IN VITRO MODELS OF LYMPHATIC ENDOTHELIAL CELLS: CYTOKINE RECEPTOR EXPRESSION PROFILING AND INCORPORATION INTO ORGANOTYPIC CULTURE MODELS

by

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ABSTRACT

Lymphatic endothelial cells (LECs) line the lymphatic vessels and lymph node sinuses. They function in balancing tissue interstitial fluid, trafficking of dendritic cells and lymphocyte movement into and out of lymph nodes, (lymph node-LECs), and can modulate self-tolerance. LECs also are important for viral pathogenesis and malignant cell migration. The discovery of LEC-specific markers has enabled the isolation and culture of LECs during the past decade, thereby increasing knowledge of LECs biology. Cytokines are small proteins secreted by cells that have pleiotropic effects on cell survival, growth, and functional activities. The expression of cytokine receptors on LECs, if present, could provide insight into the potential functions of LECs. The current study has defined the cytokine receptor expression profile, especially the IL6 family receptors, for three LEC populations: human dermal microvascular lymphatic endothelial cells (HMVEC-dLy), human lung lymphatic microvascular endothelial cells (HMVEC-LLy), and human telomerase reverse transcriptase transfected human dermal lymphatic endothelial cells (hTERT-HDLEC), either with or without viral infection mimicry through the use of poly I:C treatment. Cytokine receptor expression was examined at the RNA, protein and function levels, as a strong reference for further LECs study.

In vitro 3-dimensional (3D) cell culture is an important substitute for in vivo experiments. It is also a meaningful improvement to 2-dimensional cell culture through the modification of

culture environments to better mimic in vivo situations. Using an extracellular matrix extraction called Matrigel™ as a simplified 3D model substrate, we demonstrated that five kinds of LECs (HMVEC-dLy, HMVEC-LLy, hTERT-HDLEC, ferret lung isolated LECs and macaque jejunal LECs) can form a network-like structure on it (this process may be regarded as modeling the growth of lymphatic vessels or lymphangiogenesis). In addition, in this study we adapted an organotypic culture model containing lymphatic endothelial cells (LECs) to preliminary build a new powerful tool for in vitro study of LECs and also provide insights into the interactions between LECs and their environment.

The work represented by this thesis holds public health significances lying mainly in enriching the knowledge of human LECs, considering the importance of LECs and lymphatic biology in cancer development and immunology.

TABLE OF CONTENTS

LIST OF FIGURES

PREFACE

I would like to acknowledge my advisor Dr. Todd Reinhart for his patient guidance and professional direction not only on my research and study but also on my career development, my co-advisor Dr. Phalguni Gupta for his support, my thesis committee members Dr. Yue Chen and Dr. Frank Jenkins for their advice on this thesis, current and past Reinhart lab members Beth Juneckoa, Nicole Grant, and Dr. Stella Berendam for their kindly training and help on the lab techniques, Dr. Jayanth Venkatachari, Dr. Debjani Guha, Dr. Aki Hoji and Diana Campbell for their generous advises and help on my experiments and data analysis.

Also I would like to take this chance to thank my girlfriend, Shuangping Zheng and my parents Mrs. Bing Liu and Prof. Jun Yang for their emotional support during the past two years.

1.0 INTRODUCTION

1.1 LYMPHATIC ENDOTHELIAL CELLS (LECS): FUNCTIONS, AND RELATED CYTOKINE/CYTOKINE RECEPTOR STUDY

Lymphatic vessels are part of a second, non-circulatory, vascular system with immune functions. Lymphatic vessels absorb the fluid that leaks from blood vessels into peripheral tissues and thereby contributes to tissue homeostasis and fluid balance. They are also are intimately involved in transporting antigen and antigen presenting cells (APCs) to lymph nodes (LNs) to initiate adaptive immune responses [1]. Lymphatic vessels and LN sinuses are lined by lymphatic endothelial cells (LECs). LECs were once difficult to isolate and culture, mainly because of a lack of efficient LEC-specific surface markers. The situation has been changed since 2000, when the specific expression of LYVE-1, vascular endothelial cell growth factor (VEGF)-C receptor, VEGFR-3, Podoplanin (PDPN) was found in LECs, but not in blood vascular endothelial cells (BECs) [2-4]. These discoveries have facilitated the enrichment and study of LECs and lymphatics.

LECs function in the trafficking of dendritic cells (DCs) and lymphocytes into and out of lymph nodes (LNs) by expressing chemokines such as CCL21 and adhesion molecules such as ICAM-1. The LEC-related expression of CCL21 occurs in lymphatic vessels in multiple organs [5], which mediates the migration of antigen-loaded mature DCs from peripheral tissues to draining LNs via afferent lymphatic vessels [6]. The LECs lining the ceiling of the subcapsular

sinus are able to express chemokine receptor CCRL1 to scavenge CCL21 and maintain chemokine gradients across the sinus floor to enable the emigration of DCs [7]. After inflammatory cytokine treatment, human dermal LECs have increased expression of ICAM-1, VCAM-1, and E-selectin [8]. At the same time there also exists integrin-independent migration of DCs into LNs [9], which indicates the possible existence of other interactions between DCs and LECs such as the interaction between DC-expressed CLEC-2 and LEC-expressed podoplanin [10].

Lymphangiogenesis, which is the growth of new lymphatic vessels, is an important physiological activity of LECs. This process is extensive during embryonic or postnatal development. Lymphangiogenesis is also thought to be induced by most forms of human cancer cells leading to metastatic tumor spread through lymphatic vessels. In addition, lymphatic vessels are also observed to proliferate during inflammation [11]. The study of lymphangiogenesis can be very expansive and meaningful as it covers the field of cancer science, immunology and embryology, and the mechanisms of lymphangiogenesis have been studied for years. The most common view is that this process is mainly driven by the interaction between secreted vascular endothelial growth factors C and D (VEGF-C/D) and the cell-surface receptor VEGFR-3 on LECs [12]. Besides that, others have shown that podoplanin (a widely used LEC cell-surface marker) is necessary for lymphangiogenesis [13]. Interestingly, small interfering RNA (siRNA)-induced TLR3 activation can inhibit blood and lymphatic vessel growth [14]. Also, IL-8 promoted LECs' proliferation, tube formation, and migration without activating the VEGF signaling [15].

Cytokines are small proteins secreted by cells that have pleiotropic effects on cell survival, growth, and functional activities. The study of LEC-expressed cytokines and cellular receptors (including cytokine receptors) enrich the knowledge about LEC-associated activities especially about the immune potential of LEC, LECs that reside in LNs express MHC I molecules as well as

self-antigens expressed also in peripheral tissues but not co-stimulation molecules so they can work as tolerance-inducing antigen presenting cells (APC) to regulate T cells tolerance [16]. Besides expressing CCL family chemokines including CCL-1, 2, 5, 7, 8, 20, 21 and CXCL family chemokines CXCL-1, 3, 5, 6, 8, 9, 10, 11 to attract innate immune cells, LECs are also able to secrete different kinds of inflammatory cytokines including IL-1β, IL6, IL7, IL8, TGF-β, which directly take part in mediating immune response [17]. LECs also express several typical inflammatory factor receptors including IFNAR for IFN-α and IFNGR for IFN-γ [18] and toll-like receptors (TLRs) 1-6 and 9 [19], giving the cue that LECs have the ability not only to respond to the adaptive immune mediation signal created by other cells, but also to sense the environment and respond to infections diversely.

In this thesis, to systematically profile cytokine receptor expression by LECs, we used GeneCopoeia and Taqman based methods to perform a RNA-level scan of 84 and 14 cytokine receptors mRNAs, respectively, in untreated LECs or in a viral infection imitated environment (poly I:C treatment) in three LEC populations, human dermal microvascular lymphatic endothelial cells (HMVEC-dLy), human lung lymphatic microvascular endothelial cells (HMVEC-LLy) and human telomerase reverse transcriptase transfected human dermal lymphatic endothelial cells (hTERT-HDLEC). Then the expression of several IL6 family receptors were followed on protein level and functional level. The cytokine receptor profile study in LECs will provide a strong reference for further LECs study.

1.2 3-DIMENSIONAL TISSUE MODEL, AND 3-DIMENSIONAL CULTURE METHODS FOR LECS

Currently, cell based experiments are mainly performed at the 2-dimenssional (2D) level. Though 2D cell culture methods are convenient in their development, implementation and downstream analysis, the lack of cell-to-cell and cell-to-microenvironment interactions of 2D in vitro cell culture may set significant differences between the observations obtained from 2D-culture-based in vitro experiments and the observations obtained from in vivo experiments [20]. These differences may strongly limit the significance of observations obtained by 2D cell culture. It is thought that tissue homeostasis and the interaction among different cell types within tissues should be considered when studying the functions of individual cell types [21].

In response to the strong demand of in vivo data, and considering the high cost of in vivo study, numerous in vitro three-dimensional tissue models (or organotypic culture models, OCM) have been developed to mimic in vivo environments.

When talking about in vivo models, for LECs function, though many 3-dimensional (3D) models with LECs or ECs have been developed (most of them were developed for observing and studying EC angiogenesis), these models still have some limitations. Most models only considered the interaction between LECs/ECs with extracellular matrix (ECM). The most commonly used 3D culturing condition for LECs is plating LECs on the top of or inside Matrigel^{TM}, which is an extracellular matrix preparation isolated from the mouse Engelbreth-Holm-Swarm sarcoma [22]. Activated by Matrigel^{TM}, LECs can form a network-like structure [13, 15, 23, 24]. Though widely used, this kind of 3D model usually contains only a single cell type and the Matrigel^{TM} ECM component. Rather than considering this as a 3D tissue model, it is better to describe this method as LEC 3D culturing. Besides this, some people also believe that the "network" of LECs or ECs

on Matrigel™ is not lymphangiogenesis, as the networks do not have lumens and the networks are formed by cells realignment [22, 25]. Another limitation is that some models contain complex or unclear components. Again, taking Matrigel™ for example, as a protein mixture derived from a mouse tumor, there are more than a thousand different kinds of proteins and around 10,000 different peptides in it, the functions of most of them still not well understood [26]. Besides the Matrigel™ method, some other 3D models with LECs tend to involve in vitro culturing of tissue fragments, whose components are also very complex, such as the lymphatic ring assay which is achieved by embedding excised lymphatic duct fragments from mice into type I collagen gels [27]. The unclear components of either the cellular part or acellular parts of the 3D models may reduce the preciseness of the models as they are hard to repeat and can be hard to analyze. The last limitation of the models shown here is that some model building methods are very difficult. The fibrin bead assay is one example of the highly complex models. It involves embedding umbilical vein endothelial cell (HUVEC) coated Cytodex beads in fibrin gels, and then plating fibroblasts on top of the fibrin gels [22]. In this thesis we developed a simple, clearly composed 3D mucosal tissue model containing LECs, fibroblasts, and epithelial cells, as well as ECM collagens by modifying the method developed by our collaborators [21]. Primary analysis on the structure of the model and the survival of LECs in the model were performed. This the model could become a powerful tool for in vitro study of LEC function.

2.0 STATEMENT OF THE PROBLEM

The discovery of LEC-specific makers, including Prox-1, LYVE-1, VEGFR-3, and PDPN around the turn of the century [2-4], facilitated the efficient and reliable differentiation between LECs and blood endothelial cells (BECs). Since then, LEC research has been gradually increasing. This LEC-focused research has greatly increased our understanding of lymphatics. LECs are not only the construction unit of lymph vessels, but they also have important functions including but not restricted to trafficking of DCs and lymphocytes [7-10], mediating cancer metastasis [28-30], mediating inflammation [11, 17-19], and mediating self-tolerance [16, 31-33]. At the same time, much current research still uses LEC and BEC mixed endothelial cell (EC) populations as study models [34-37]. However, meaningful observations have been obtained through the usage of mixed EC populations. For example, it has been meaningful to use mixed EC populations in studies of cancer metastasis, because in the broad view cancer metastasis involves both blood and lymph vessels. With regard to the study of LECs, though, the mixed usage of EC populations may set barriers for the further acquisition and interpretation of studies of LEC cell-type-specific information**. The objectives of the study were to (1) determine the cytokine receptor expression profile of LECs under both mock treatment and viral infection imitated environments, and (2) develop 3D lung mucosal tissue models containing LECs.**

2.1 SPECIFIC AIM 1: DETERMINE LEC CYTOKINE RECEPTOR EXPRESSION PROFILE

Cytokines have a wide range of biological functions including regulating cellular survival, differentiation, proliferation, and anti-infectious or inflammatory activities [38]. The study of cytokine receptor expression profiles in LECs can give insights into LECs' immune functions and help to understand how LECs respond to the changes in their environment. Transcriptional scans of murine LECs isolated from inflamed and resting LNs have been performed previously [18], and although several studies have examined the interactions between cytokines and cytokine receptors in human LECs [17], the systematic study of human LEC cytokine receptor expression profiles is still lacking. In the study here, systematic transcriptional scanning was performed for two commonly used human LEC primary cell populations, HMVEC-dLy and HMVEC-LLy, as well as a long life-span human LEC cell line, hTERT-HDLEC. The cytokine receptor mRNA expression profiles of LECs were obtained under both mock and viral infection imitated, poly I:C treated, environments. In addition, several IL6 family cytokine receptors were studied further at the protein and functional levels.

