance W, Gileadi U, Prota G, Holder KA, ... Jackson DG (2017). Dendritic cells enter lymph vessels by hyaluronan-mediated docking to the endothelial receptor LYVE-1. *Nature Immunology*, 18, 762–770. [PubMed: 28504698]
- Joukov V, Pajusola K, Kaipainen A, Chilov D, Lahtinen I, Kukk E, ... Alitalo K (1996). A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. *EMBO Journal*, 15, 290–298. [PubMed: 8617204]
- Karaman S, & Detmar M (2014). Mechanisms of lymphatic metastasis. *The Journal of Clinical Investigation*, 124, 922–928. [PubMed: 24590277]
- Klupp BG, Hengartner CJ, Mettenleiter TC, & Enquist LW (2004). Complete, annotated sequence of the pseudorabies virus genome. *Journal of Virology*, 78, 424–440. [PubMed: 14671123]
- Kumar S, Stecher G, & Tamura K. (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, 33, 1870–1874. [PubMed: 27004904]
- Kuwai T, Nakamura T, Sasaki T, Kitadai Y, Kim JS, Langley RR, ... Fidler IJ (2008). Targeting the EGFR, VEGFR, and PDGFR on colon cancer cells and stromal cells is required for therapy. *Clinical and Experimental Metastasis*, 25, 477–489. [PubMed: 18324358]

- LaRochelle WJ, May-Siroff M, Robbins KC, & Aaronson SA (1991). A novel mechanism regulating growth factor association with the cell surface: Identification of a PDGF retention domain. *Genes & Development*, 5, 1191–1199. [PubMed: 2065974]
- Liu NF, & Zhang LR (1998). Changes of tissue fluid hyaluronan (hyaluronic acid) in peripheral lymphedema. *Lymphology*, 31, 173–179. [PubMed: 9949388]
- Liu J, Meisner D, Kwong E, Wu XY, & Johnston MR (2009). Translymphatic chemotherapy by intrapleural placement of gelatin sponge containing biodegradable paclitaxel colloids controls lymphatic metastasis in lung cancer. *Cancer Research*, 69, 1174–1181. [PubMed: 19176391]
- Lund AW, Medler TR, Leachman SA, & Coussens LM (2016). Lymphatic vessels, inflammation, and immunity in skin cancer. *Cancer Discovery*, 6, 22–35. [PubMed: 26552413]
- Luong MX, Tam J, Lin Q, Hagendoorn J, Moore KJ, Padera TP, ... Jain RK (2009). Lack of lymphatic vessel phenotype in LYVE-1/CD44 double knockout mice. *Journal of Cellular Physiology*, 219, 430–437. [PubMed: 19170073]
- Lynskey NN, Banerji S, Johnson LA, Holder KA, Reglinski M, Wing PA, ... Sriskandan S (2015). Rapid lymphatic dissemination of encapsulated group A streptococci via lymphatic vessel endothelial receptor-1 interaction. *PLOS Pathogens*, 11, e1005137. [PubMed: 26352587]
- Ostman A, Andersson M, Betsholtz C, Westermarck B, & Heldin CH (1991). Identification of a cell retention signal in the B-chain of platelet-derived growth factor and in the long splice version of the A-chain. *Cell Regulation*, 2, 503–512. [PubMed: 1782212]
- Pollock RA, & Richardson WD (1992). The alternative-splice isoforms of the PDGF A-chain differ in their ability to associate with the extracellular matrix and to bind heparin in vitro. *Growth Factors*, 7, 267–277. [PubMed: 1306682]
- Robbiani DF, Finch RA, Jäger D, Muller WA, Sartorelli AC, & Randolph GJ (2000). The leukotriene C(4) transporter MRP1 regulates CCL19 (MIP-3 γ , ELC)-dependent mobilization of dendritic cells to lymph nodes. *Cell*, 103, 757–768. [PubMed: 11114332]
- Roberti MP, Arriaga JM, Bianchini M, Quintá HR, Bravo AI, Levy EM, ... Barrio MM (2012). Protein expression changes during human triple negative breast cancer cell line progression to lymph node metastasis in a xenografted model in nude mice. *Cancer Biology & Therapy*, 13, 1123–1140. [PubMed: 22825326]
- Saitou N, & Nei M (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, 4, 406–425. [PubMed: 3447015]
- Singla AK, Bondareva A, & Jirik FR (2014). Combined treatment with paclitaxel and suramin prevents the development of metastasis by inhibiting metastatic colonization of circulating tumor cells. *Clinical and Experimental Metastasis*, 31, 705–714. [PubMed: 24965002]
- Stacker SA, Caesar C, Baldwin ME, Thornton GE, Williams RA, Prevo R, ... Achen MG (2001). VEGF-D promotes the metastatic spread of tumor cells via the lymphatics. *Nature Medicine*, 7, 186–191.
- Teoh D, Johnson LA, Hanke T, McMichael AJ, & Jackson DG (2009). Blocking development of a CD8+ T cell response by targeting lymphatic recruitment of APC. *The Journal of Immunology*, 182, 2425–2431. [PubMed: 19201897]
- van de Pavert SA, & Mebius RE (2010). New insights into the development of lymphoid tissues. *Nature Reviews Immunology*, 10, 664–674.
- Venkatesh B, Lee AP, Ravi V, Maurya AK, Lian MM, Swann JB, ... Warren WC (2014). Elephant shark genome provides unique insights into gnathostome evolution. *Nature*, 505, 174–179. [PubMed: 24402279]
- Weigel PH, & Baggenstoss BA (2017). What is special about 200kDa hyaluronan that activates hyaluronan receptor signaling? *Glycobiology*, 27:868–877. [PubMed: 28486620]
- Yan YH, Yu F, Zeng C, Cao LH, Zhang Z, & Xu QA (2016). CCL17 combined with CCL19 as a nasal adjuvant enhances the immunogenicity of an anti-caries DNA vaccine in rodents. *Acta Pharmacologica Sinica*, 37, 1229–1236. [PubMed: 27546005]
- Zuckerkandl E, & Pauling L, (1965). Evolutionary divergence and convergence in proteins In Bryson V, & Vogel HJ, (Eds.), *Evolving genes and proteins* (pp. 97–166). New York: Academic Press.

ATGGAAAAGCATAGGATTCTCCCATCAATTCTTTTGAGCTGGAGTTTGCTGTTGTTGGTT
 M E K H R I L P S I L L S W S L L L L V 20
 CAGACTGATGAAGATCTTCTTGATGGCAGCTGTCGGATTTTGGGTGTGTTCCATATCGGT
 Q T D E D L L D G S © R I L G V F H I G 40
 ATCAATAACAGATACAATATGTCGCTGGAGGCAGCTCACGCCAACTGCCGATCACTCGGG
 I N N R Y N M S L E A A H A N © R S L G 60
 GTGAAATTGGCAACACAAGCTCAGGTTGAGGCTGCCAATCTGGCTGGATTTTCAGGTTTGC
 V K L A T Q A Q V E A A N L A G F Q V © 80
 AGATTCGGTTGGGTGGATGATGGATAACCCCGTAATATCAAGAGTCACTCCAATGATAAAT
 R F G W V D D G Y P V I S R V T P M I N 100
 TGTGAAATAACCAAAACAGGAGTGATAAAAATGATGGATATCAACCGTCTGTATGATGGC
 © G N N Q T G V I K M M D I N R L Y D G 120
 TTTTGCATCAGTATCAAAGATGCCCATAAAGGTGAATGCTTGTGAGCCACTACTGCAAATC
 F © I S I K D A H K V N A © E P L L Q I 140
 ACAGCCACAGGAATCAGTATGATCGAAACAGCGTCGCCAACTCTCCTGGATCTCACACAT
 T A T G I S M I E T A S P T L L D L T H 160
 GCGGAGACCGTATCGGTAAAGCCCTCTGAAACTACCCCTCAACTAGAGACTGAAACACAT
 A E T V S V K P S E T T P Q L E T E T H 180
 GGACTTCCAGGAGCAAATACCGAGCTGCCAATTTCCAGTGCAAACCCCGTACCTCGGCA
 G L P G A N T E L P I S S A N P S T S A 200
 ACAAAGCCCCTCATCTCAACTACAACATCCCTCATCTCAACCACAGAATTATTCTTAGCA
 T K P L I S T T T S L I S T T E L F L A 220
 GTGATGAACAACAAGACCAACCGTTTGGTGGTAATAGTTCTGACGGTGTTAGCTGCCATT
 V M N N K T N R L V V I V L T V L A A I 240
 TTGATAATAGTCACTGTGGTCATCATCGTTTGCCATTTGACAAGGAAAAGGTGGAACACC
 L I I V T V V I I V C H L T R K R W N T 260
 TTTGATTTTGAAGCTGTACCACCAAAAGAAATCGTAGAGATTGAAGAGTGGAACCATAAC
 F D F E A V P P K E I V E I E E W N H N 280
 TTGGTAGACAATGGGAACGATTAG
 L V D N G N D 287