2.2 SPECIFIC AIM 2: DEVELOP 3D CULTURE METHODS OF LECS

Three-dimensional (3D) culture of cells has become a trend in the field of cellular biology. Most 3D culture methods enable cell-cell and cell-environment interactions during culture [20], which mimics the real, in vivo situation for the cells under study and increases the value of the in vitro observations. Though a number of 3D culture models that include LECs or ECs have been

developed, they have disadvantages that include either over complication in their constructions (e.g., 3D culture following in vivo isolation of lymph vessels) or uncertainty in their components (e.g.., the matrix model component Matrigel™). In this thesis, a simple but efficient 3D culture method with LECs was developed that consisted of a 3D lung mucosal tissue model containing implanted LECs. It imitated the structure of lung mucosal tissue as well as supported the survival of LECs. With further modifications, the tissue model containing LECs can be further used for infection or cancer models. In addition, the most commonly used 3D culture method of LEC, culturing LECs on Matrigel™, was also performed in parallel in the thesis.

Altogether, the thesis will work as a good reference for further LEC study by providing the data of cytokine receptor expression profile in LECs and the method of an in vitro 3D tissue model containing LECs.

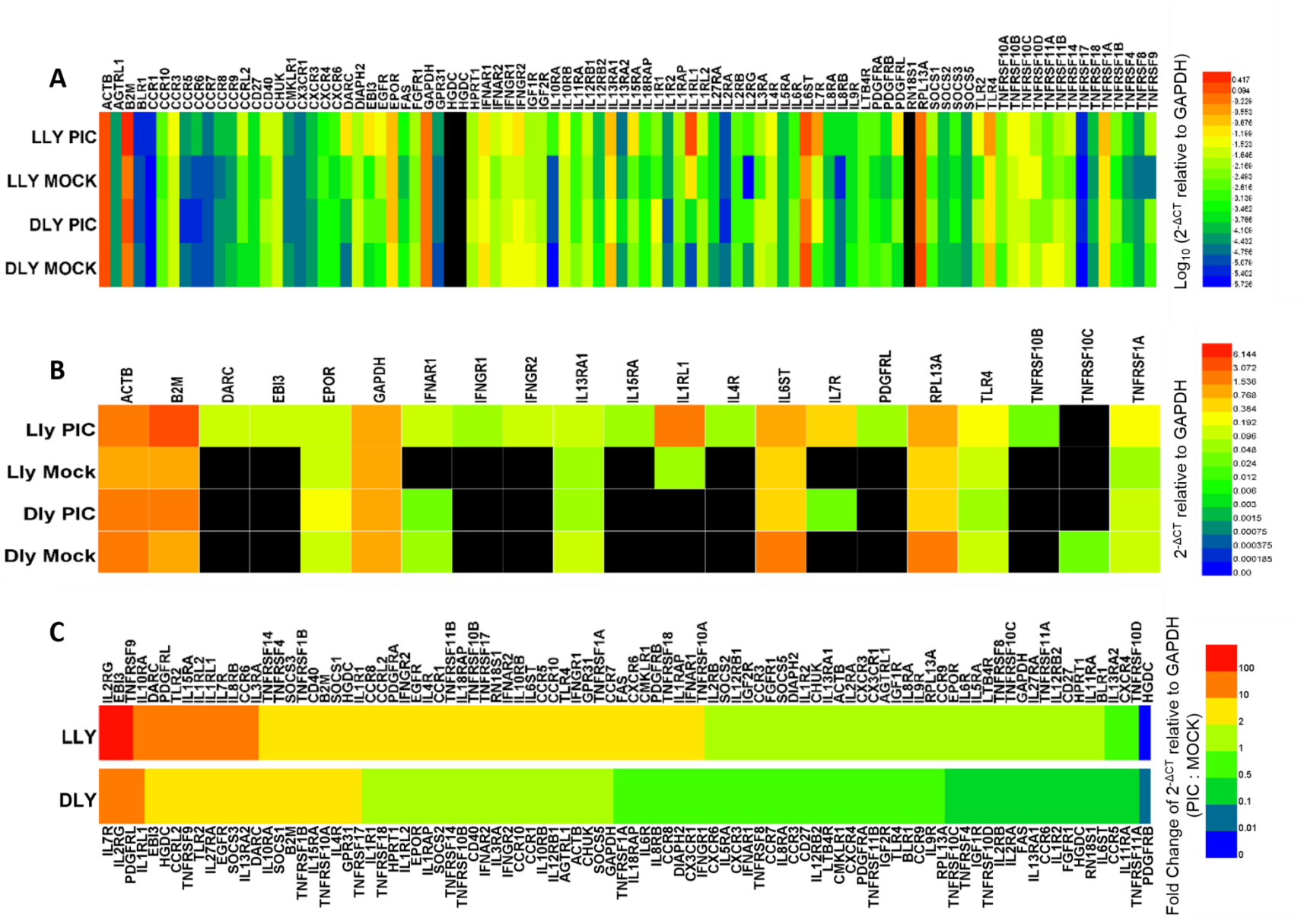
3.0 STATEMENT OF CONTRIBUTION

In the thesis, GeneCopoeia real-time RT-PCR array experiments and related $2^{-\Delta CT}$ values were performed and calculated, respectively, by Dr. Stella Berendam. The analysis, interpretation and presentation of GeneCopoeia data, and all the rest contents of thesis are the independent work of the thesis author, Fan Yang.

4.0 RESULTS

4.1 CYTOKINE RECEPTOR EXPRESSION PROFILES OF HUMAN LECS

To understand better the abilities of LECs to sense the local immune environment and cytokine milieu, we screened 2D cultured HMVEC-dLy and HMVEC-LLy cell populations with poly I:C (PIC) or without (MOCK) and examined the total RNAs from these cells for the expression of 84 cytokine receptor related genes using the ExProfile^{TM} Human Cytokine Receptor Related Gene qPCR Array (GeneCopoeia) (Figure 1). In the microarray, ATCB (β-actin), B2M (β-2-Microglobin), Ribosomal Protein L13a (RPL13A), Hypoxanthine Phosphoribosyl transferase 1 (HPRT1) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as endogenous controls. In addition, two HGDCs served as genomic DNA controls to detect genomic DNA contamination. In Figure 1A, all genes except for RN18S1 have higher $2^{-\Delta CT}$ values (relative to GAPDH) than HGDCs and could be regarded theoretically as having positive expression. According to that we conclude that at the RNA level LECs have a wide range of the expression of cytokine receptors though most of them are at a relatively low level (Figure 1A), and poly I:C can change the expression of several cytokine receptor related genes (Figure 1C). Through a narrowing of the range by setting the cutoff value of $2^{-\Delta CT}$ relative to GAPDH at 0.05, we identified the most abundantly expressed cytokine receptor mRNAs in Figure 1B.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LLY MOCK
DLY PIC
DLY MOCK
Log10 (2-ΔCT relative to GAPDH)
B
Lly PIC
Lly Mock
Dly PIC
Dly Mock
2-ΔCT relative to GAPDH
C
LLY
DLY
Fold Change of 2-ΔCT relative to GAPDH (PIC : MOCK)

Figure 1. HMVEC-dLy (Dly) or HMVEC-LLy (Lly) expression of cytokine receptors with or without the stimulation by poly I:C—GeneCopoeia real-time RT-PCR assay.

(A) The heat map shows the expression levels of 84 cytokine receptor related mRNAs and 7 control genes in poly I:C treated(25μg/ml, 24h) (PIC) or untreated (MOCK) HMVEC-dLy (Dly) or HMVEC-LLy (Lly) cells. Different colors represent the values of $\log_{10}(2^{-\Delta CT}$ relative to GAPDH). (B) The heat map shows a collection of abundantly expressed cytokine receptor mRNAs in (A). Cutoff value of $2^{-\Delta CT}$ relative to GAPDH is set to 0.05. Different colors represent the values of $2^{-\Delta CT}$ relative to GAPDH, and black blocks represent data lower than 0.05. (C) Fold-change form of (A). Different colors represent the ratios of the values of $2^{-\Delta CT}$ relative to GAPDH of poly I:C treated samples to the values of $2^{-\Delta CT}$ relative to GAPDH of mock treated samples. Data represent one experiment. The heat map was made using Heatmap Illustrator (Huazhong University of Science and Technology, China).

Among the relatively highly expressed cytokine receptors related genes were the Duffy Antigen Receptor for Chemokines (DARC) or Atypical chemokine receptor 1 (ACKR1), which is a glycosylated membrane protein having affinities to more than 20 inflammatory chemokines. It is mainly expressed in blood cells and endothelial cells, mainly functioning in non-signal-induced binding with chemokines to regulate chemokine gradients. [39] ACKR1 is utilized by malarial parasites *Plasmodium vivax* and *Plasmodium knowlesi* as a receptor [40, 41]. A pervious study has shown that ACKR1 functions to inhibit tumor growth and metastasis [42]. DARC expression was mainly observed in the DARC expression was mainly observed in the HMVEC-LLy cells treated with poly I:C.

Epstein-Barr virus-induced gene 3 (EBI3) was originally found as a soluble hematopoietin receptor that were upregulated by EBV infection [43]. It is now known as a unit forming the heterodimer of the IL12 cytokine family members IL12, IL27, IL35 [43-45]. The expression of EBI3 was also mainly observed in the HMVEC-LLy cells treated with poly I:C .

Erythropoietin receptor (EPOR) which is mainly expressed on erythrocytic progenitors and precursors in bone marrow to mediate red blood cell production [46]. It is highly expressed in all samples and slightly upregulated (1.46 folds change detected by GeneCopoeia, Figure 3) in HMVEC-dLy cells after poly I:C treatment.

Three members of the tumor necrosis factor receptor superfamily were relatively highly expressed by LECs, including TNFRSF1A or TNFR1, TNFRSF10B or Death Receptor 5 (DR5), and TNFRSF10C or TRAIL receptor 3 (TRAILR3) (Figure 1B). TNFRSF1A, which is widely expressed in a number of cell types [47] is expressed by all four LECs populations, with a slight increase (2.5 folds change detected by GeneCopoeia, Figure 3) in expression observed in HMVEC-

LLy cells after poly I:C treatment. TNFRSF10B was abundantly expressed in poly I:C treated HMVEC-LLy cells and TNFRSF10C was expressed more in poly I:C treated HMVEC-dLy cells.

IL13RA1 and IL4R(α) which together form the receptor complex of IL13 and IL4 [48] were both expressed (Figure 1B). IL13RA had relatively high expression in all four samples, with expression upregulated (1.47 fold change, Figure 3) in HMVEC-LLy cells and downregulated (0.36 fold change, Figure 3) in HMVEC-dLy cells after poly I:C treatment. High IL4R expression was only observed in HMVEC-LLy cells treated with poly I:C.

IL15RA (IL15Rα), together with IL-2Rβ and γ, forms the IL15 receptor [49]. IL15Rα expression was only abundant in the HMVEC-LLy population treated with poly I:C.

IL7R, together with IL2RG (IL2Rγ) which is a shared receptor by IL-2, 4, 7, 21[50, 51], forms the IL7 signaling receptor. IL7R expression was upregulated in both HMVEC-LLy and HMVEC-dLy populations after poly I:C stimulation.