FIGURE 1.

Nucleotide and deduced amino-acid sequences of elephant shark (*C. milii*) LYVE-1 cDNA. The *C. milii* LYVE-1 cDNA encodes a 287-amino-acid type I membrane glycoprotein containing a 15-amino-acid putative signal peptide, a 211-amino-acid cell-surface domain which includes a 79-amino-acid Link module and a 23-amino-acid transmembrane domain, and a 34-amino-acid cytoplasmic domain. Two potential N-linked glycosylation sites in the Link module are boxed. The half-cystine residues are marked by circles. The boundaries of

the exons of *C. milii* LYVE-1 cDNA are distinguished by the shaded and unshaded sequences

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

FIGURE 2.

Alignment of the amino acid sequence of *C. milli* LYVE-1 with those of other vertebrate homologs. The deduced amino acid sequence of *C. milli* LYVE-1 is aligned with those of other vertebrate homologs (human, chimpanzee, dog, cow, rat, mouse, chicken, bird, frog, coelacanth, whale shark, salmon, and zebrafish). The arrow indicates the identified N-terminal amino acid residue in human and cow LYVE-1. The Link module, which is present in all vertebrates, is over-lined in red. The amino acid residues (Tyr-87, Ile-97, Arg-99, Lys-105, and Lys108) in human LYVE-1 Link module, which have been identified to

interact with HA (Banerji, Hide, James, Noble, & Jackson, 2010), are boxed in red. The AAAR domain present in other vertebrate homologs is over-lined in green. The half-cystine residue (which is equivalent to Cys-201 in human LYVE-1) next to the AAAR domain is involved in forming the intermolecular disulfide bond (Huang et al., 2003). This disulfide bond is believed to play a role in the ligand (CRS motif) binding activity of the AAAR domain and is hypothesized to be an additional component of the AAAR domain. The *C. milli* LYVE-1 AAAR domain, which does not have such a half-cystine residue nearby, is a primordial domain. The homologous acidic amino acid residues (Glu and Asp) in the AAAR domains and half-cystine residues (which are equivalent to Cys-201 in human LYVE-1) next to the AAAR domain are boxed in green. The transmembrane domain is underlined. Identical half-cystine residues in all vertebrate LYVE-1 species are indicated by ▲. Half-cystine residues, which are only present in other vertebrate homologs, are indicated by △. Two potential N-linked glycosylation sites are marked by *. The bar indicates the absence of the corresponding amino acid residues in *C. milli* LYVE-1 and other vertebrate homologs

FIGURE 3.

Structural domain comparison of vertebrate LYVE-1 homologs. Human LYVE-1 (panel a) and bird LYVE-1 (panel b) possess a Link module with two disulfide bonds and one disulfide bond in the N-terminal and C-terminal extension of the Link module, and an AAAR domain with one half-cystine residue (equivalent to Cys-201 in human LYVE-1) nearby. It is believed to play a role in the ligand binding activity of the AAAR domain by forming the intermolecular disulfide bond. The disulfide-bond structural features seen in the Link modules of human and bird LYVE-1s are also present in the homologs from elephant

shark (panel c) coelacanth (panel d), whale shark (panel e), and other mammals (Figure 2) but not from salmon and zebrafish (panels f and g, respectively) which only contain two disulfide bonds in the Link module. This suggests that the Link modules in salmon and zebrafish LYVE-1 are primordial modules in evolution. *C. milii* LYVE-1 has an AAAR domain but without a half-cystine residue nearby (equivalent to Cys-201 in human LYVE-1). This AAAR domain of *C. milii* LYVE-1 is a primordial domain in evolution as compared to those in mammals and bird. The two N-linked glycosylation sites are localized at both sides of the Link module in all vertebrate LYVE-1 homologs

FIGURE 4. A phylogenetic tree of LYVE-1 orthologs, which possess Link modules and/or AAAR domains, from jawed vertebrates and the evolutionary emergence of lymph nodes in these vertebrates. The + and – represent the presence and absence of Link modules and AAAR domains in LYVE-1 orthologs and of lymph nodes in these vertebrates

FIGURE 5.

VEGF and PDGF peptides as novel adjuvants enhance immunity of pseudorabies virus vaccine. Pigs were vaccinated intramuscularly at day 0 with 1 ml of PRV/Marker Gold* alone, PRV/Marker Gold* alone $\pm$ 200 μ g PDGF peptide (PDGF) or VEGF peptide (VEGF) or normal saline (vaccine alone) and challenged with virulent wild-type virus (TNL strain) at day 28 (week 4) post vaccination as marked by an arrow. Pigs, which were not vaccinated, were used as control. *The titer of 512 (average number from six pigs) is the maximal limit of the assay and the exact titer should be higher. The survival rates of pigs were determined at week 8. All animals in the control group died after challenge with virulent virus. Animals that were vaccinated in the presence and absence of PDGF peptide or VEGF peptide all survived even after challenge with virulent virus

TABLE 1

Roles of the AAAR domain and Link module of mammalian LYVE-1 (CRSBP-1) in lymphatic trafficking and metastasis

	AAAR domain	Link module
Ligands	CRS-containing growth factors/cytokines/ oligopeptides (Kd~1 nM)	HA (MW: 1-2 × 10 ³ kDa) (Kd~30 nM)
Lymphatic trafficking	Lymphatic entry ^{a,b} (adaptive immunity)	Lymphatic docking ^{d,e} (bacterial dissemination to lymph nodes)
Lymphatic metastasis	Promotion ^c	Inhibition ^f

^aCCL19 and CCL21, which are putative ligands for CRSBP-1, are required for the direct lymphatic entry of both naive T lymphocytes and antigen-presenting dendritic cells (Gunn et al., 1999; Robbiani et al., 2000).

^bCRS-containing oligopeptides (PDGF and VEGF peptides) function as adjuvants to enhance adaptive immunity of vaccines as demonstrated in this work.

^cThe expression levels of PDGF-BB, a CRSBP-1 ligand, in tumors are correlated with their tendency to promote lymphatic metastasis (Cao, 2005; Cao et al., 2004).

^dEncapsulated group A streptococcus spreads to draining lymph nodes by capsular HA-mediated docking to LYVE-1 on lymphatic vessels (Lynskey et al., 2015).

^eDendritic cells and activated T lymphocytes enter lymphatic vessels by cell-surface HA-mediated docking to LYVE-1 on lymphatic vessels (Johnson et al., 2017). The role of the HA-mediated docking to lymphatic vessels of dendritic cells in adaptive immunity is currently not clear.

^fHA binding to the Link module inhibits the activity of PDGF-BB to induce opening of lymphatic intercellular junctions in mice as determined by FITC-dextran intradermal injection (Huang et al., 2006). Administration with high-dose HA inhibits metastasis of melanoma cells to the lung in syngeneic mice (Hirose et al., 2012)