Two members of Toll-like receptor superfamily were highly expressed by LECs, including TLR4 and Interleukin 1 Receptor-Like 1 (IL1RL1). The high expression of TLR4 was observed in all four samples, which is consist with our pervious results [19]. The expression of TLR4 in HMVEC-dLy cells was downregulated (0.48 fold change, Figure 3) by poly I:C treatment, whereas the expression of TLR4 in HMVEC-LLy cells was upregulated (2.75 fold change, Figure 3) by poly I:C. The expression of IL1RL1 was observed mostly in HMVEC-LLy cells and it was highly upregulated (23.9 fold, Figure 3) by poly I:C treatment.

A member of type I interferon receptors, Interferon (Alpha, Beta and Omega) receptor 1 (IFNAR1) which forms type I IFN receptor when combined with IFNAR2 [52], were abundantly expressed by LECs (Figure 1B). And two members of type II interferon receptors, interferon gamma receptor 1 (IFNGR1) and interferon gamma receptor 2 (IFNGR2), were also highly

expressed by LECs (Figure 1B). IFNAR1 had boarder and higher expression level compared with other two interferon receptors. Interesting, the expression of IFNGR1 was upregulated (2.69 fold change, Figure 3) after poly I:C treatment in HMVEC-LLy cells but downregulated (0.74 fold change, Figure 3) in HMVEC-dLy cells, whereas IFNGR1 and IFNGR2 were relatively highly expressed in HMVEC-LLy cells treated with poly I:C.

Platelet-Derived Growth Factor Receptor-Like (PDGFRL) and platelet-derived growth factor receptor beta (PDGFRB) have high similarity with the respect of their coding sequences [53]. Again, the highest expression was observed in HMVEC-LLy cells treated with poly I:C.

There overall was high expression of interleukin 6 signal transducer (IL6ST or gp130) in all four LEC samples. IL6ST is a signal transduction receptor shared by interleukin (IL)-6 family cytokines including IL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), cardiotrophin-like cytokine (CLC), neuropoietin (NPN), IL-27, and IL-31 [54]. Given the high expression of IL6ST, we next included several IL6 family cytokine receptors in a focused real-time RT-PCR follow-up analysis.

Using Taqman real-time RT-PCR we followed up on the screening analysis and extended the analyses to include 14 cytokine receptor mRNAs (eight non-IL6 family receptors, including CCR6, DARC, IL2RG, IL7R, TLR3, IFNAR1, EPOR, PDGFRA, and six IL6 family receptors, including IL6ST, IL6Ra, CNTFR, LIFR, OSMR, IL11Ra) in HMVEC-dLy, HMVEC-LLy, and hTERT-HDLEC cell populations (Figure 2). The results obtained from the GeneCopoeia real-time RT-PCR array generally were similar with the results obtained from Taqman assay (Figure 3). A number of differences were obtained, though. Compared with the Taqman data, the GeneCopoeia array results showed an opposing effect of poly I:C treatment on the expression of IL6ST in HMVEC-dLy cells, lower expression of DARC in HMVEC-dLy cells, and higher

expression of EPOR and an opposite effect of poly I:C treatment on EPOR expression in both HMVEC-LLy and HMVEC-dLy cell populations. The differences between these findings might result from the reduced specificity of the SYBR-green-based real-time RT-PCR technique used in the GeneCopoeia real-time RT-PCR array, and the fact that the array screening was performed only once. Also, the acquisition of a new aliquot of HMVEC-dLy cells (new low passage cells from the same company) after the performance of the GeneCopoeia array and before the performance of the Taqman assays could have contributed to the differences obtained with the two approaches.

Surprisingly, we found using Taqman assays and comparing the different LEC populations, that the HMVEC-dLy, HMVEC-LLy, and hTERT-HDLEC cells overall have similar expression patterns for the 14 cytokine receptor mRNAs in both untreated and poly I:C treated environments. (Figure 2). Poly I:C treated HMVEC-LLy and HMVEC-dLy cells expressed CCR6 mRNA to low levels and we did not observe CCR6 expression in the three untreated LEC populations (Figure 2A).

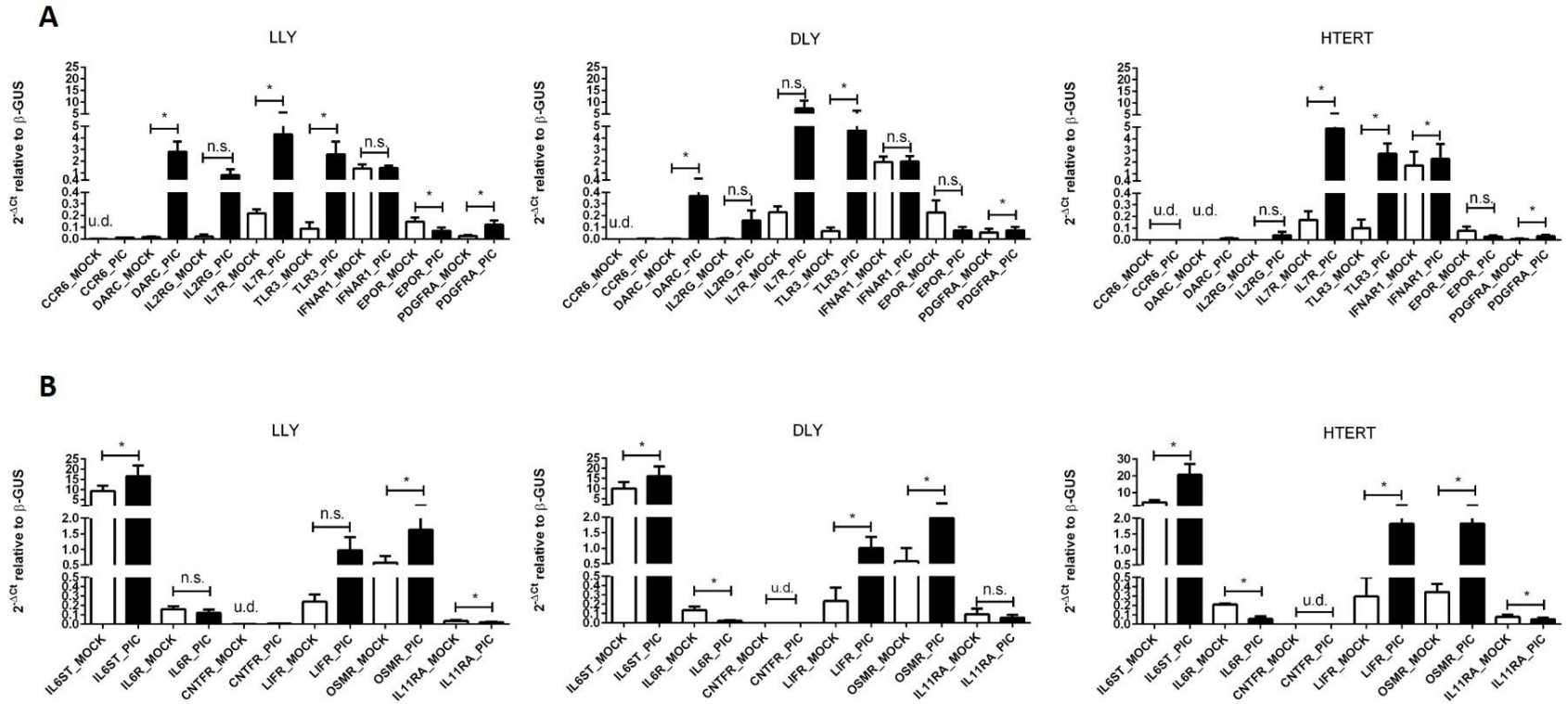
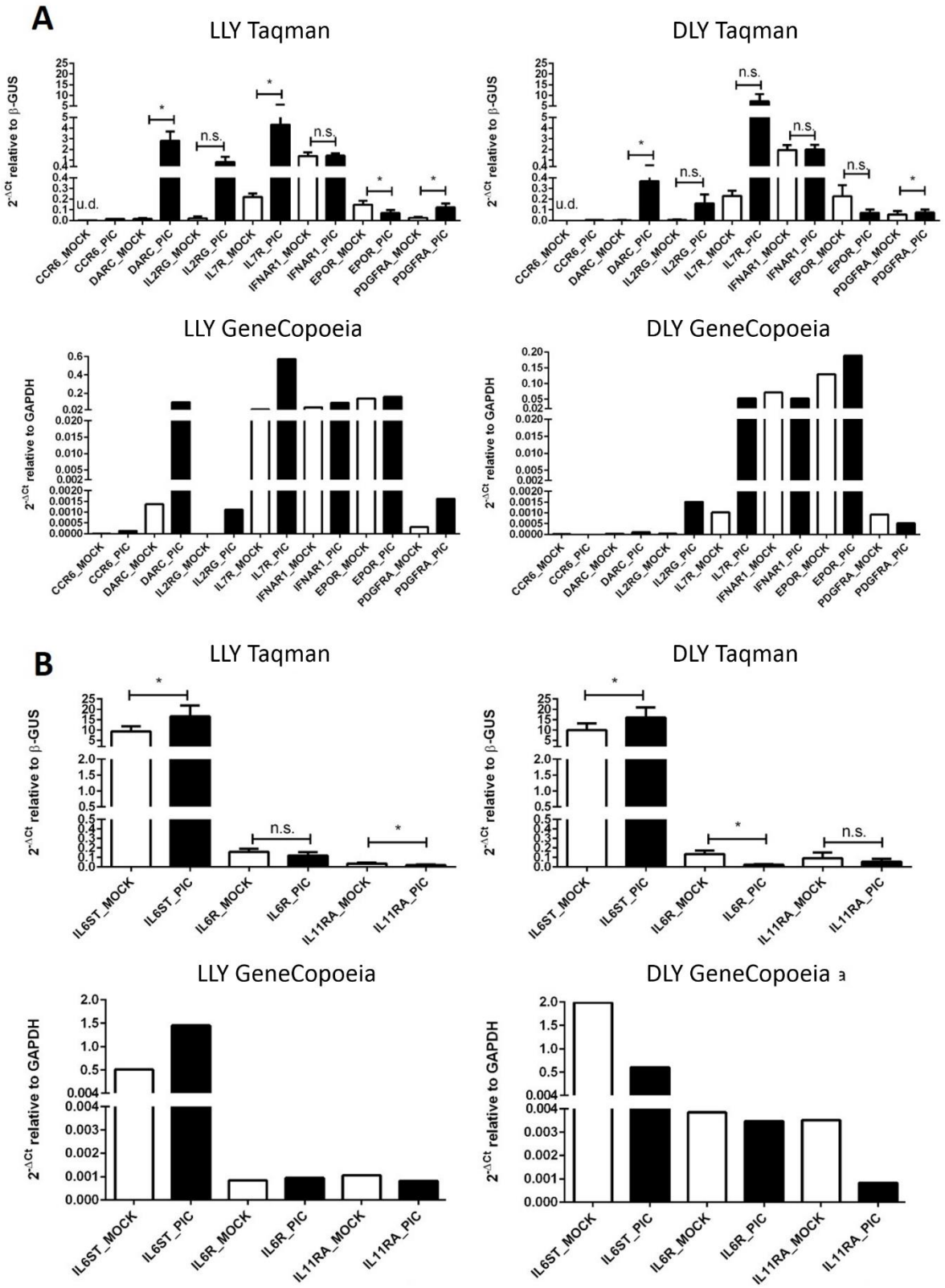


Figure 2. HMVEC-dLy (DLY) , HMVEC-LLy (LLY) and hTERT-HDLEC (HTERT) expression of cytokine receptors with or without the stimulation by poly I:C—Taqman assay.

The graphs show the values of 2-ΔCT relative to β-GUS obtained by Taqman assay in poly I:C treated (PIC) (balck) or untreated (MOCK) (white) HMVEC-dLy (DLY) , HMVEC-LLy (LLY) and hTERT-HDLEC (HTERT). (A) Eight non-IL6 family cytokine receptors expression profile in the three LEC populations. (B) Six IL6 family cytokine receptors expression profile in the three LEC populations. Symbol * means significant difference ($P<0.05$) is observed between the two groups, and n.s. means nonsignificant ($P>0.05$). Paired t-tests ware perform to calculate P-values. U.d. means "undetected" which indicates "the CT value of its genome DNA control (No RT control) subtract the CT value of the indicated sample ≤ 3". The function of the Taqman primers and probes of the undetected groups were examed on positive control samples (RNA isolates form human cerebral cortex for CNTFR; RNA isolates from human CCR6 transfected murine L1.2 cells for CCR6). Error bars indicate SD. The data were compiled from three independent experiments.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2^-ΔCt relative to β-GUS
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u.d.
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CCR6_MOCK
CCR6_PIC
DARC_MOCK
DARC_PIC
IL2RG_MOCK
IL2RG_PIC
IL7R_MOCK
IL7R_PIC
IFNAR1_MOCK
IFNAR1_PIC
EPOR_MOCK
EPOR_PIC
PDGFRA_MOCK
PDGFRA_PIC
IL6ST_MOCK
IL6ST_PIC
IL6R_MOCK
IL6R_PIC
IL11RA_MOCK
IL11RA_PIC

Figure 3. Comparison between Taqman and GeneCopoeia data.

The graphs show the values of $2^{-\Delta CT}$ relative to β-GUS obtained by Taqman assay (Taqman) or the values of $2^{-\Delta CT}$ relative to GAPDH obtained from GeneCopoeia assay (GeneCopoeia) in poly I:C treated (PIC) or untreated (MOCK) HMVEC-dLy (DLY) , HMVEC-LLy (LLY). Figure (A) shows The Comparison between Taqman and GeneCopoeia data for seven non-IL6 family cytokine receptors. Figure (B) shows The Comparison between Taqman and GeneCopoeia data for three IL6 family cytokine receptors. Symbol * means significant difference ($P<0.05$) is observed between the two groups, and n.s. means nonsignificant ($P>0.05$). Paired t-tests ware perform to calculate P-values. Error bars indicate SD. The data were compiled from three (Taqman assay) or one (GeneCopoeia assay) independent experiment(s).

Platelet-derived growth factor-receptor α (PDGFRA/PDGFR-α) was expressed in the three LEC populations and the expression was upregulated after poly I:C treatment (Figure 2A). PDGFR-α forms heterodimers with platelet-derived growth factor-receptor β (PDGFR-β) to recognize all five PDGFs (PDGF-AA, AB, BB, CC, and DD), it can also form homodimers to recognize PDGF-AB and BB [55, 56]. The expression of PDGFR-α in LECs gives LECs the potential to respond to all PDGFs. In addition to the well-studied vascular endothelial growth factors (VEGFs), the interactions between PDGFs and ECs have been given growing attention in recent years. A recent study has shown that some tumors can express PDGFs [57]. In addition, PDGF-AA, AB, BB, CC are able to function on lymphatic vessels and induce VEGF-C/-D/VEGFR-3 independent lymphangiogenesis in vivo [58]. The observation that PDGFR-α is expressed in LECs and is upregulated by poly I:C treatment at the mRNA level (Figure 3) strengthens the evidence of LECs' ability to interact with PDGFs and give cue about the viral infection may increase the impact of PDGFs on LEC and possibly increase PDGFs induced lymphangiogenesis.

Erythropoietin (EPO) can induce erythropoiesis of hematopoietic precursor cells [59]. EPOR is inducible in multiple cell types and EPO can affect cell types such as cardiac cells [59], neuronal cells [60] and endothelial cells [61, 62]. EPOR expression in human LECs was shown previously [28]and our data confirm this finding. It was also shown that EPO can induce the

lymphangiogenesis of LECs [28]. Here we have provided additional evidence that LECs express EPOR (Figure 2A).

4.2 TRANSCRITIONAL AND CELL SURFACE EXPRESSION OF IL-6 FAMILY CYTOKINE RECEPTORS

The follow-up Taqman analyses of IL6 family receptor mRNA levels confirmed the abundant expression of IL6ST in all four LEC populations (Figure 2B). This is consistent with the evidence of IL6ST's wide expression [63]. As for the expression of the specific receptors that are able to form receptor complexes with IL6ST for the specific recognition of IL6 family cytokines, IL6Rα, LIFR, OSMR, IL11Rα but not CNTFRα were observed to be expressed at the mRNA level in HMVEC-dLy (DLY), HMVEC-LLy (LLY) and hTERT-HDLEC (HTERT) LEC populations (Figure 2B). Poly I:C increased the expression of IL6ST, LIFR, OSMR and decreased the expression of IL6RA and IL11RA in the three LEC populations. Interestingly, IL6ST, LIFR and OSMR are all signal transducing receptors, whereas IL6Rα and IL11Rα are non-signaling receptors [64]. IL6 and IL11 signal through IL6ST homodimers after initially binding with their own α-receptor (IL6Rα or IL11Rα, respectively), whereas human herpes virus 8 (HHV8) secreted viral IL-6 (vIL6) can directly signal through IL6ST homodimers without IL6Rα [65]. LIF, OSM, CT-1 directly bind and signal through the heterodimer of IL6ST/ LIFR. CNTF and CLC signal through the heterodimer of IL6ST/LIFR after binding to CNTF α-receptor (CNTFRα). OSM can also signal by binding with the heterodimer of IL6ST/OSMR [64]. IL-27 has been shown to signal through the gp130/IL27R heterodimeric receptor [66]. The Taqman data indicate that poly I:C

stimulation might increase LEC potential to respond to LIF and OSM but decrease the potential to respond to IL6 and IL11.

To extend the mRNA analyses, we used flow cytometry to examine cell surface expression of several IL6 family cytokine receptors (Figure 4). The cell surface expression of the indicated proteins were detected using flow cytometry, with gating strategies based on unstained and isotype antibody control staining (Figure 16). Different from the mRNA level results (Figure 2), only IL6ST was observed to be highly expressed on the cell surface for all LEC populations. In contrast, IL6R, OSMR, LIFR and IL27R had very low or no surface expression for the three untreated LEC populations. Poly I:C treatment led to slight shifts of positively stained LECs (red) compared to isotype antibody stained cells (blue) for IL6Rα, LIFR, OSMR and IL27R for all the three LECs populations. Different from the mRNA results, poly I:C treatment here slightly reduced the percent of IL6ST positive cells in the three populations. As a positive control, all cell populations stained strongly for podoplanin with nearly all cells showing strong staining (Figure 4).

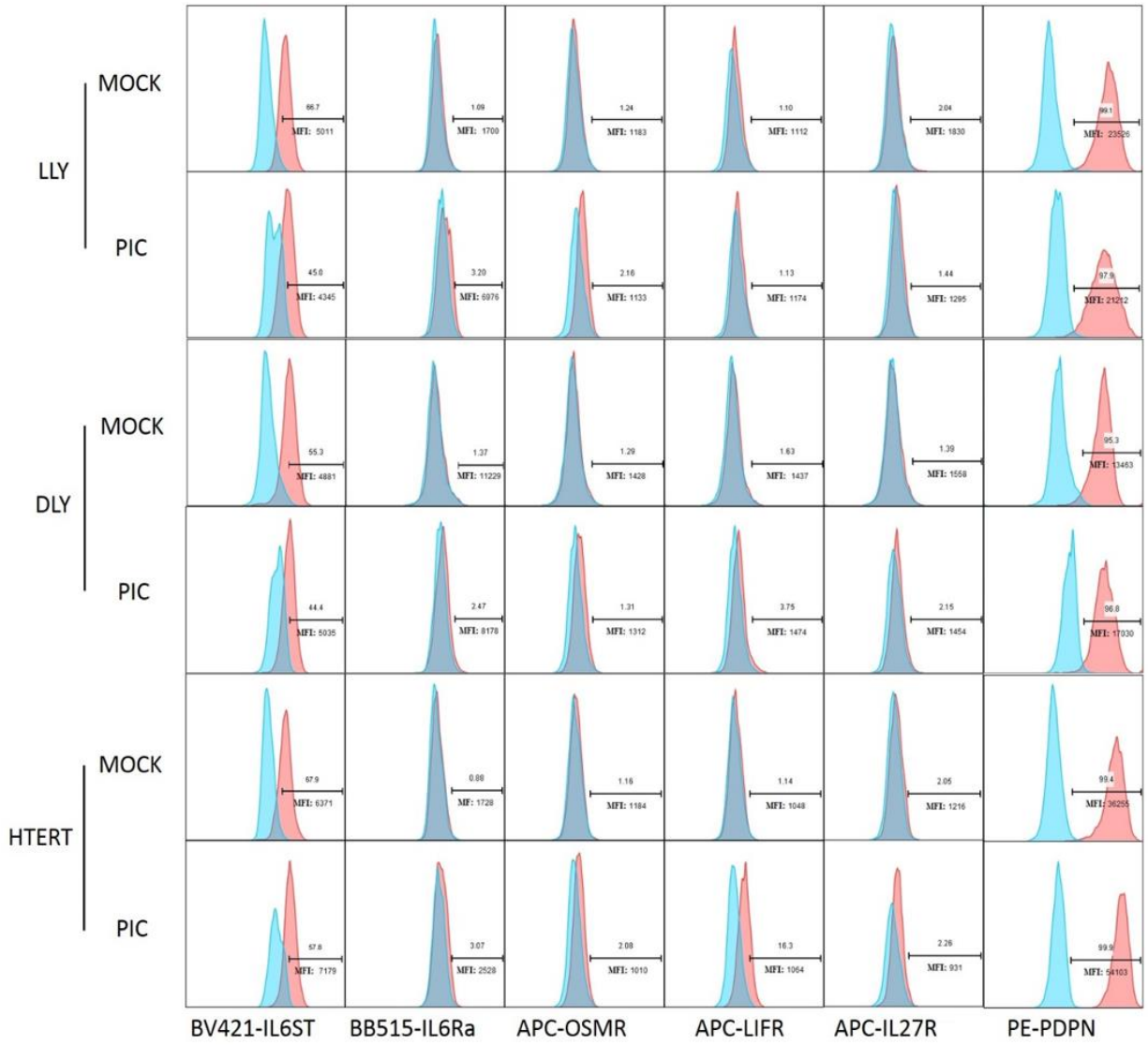

Figure 4. HMVEC-dLy (DLY) , HMVEC-LLy (LLY) and hTERT-HDLEC (HTERT) expression of IL6 family cytokine receptors with or without the stimulation by poly I:C—flow cytometry assay.

Surface expression of five IL6 family cytokine receptors IL6ST, IL6R(a), OSMR, LIFR, IL27R and one LEC surface marker Podoplanin(PDPN) in poly I:C treated (PIC) or untreated (MOCK) HMVEC-dLy (DLY), HMVEC-LLy (LLY) and hTERT-HDLEC (HTERT) were examined using flow cytometry. Red curves indicate positive staining, while blue curves indicate isotype control. The gates represent the percentage of cells positive for cytokine receptor expression (red) and were set at 1% of the isotype controls (blue). Figure shows representative data out of at least two independent repeats.

4.3 FUNCTIONAL RESPONSE OF LECS TO IL-6 FAMILY CYTOKINES

IL6 is one of the most important and pleiotropic pro- and anti-inflammatory factors [67] . The ubiquitous expression of IL6ST, and the wide expression of IL6 as well as the existence of soluble IL6Rα, enable IL6 to interact and function on many cell types including endothelial cells [68]. Previously, IL6 was shown to induce the expression of VEGF-C in "conditionally immortalized" murine LECs through Src-mediated ERK1/2 and p38MAPK [69];

Leukemia inhibitory factor (LIF) is a member of the IL6 family of cytokines and is commonly used in stem cell culture media to inhibit the spontaneous difference of embryonic stem cells [70]. It has been shown to have contradictory effects on endothelial cells. LIF inhibits the angiogenesis and proliferation in vitro of bovine aortic endothelial cells and bovine microvascular endothelial cells [71-73], whereas when combined with basic FGF, LIF was noted to enhance the formation of capillary-like structures of embryonic endothelial cell [74].

OSM is also a member of the IL6 family of cytokines with multiple functions in hematopoiesis, mesenchymal stem cell differentiation, liver regeneration, heart remodeling, nociception, inflammation and metabolism [75]. Regarding the interaction between OSM and endothelial cells, OSM was shown to induce the expression of cytokines including IL6, CXCL-1, -2, -5, and adhesion molecules including ICAM-1 and VCAM-1 in human umbilical vein endothelial cells (ECs) [76]. OSM also induces cytokine CCL21 expression in human lung microvascular ECs and human dermal microvascular ECs [77], and affects the transcription of CCL20, CCL21, CXCL10, CXCL12, ICAM-1, VCAM-1 in HMVEC-dLy, HMVEC-LLy and hTERT-HDLEC [19].

To examine further the expression and function of IL6 family cytokine receptors by LECs, three IL6 family cytokines (human recombinant IL6, LIF, and OSM) were used to stimulate

HMVEC-dLy (DLY) , HMVEC-LLy (LLY) and hTERT-HDLEC (HTERT) cells. STAT3 is a proximal signaling molecule activated by IL6 that was once called acute-phase response factor (APRF) and was firstly defined as a nuclear factor that can be activated by IL-6 [78]. All IL6 family cytokines have the potential to active STAT3 because they all share the IL6ST (gp130) signaling receptor [63]. In addition, OSM [75] and LIF [79] STAT3 activation have been well-studied. To determine whether LECs are responsive to IL6 family cytokines, immunoblotting was performed for total STAT3 and phosphorylated STAT3 (Tyr705) to examine LEC responsiveness (Figure 5).

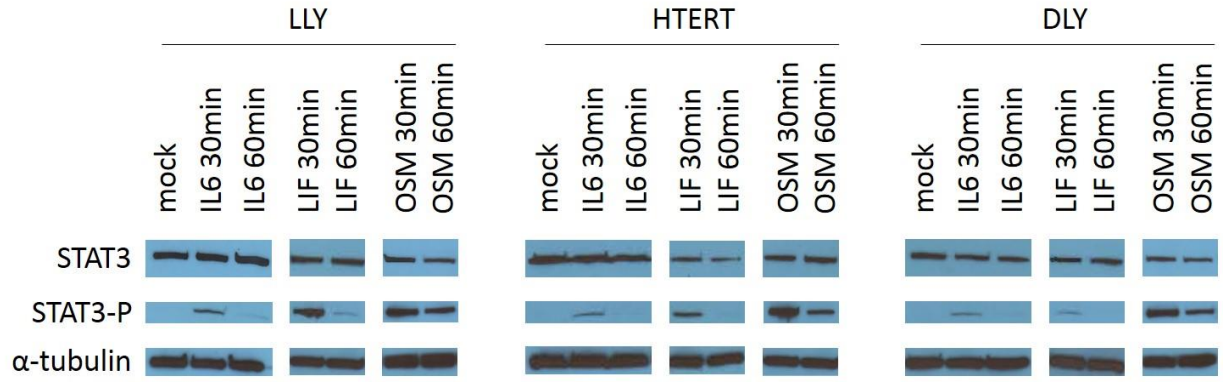

Figure 5. Multiple IL6 family cytokines (IL6, LIF and OSM) induce the phosphorylation of STAT3 in Dly, Lly and hTERT-LEC.

Human recombinant IL6, LIF, or OSM (10 ng/ml) were used to treat HMVEC-dLy (DLY) , HMVEC-LLy (LLY) and hTERT-HDLEC (HTERT) cells for 30 or 60min. Immunoblotting was performed to detect the phosphorylation (Tyr705) of STAT3. In parallel, the loading control a-tubulin and total STAT-3 were detected. Figure shows representative data out of at least two independent repeats.

Immunoblotting for STAT3-P (Figure 5), revealed that IL6, LIF, OSM were all able to activate STAT3 in HMVEC-dLy, HMVEC-LLy and hTERT-HDLEC cell populations. The activation had different patterns across ligands and LEC populations. The amounts of STAT3-P were universally higher at 30min of treatment compared to 60min of treatment for all ligands. In addition, OSM treatment induced the strongest and longest STAT-P expression in HMVEC-dLy,

HMVEC-LLy and hTERT-HDLEC compared with other two ligands. Furthermore, IL6 or LIF induced STAT3-P expression usually resolved after 60min of treatment, except for LIF induced STAT3-P expression in HMVEC-LLy, which was sustained after 60min of treatment. The quick drop-off of STAT3-P has been proved before as the result of SOCS3 regulation [80]. Importantly, these data provide strong evidence that LECs can respond to IL6 directly without addition of IL6R, particularly given that there are reports stating the lack of IL6R expression in endothelial cells [81-83].

4.4 DEVELOPMENT OF ORGANOTYPIC CULTURE MODELS CONTAINING LYMPHATIC ENDOTHELIAL CELLS

4.4.1 Network formation by LECs on Matrigel™

It has been reported that LECs can form a network-like structure when cultured on the Matrigel™ extracellular matrix (ECM) substrate [13, 15, 23, 24]. We used this "capillary tube formation assay" described in [13] to begin to examine the effects that 3D culture might have on LEC phenotype and function and to study the factors affecting LEC network formation. We note that there are no assays that well represent and recapitulate LEC "function", although this network formation assay provides some level of functional analysis. We seeded hTERT-HDLECs on the top of Matrigel™ coated plates (Figure 6A) and observed rapid network formation (Figure 6A-E). Up to 16h after plating, we observed a rapid process of network formation (Figure 6A-D). Around 16h (Figure 6D), the networks were fully formed and additional re-organization stopped. At 18h the outline of the network started to dim, and some network components started to disappear

suggesting a dissolution of the network (Figure 6E). After 3-4 days, even with media being changed every two days, the network ultimately aggregated and involuted (data not shown).

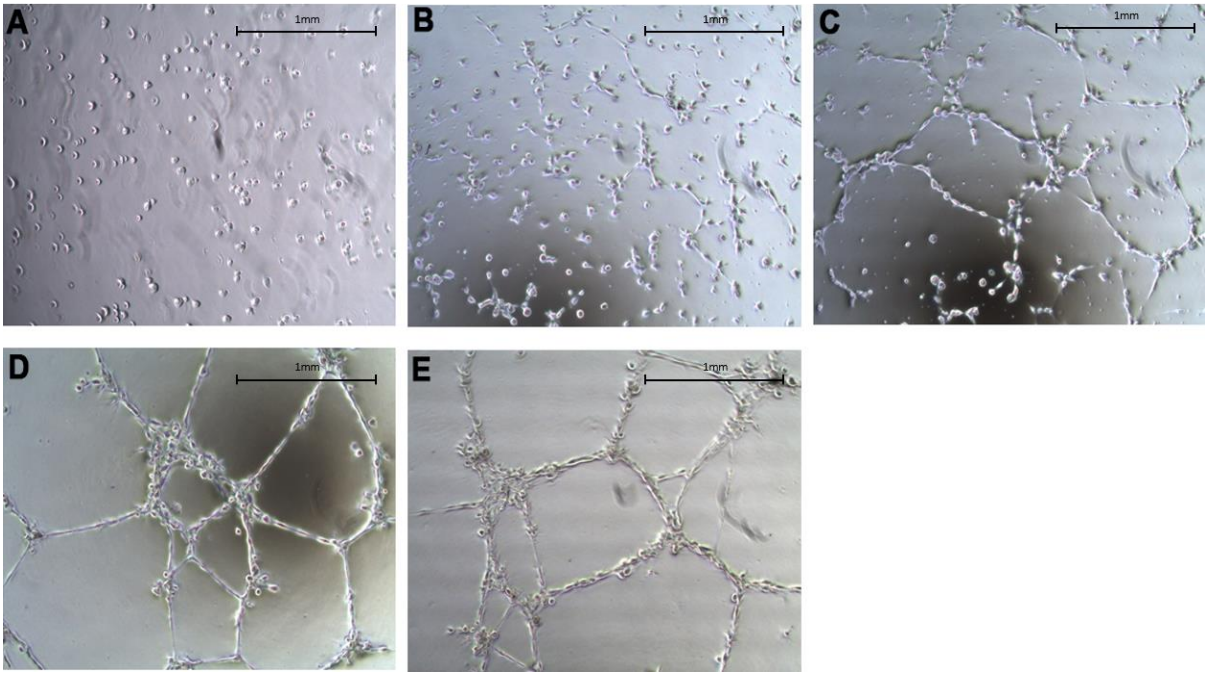

Figure 6. hTERT-HDLEC formed a network-like structure on MatrigelTM coated plates.

Matrigel™ was plated on 24-wells plates for 30 minutes. Then hTERT-HDLECs were seeded on the top of Matrigel™. Pictures were taken at 0h(A), 2h(B), 4h(C), 16h(D), 18h(E) under microscopy after hTERT-HDLEC was seeded. Pictures show one representative experiment out of at least three independent repeats.

We also performed this assay on additional populations of primary LECs, including HMVEC-dLy, HMVEC-LLy, ferret lung LECs (FeLg-LEC) and macaque jejuna LECs (R24J), these last two derived by Dr. Stella Berendam in our laboratory [84]. Although to differing degrees, all of these LECs formed network-like structures on Matrigel™ (Figure 7). The networks formed by HMVEC-dLy (Figure 7A) and FeLg-LECs (Figure 7D) were similar to the networks formed by hTERT-HDLECs (Figure 6D). At 16h HMVEC-LLy (Figure 7B) and R24J LECs (Figure 7C) only started to form connections and the network structure looked more like hTERT-HDLEC networks at 4h (Figure 6C). This might result from the different growth or migration rates of different cell types.

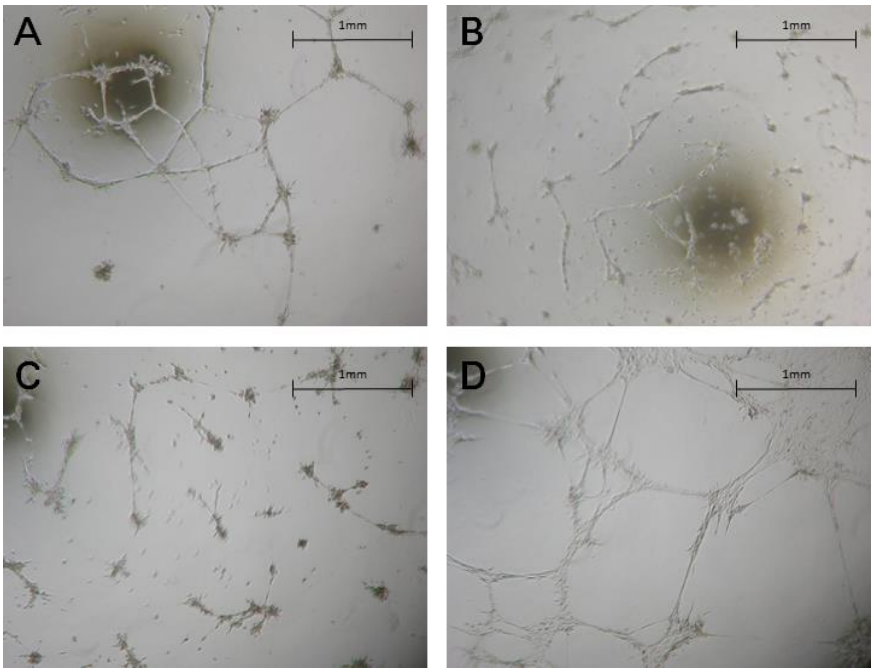

Figure 7. Other kinds of LECs also form the network structure on MatrigelTM.
After being seeded for 16h, Human dermal microvascular lymphatic endothelial cells (HMVEC-dLy, Fig A), human lung lymphatic microvascular endothelial cells (HMVEC-LLy, Fig B), ferret lung isolated LECs (FeLg-LEC, Fig C) and macaque jejuna isolated LECs (R24J, Fig D) all formed network-like structures to different degrees on Matrigel™ coated plates. Pictures show one representative experiment out of at two independent repeats.

To examine whether network formation on Matrigel™ is unique to LECs, we cultured the human bronchial epithelial cell line 16HBE14o- and the human lung fibroblast cell line MRC5 on Matrigel™ for 16h. These two cell types did not form networks. This result indicated that network formation on Matrigel™ observed with LECs is not a property shared by all cell types.

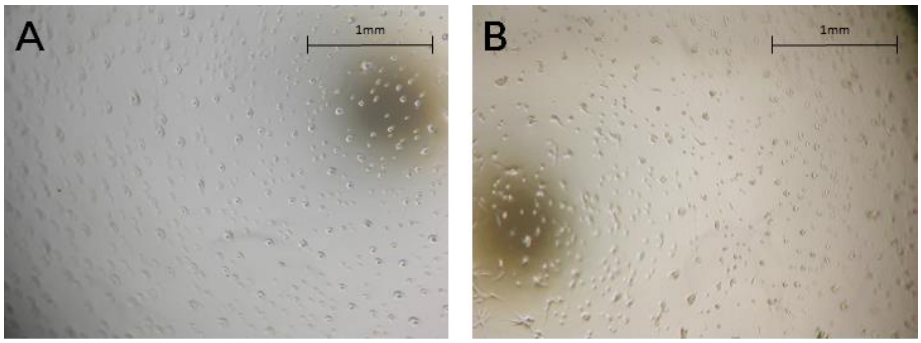

Figure 8. Human epithelial cells and lung fibroblasts do not form the networks on Matrigel™.

After being seeded for 16h, human bronchial epithelial cells (16HBE14o-, Fig A) and human lung fibroblasts (MRC5, Fig B) did not form networks on Matrigel™ coated plates. Pictures show one representative experiment out of at least two independent repeats.

For better tracking of LECs in these and downstream models, we used a lipophilic live cell dye called CM-dil to fluorescently label hTERT-HDLECs (dil-hTERT-LECs). We performed the network formation assay on Matrigel™ with dil-hTERT-LECs and found that the dye efficiently labeled LECs without visibly affecting their network forming activity on Matrigel™ (Figure 9).

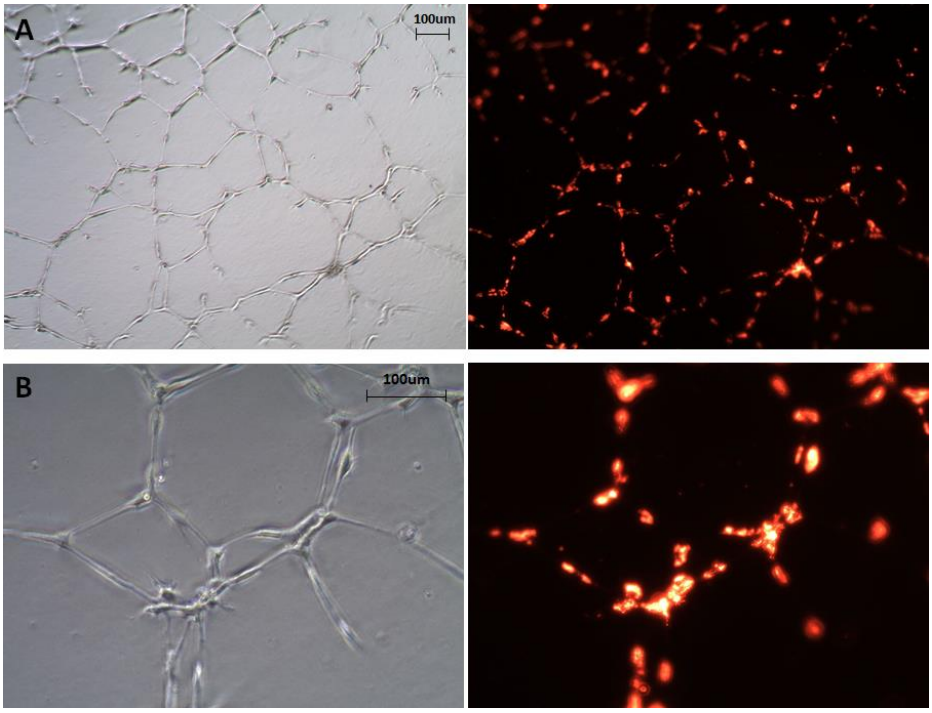

Figure 9. CM-dil labeled hTERT-HDLECs (dil-hTERT-LECs) formed the networks on Matrigel™.

After being seeded on Matrigel™ coated plates for 16h, CM-dil labeled TERT-HDLECs were observed to form network-like structures under both white filed (left) and TRIC channel (right). (A) 40X magnification. (B) 100X magnification. Pictures show one representative experiment out of at least two independent repeats.

4.4.2 Develop of 3D mucosal tissue models containing LECs

To provide a strong tool to study LECs in a tissue-like environment in vitro, we adapted a 3D lung mucosal tissue model with the long life-span LEC hTERT-HDLEC population inside by modifying an established tissue model [21]. We held the hypothesis that the 3D lung mucosal tissue model will give LECs an in vivo-like environment that will support LEC survival and immune potential and that might provide the ability, like Matrigel™, to support LEC network forming.

The tissue models were created in transwells set on 6-wells plates. The basic models developed [21] were made of three layers (from bottom to top): a collagen layer as a base, a

collagen and fibroblast (MRC5) mixture layer, and an epithelial (human bronchial epithelial cells, 16HBE14o$^-$) layer. The tissue models were made by adding layer by layer from the bottom to the top with different incubation periods. After all layers were added, a 7-days air exposure was performed to mimic the physiological conditions in lung with the air/liquid interface. (Figure 10 A and B "basic").

Two kinds of models containing LEC were attempted: (1) hTERT-HDLECs were mixed within the collagen & fibroblasts (MRC5) mixture layer, and then the model was cultured as basic model (Figure 10 A and B "containing LECs (inside)"); (2) hTERT-HDLECs were seeded on the top of collagen & fibroblasts (MRC5) mixture layer 7 days after the seeding of the mixture layer, and then the model was cultured for 3-4 days before adding 16HEB cells (Figure 10 A and B "containing LECs (inside)"). For obtaining the best culture condition, two kinds of media were tried: "DMEM" media, which is a basic media containing basic nutrition, and "EGM2" media, which indicates a 1:1 mixed media of DMEM and EGM2-MV here (among them EGM-2MV is the media used for LECs culture and contains several growth factors in addition to the basic nutrition).

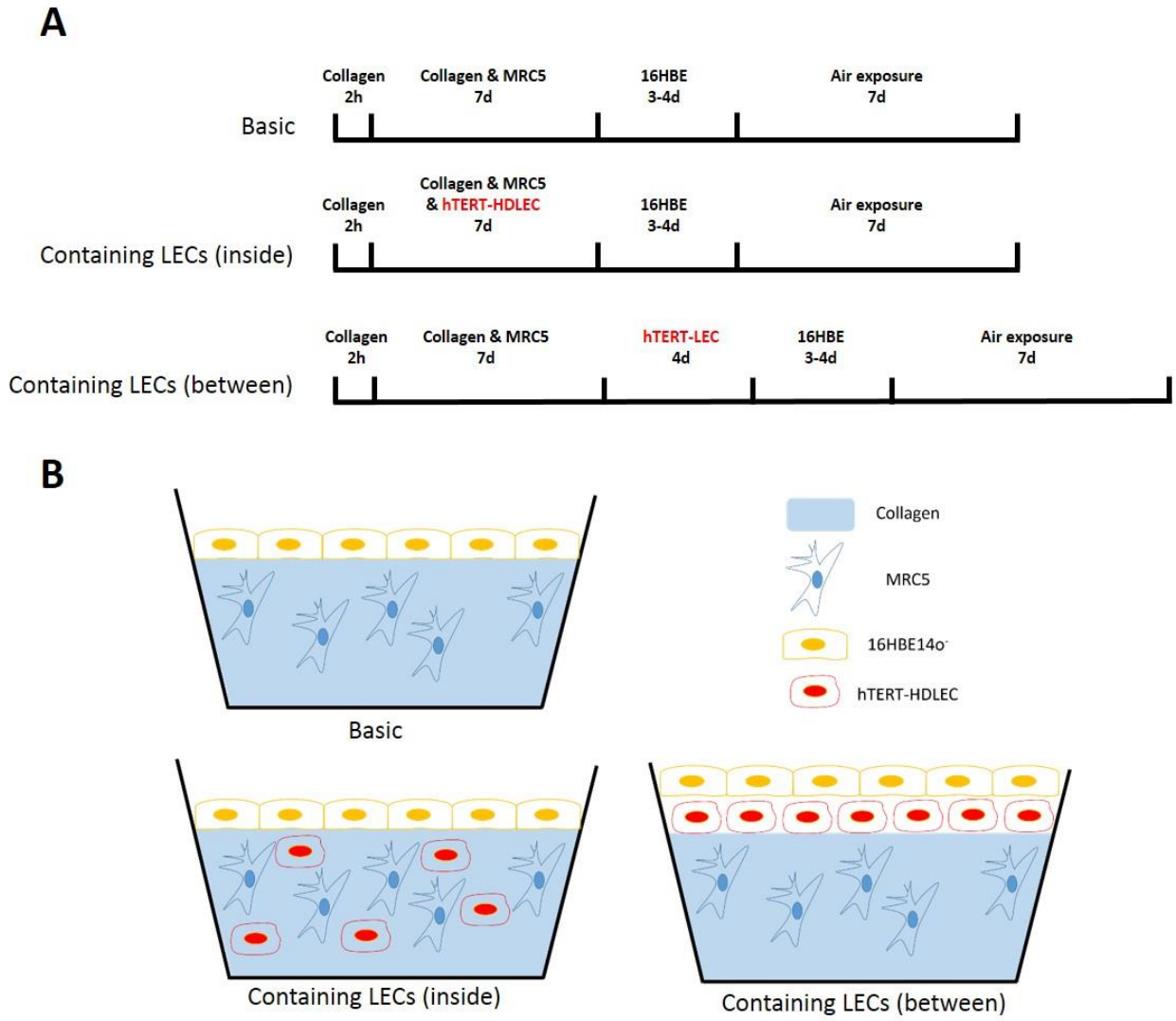

Figure 10. Setup of the 3D tissue model.

(A) Flowcharts of 3D models setup. From left to right, the flow chart represents the building process of the three kinds of 3D lung mucosal tissue model: incubation time and the main component/operation of each layer are shown. (B) Schematic diagrams of 3D models setup: models are built in transwells, different components are indicated.

Immunohistochemistry was done to analyze the structure of the basic 3D tissue models. As an imitation of real in vivo tissue, the basic in vitro lung mucosal tissue model has an epithelial layer, and stromal components on top of the membrane of a transwell insert (Figure 11). As for the models with LECs, Figure 12B shows the culture process of a model containing LECs

(between) when cultured in DMEM media under microscope. It indicates even under the basic nutrition environment, the model looked very healthy during the culture process (Figure 12).

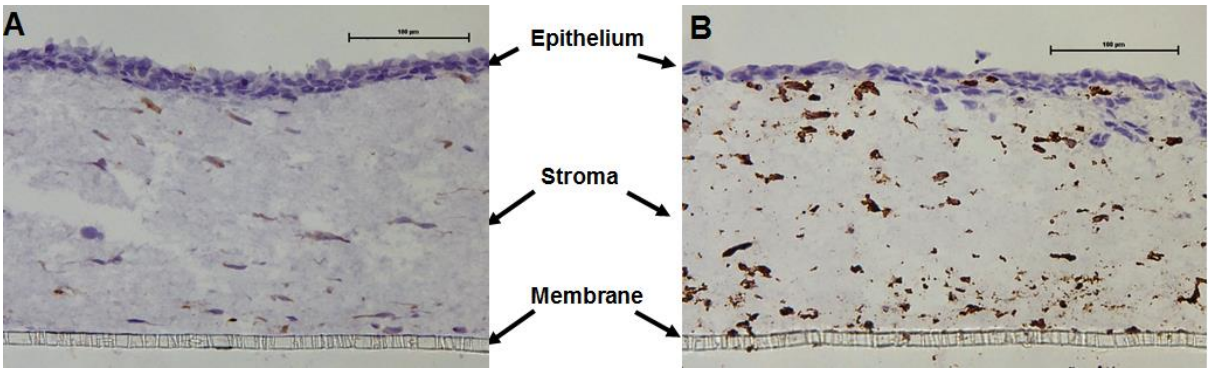

Figure 11. 3D lung tissue model without LECs.

Immunohistochemical staining (with hematoxylin counterstaining) of a 10-µm cryosection of the 3D lung tissue model without LECs is shown (magnification X200). (A) IHC using isotype control. (B) IHC for vimentin (MRC5 marker). The distribution of MRC5 (brown) in the stroma is indicated in (B). The epithelial layer (16HBE14o-) and the membrane are also indicated by arrows. Pictures show one representative experiment out of at least two independent repeats.

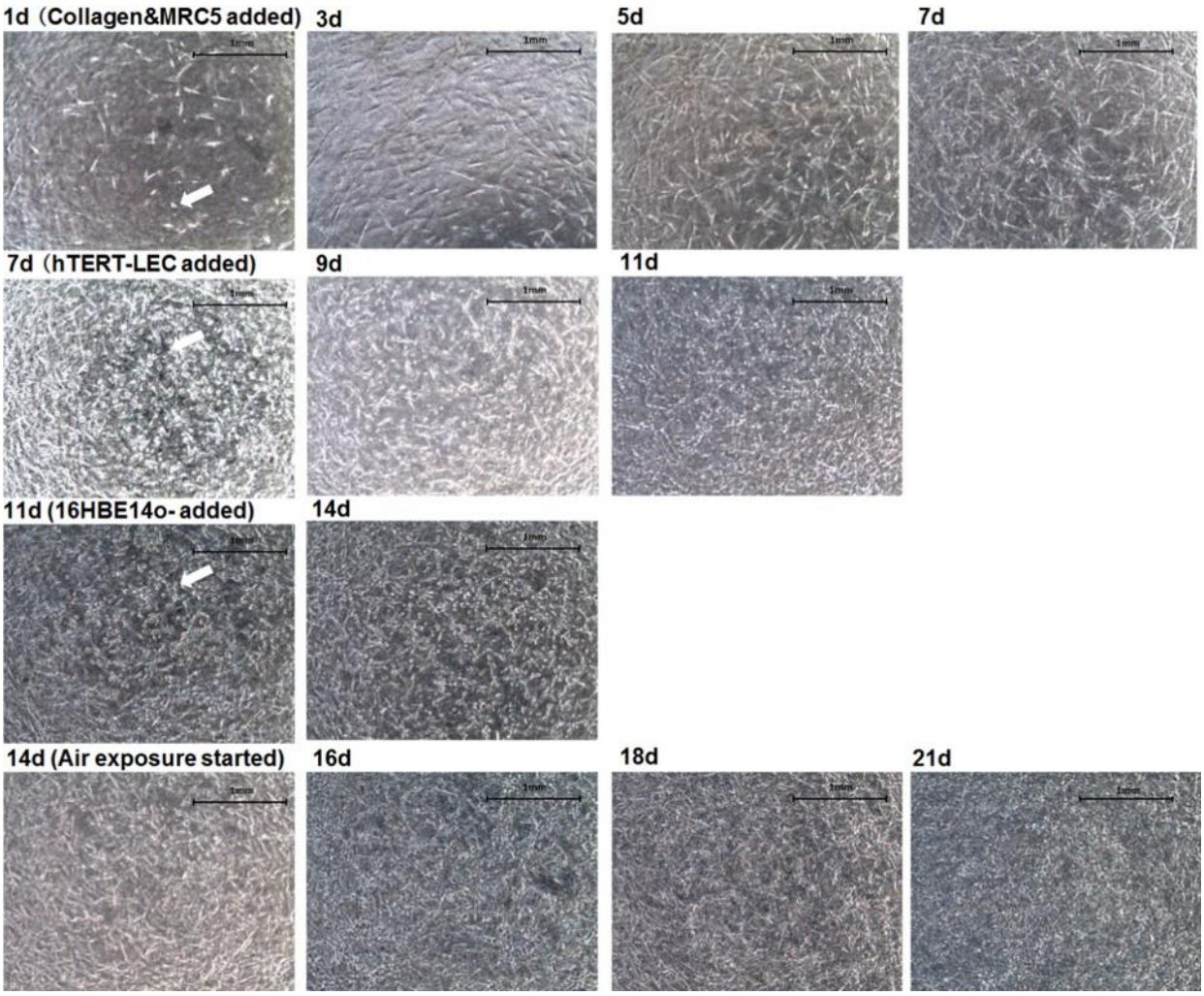

Figure 12. Growth process of the 3D lung mucosal tissue model containing LECs (between) cultured in DMEM media.

Growth process of a tissue model containing LECs (between) when cultured in DMEM. Phase contrast microscopy images of the middle part of the 3D lung mucosal tissue model with LECs during the culturing process are shown (magnification X40). The first row of images show the growth of MRC5 in the collagen-and-fibroblasts mixture layer after the layer is seeded on the top of the collagen layer at day 1. The second row of images show the growth of hTERT-LEC after addition at day 7. The third row of images show the growth of 16HBE14o- cells after added at day 11. Newly added cells are indicated. In each row of pictures, the healthy growth of newly added cells can be observed (the shape changes and amount increase of newly added can be observed). The fourth row of images show the 7-days-length air exposure. Pictures show one representative experiment out of three independent repeats.

Then Taqman real-time RT-PCR showed that at RNA level the tissue models expressed two kinds of growth factors (VEGF-A, VEGF-C) in all culture conditions (Figure 13). The expression of VEGF-A and VEGF-C in the tissue models gave primary evidences that the models

have potentials to support the survival of LECs because VEGF-A and VEGF-C were reported to be essential for the survival of LECs [3].

In Figure 13, two patterns can be observed: (1) Comparing between the models having the same structure (e.g. comparing between "DMEM+LEC(inside)" and "EGM+LEC(inside)"), the models cultured in DMEM (e.g. "DMEM+LEC(inside)") always had higher expression levels of VEGF-A and VEGF-C than the models cultured in "EGM" (e.g. "EGM+LEC(inside)"). This may be because that the growth factors in "EGM" (which contains VEGF-A but not VEGF-C) have performed feed-back inhibitions on the expressions of related growth factors including VEGF-A and VEGF-C in the model. (2) Comparing among the models cultured in the same media (e.g. comparing among "DMEM+LEC(inside)", "DMEM+LEC(outside)" and "DMEM-LEC"), the models containing LECs (inside) (e.g. "DMEM+LEC(inside)") always had either the highest VEGF-A expression levels (Figure 13A) or the lowest VEGF-C expression levels (Figure 13B), while the VEGF-A and VEGF-C expression levels in models containing LECs (between) (e.g. "DMEM+LEC(between)") and models not containing LECs (e.g. "DMEM-LEC") were similar (Figure 13A and B). Combining with the data showing in 2D culture hTERT-HDLECs expressed high-level VEGF-A but very low-level VEGF-C (Figure 13C), the data can be interpreted as the model containing LEC (inside) gave LECs the best survival status to (1) contribute the expression of VEGF-A of the whole model, as well as (2) to pull down the expression of VEGF-C of the model because the adding of LEC diluted the percent of VEGF-C RNA in the whole RNA of the model.

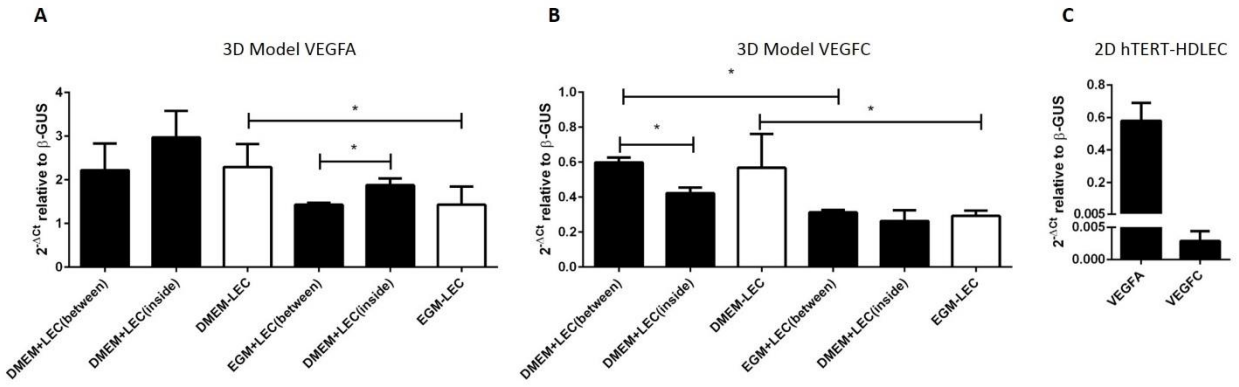

Figure 13. The 3D tissue models are able to express growth factors to support the survival of LECs.

After 7 days air exposure. total RNA were extracted from the tissue models. The histograms show the values of 2-ΔCT relative to β-GUS obtained by Taqman assay. (A) The expression of VEGFA in different types of tissue models. (B) The expression of VEGFC in different types of tissue models. (C) The expression of VEGFA and VEGFC in 2D cultured (monolayer) hTERT-HDLECs. "DMEM": tissue model was cultured in DMEM media; "EGM": tissue model was cultured in a 1:1 mixed media of DMEM and EGM2-MV; "+" or "-": model contains or does not contain hTERT-HDLEC; "LEC(between)": hTERT-HDLECs were seeded between stroma and epithelial layer; "LEC(inside)": hTERT-HDLECs were seeded inside stroma layer. Data represent more than two (model) or one (2D hTERT-HDLEC) independent experiment(s). Symbol * means significant difference ($P<0.05$) is observed between the two columns. (Paired t-test was done to calculate P-values). Error bars indicate the SD value.

To analyze the distribution, morphology and survival status of LECs in the model, hTERT-HDLECs were pre-stained with the lipophilic live cell dye CM-dil before being added into the tissue models. Then live or fixed models were analyzed using fluorescent microscopy (Figure 14, Figure 15).

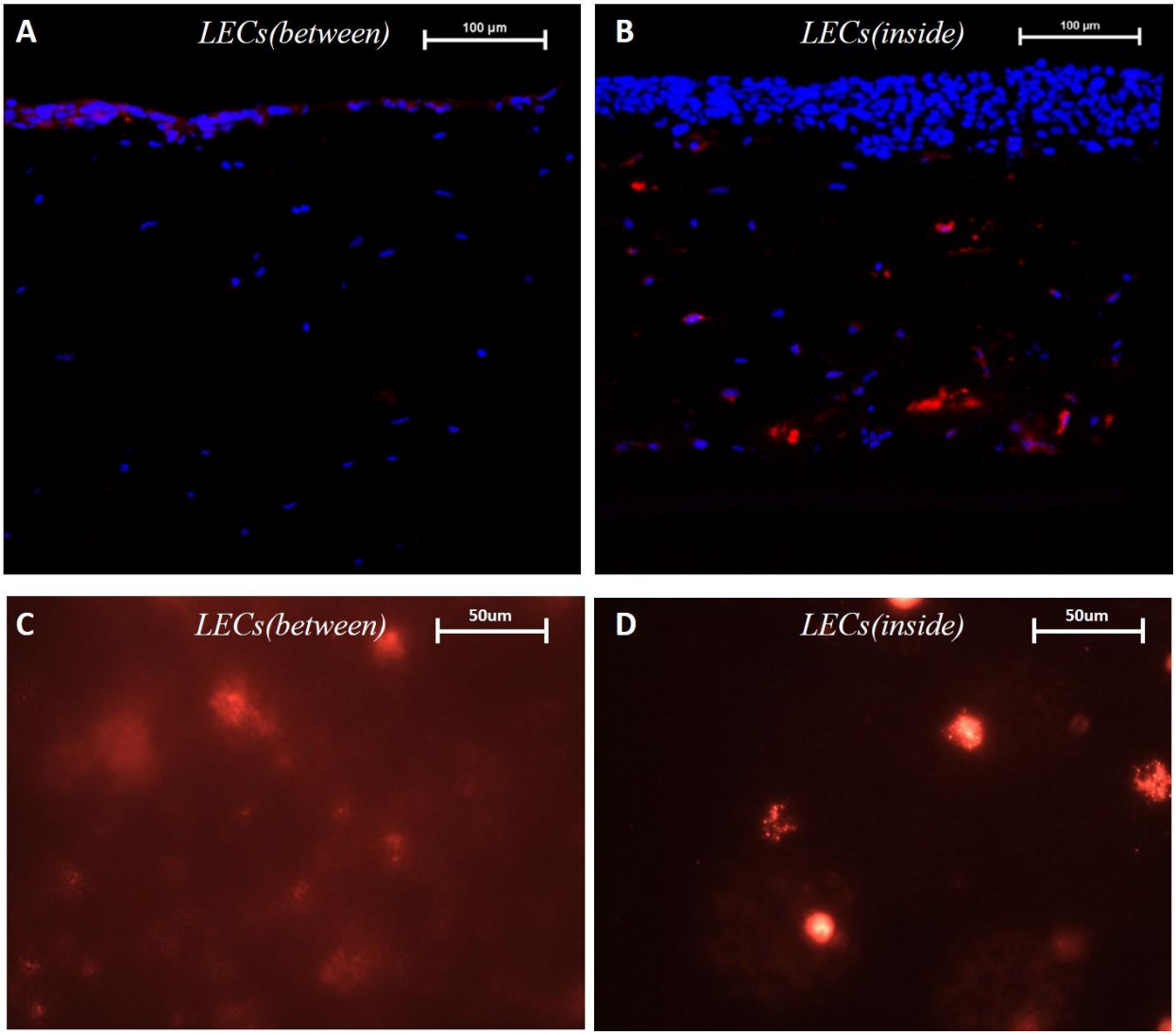

Figure 14. 3D lung tissue models containing LECs: Section views of fixed tissues and Top views of *live tissues.*

After 7 days air exposure, DMEM media cultured tissue models with LECs(between) (A,C) and tissue models with LECs(inside) (B,D) were analyzed for their structures. (A-B) 10-µm cryosections of 4%PFA fixed 3D lung tissue model with CM-Dil per-stained hTERT-HDLECs (red) were stained with DAPI (blue) and imaged under fluorescent microscopy (magnification X200). (C-D) live tissue models with CM-Dil per-stained hTERT-HDLECs (red) were directly imaged under fluorescent microscopy (FITC channel, magnification X200). Pictures show one representative experiment out of at least two independent repeats.

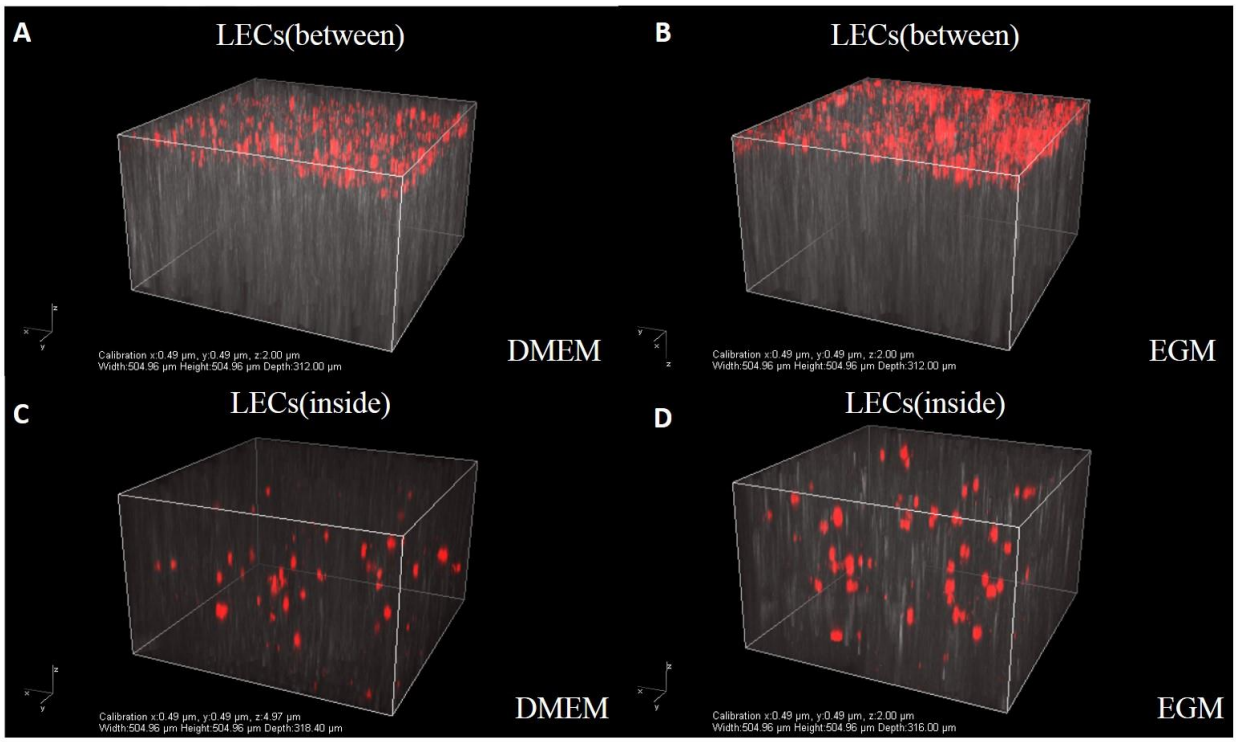

Figure 15. 3D lung tissue models containing LECs: 3D views of live tissues.

After 7 days air exposure, DMEM media cultured (A and C) or EGM media cultured (B and D) tissue models that contain LECs (inside) (C and D) or LECs (between) (A and B) were analyzed for their structures under fluorescent confocal microscope (Z-stack, under bright field and TRIC channel). Figures are representative data out of at least two independent repeats.

In the images of live tissues (under both phase contract fluorescent microscope and confocal microscope), clear round cell shapes of CM-dil labeled hTERT-HDLECs were observed in models containing LECs (inside) cultured in both media (Figure 14C, Figure 15C and D); While for models containing LECs (between), red spots that are smaller than the size of normal cells were observed (Figure 14D, Figure 15A and B), these small red spots may be regarded as either dead LEC particles or lipid containing organelles of live LECs. For both the models containing LECs (between) (Figure 15A and B) and the models containing LECs (inside) (Figure 15C and D), "EGM" culture condition (Figure 15B and D) always enabled the tissue model to have more "cells".

In the images of fixed tissues (Figure 14A and B), a mixture of LECs and epithelial cells was observed in models containing LECs (between), this is reasoned from the culture method of seeding two sets of cells on top of each other. For the models containing LECs (inside), several red-shaped LECs containing blue-stained nucleus was observed, indicating the survival of LECs in the model.

Besides the histological analysis, flow cytometry was also performed to analyze the survival of LECs in the model. Whole cells were isolated form the model, then cell type specific staining and live-dead staining followed by flow cytometry were done (Figure 18). The data is not representative and convincing because of the strong background binding of isotype control antibodies.

According the data obtained by now, the culture method of including hTERT-HDLECs inside the stroma layer (LECs (inside)) supported better the survival of LECs than the culture method of putting LECs between the stroma layer and epithelial layer (LECs (between)). The 1:1 mixed media of DMEM and EGM2-MV supports better the growth of LECs in the model than using DMEM only. Though the LECs were observed being alive in the tissue model, different for the original hypothesis, no evidence has been obtained to show the LECs were enabled network forming in the model. In addition, further utilization of the model is needed to show the efficiency of the model.

5.0 DISCUSSIONS

Lymphatic endothelial cells have become a relatively new research topic since the discovery of LEC-specific cell surface markers at the turn of the century [2-4], which has enabled the isolation of LECs from BECs in mixed EC populations. LECs were shown to function more than simply as a lining for the lymphatic vessels and lymph node sinuses. They also have important functions directly responding to as well as mediating other cells' responses to infections and cancers [7-11, 16-19, 28-33]. The work in this thesis provides a foundation for further LEC research in two aspects: (1) the cytokine receptor expression profiles of LECs; and (2) development of 3D tissue models containing LECs.

5.1 CYTOKINE RECEPTOR EXPRESSION PROFILES OF LECS

This project included a cytokine receptor expression profile study of human LECs. Cytokines are small molecules secreted by cells and function on the producer (autocrine) or other (paracrine) cells. Cytokines have pleiotropic effects on cell survival, growth, and functional activities. Increasing the knowledge of cytokine or cytokine receptor expression by LECs will contribute to a better understanding of the potential functions and activities of LEC. In this thesis project, the observations that at the mRNA level LECs have a wide-range of expression of cytokine receptor mRNAs, although most are at relatively low levels (Figure 1 and Figure 2), provided indications as to the possible responsiveness of LECs to their environments. These findings reveal insight into the functions LECs may have related to cytokine/receptor interactions. Moreover, similar

with our laboratory's previous observation that poly I:C treatment changed the expression levels of cytokines in LECs [19], poly I:C treatment changed the expression of multiple cytokine receptor related mRNAs at both the mRNA and protein levels (Figure 1C and Figure 4). These observations support the existence of signaling and transcriptional regulation pathways, such as the MyD88-dependent pathway [85], that link TLR3 ligand sensing and the modulation of expression of cytokine receptors.

In addition to TLR3, our previous study [19] and this thesis work (Figure 1) have shown the expression of TLR 1-6 and 9 in LECs. The expressions of TLRs, major members of the family of pattern recognition receptors, together with the evidences that LECs express the malarial parasite receptor DARC (Figure 1), HIV-1 co-receptors CXCR4, CCR5 (Figure 1) [86-89], and *Mycobacterium tuberculosis* receptor mannose receptor (MR) [90, 91], indicate that LECs have the potential to sense different pathogens. Whether these receptors are used by pathogens to enter or traverse LECs, or by LECs to capture and eliminate pathogens, is not clear and will require further study.

LECs express CCR6 to low levels (Figure 1 and Figure 2). Combined with the evidence that LECs can express CCL20 to high levels [17], the possibility may exist as the highly expressed CCL20 in LECs strongly inhibit the CCR6 expression thorough an autocrine loop. Interestingly, LN LECs are able to express chemokine receptor CCRL1 to regulate CCL21 secreted by themselves and other cells, allowing them to regulate the CCL21 gradient, providing evidence for the possible existence of other untypical autocrine loops controlling LECs. In addition, CCR6 was recently found to be a new co-receptor for HIV-1. CCR6 transfection enable SIV and HIV infection to the originally HIV-resisted CD4-transduced NP-2 cell (NP-2/CD4) [92]. To date, direct evidence showing infection of LECs by HIV-1 has been lacking. It may be because LECs

have not been detected as having expression of CD4 [86, 88], the main receptor of HIV. At the same time several HIV co-receptors have been shown by others, or in this thesis, to be expressed by LECs or ECs, including CXCR4 (Figure 1A) [86-88] or CCR5 (Figure 1A) [89]. These co-receptors might mediate several interactions between LECs and HIV proteins to mediate a fully infectious effect of HIV on ECs, such as: (1) HIV-1 gp120 protein induced cytotoxic effects on human lung endothelial cells [86]; (2) hyperpermeability effects on lymphatic cell monolayers [88]; or (2) HIV Tat stimulation of angiogenesis of vascular endothelial cells [93].

LECs express growth factors including VEGF-A and VEGF-C (Figure 13), and PDGFRs (Figure 1Figure 2). Among them VEGF-C and its receptor VEGFR3 are the best studied with regard to LECs, and most observations about lymphangiogenesis focus on the interference of the VEGF-C-VEGFR3 interaction and LECs [2, 3, 15, 22, 28, 30, 69, 71]. Interestingly, relatively low expression of VEGF-C by LECs was observed in this project (Figure 13). It is possible that the VEGF-C-driven lymphangiogenesis of LECs is mainly regulated by the environmentally secreted VEGF-C (i.e., production by other cell types in tissues). This study also draws attention to the expression of PDGFRs in LECs. The expression of PDGFRα (Figure 1 and Figure 2) provides LECs the potential to respond to all PDGFs, some members of which have been shown to function in lymphangiogenesis [58]. In addition, poly I:C increased expression of PDGFRA mRNA in LECs, potentially explaining, in part, the possible viral infectious effects on lymphangiogenesis.

The expression of cytokine receptors will enable LECs to respond to subsets of cytokines and the collection of expressed and signaling receptors will control the responses of LECs and in turn their modulation of the local milieu. Among the interleukin family receptors, because of the in depth knowledge about IL6 family cytokines' extreme pleiotropy in mediating inflammatory

activities, cell growth, cell survival and angiogenesis [68, 76, 79, 94], and given the abundant expression of IL6ST, the IL6 cytokine family receptors were studied in detail as part of this thesis.

Currently, knowledge of the interaction between LECs and IL6 family receptors exists, but has been limited to the observation that IL6 can induce VEGF-C expression in an immortalized murine LEC cell line [69], as well as our previous observation that OSM affected the transcriptional expression of several cytokines and adhesion molecules in HMVEC-dLy, HMVEC-LLy and hTERT-HDLEC cell populations [19]. At the same time, there is almost no information about the interactions between LIF and LECs, though there was an interesting story about the opposing (sometimes enhancing, while sometimes inhibitory) impacts of LIF on the growth of ECs [71-74]. Following the initial screening assay, this thesis aimed at providing more convincing and direct evidence of the potential interactions between IL6 family cytokines and LECs.

Based on the mRNA, surface staining, and functional results on IL6 family cytokine receptor expression and function in HMVEC-dLy, HMVEC-LLy, and hTERT-HDLECs, we showed that the LECs express IL6Rα, LIFR, OSMR, and IL11Rα but not CNTFRα mRNAs (Figure 2). The LECs abundantly expressed IL6ST protein on cell surface, but the cell surface expression of LIFR, OSMR and IL27R proteins were very low (Figure 4). Interestingly, LECs nevertheless could respond to IL6, LIF, and OSM ligands treatment as evidenced by phosphorylation of STAT3 (Figure 5). These findings provided evidence of functional IL6ST, IL6Rα, LIFR and possibly OSMR (because without OSMR, OSM can still signal through LIFR/IL6ST complex [64, 94]). The inconsistencies between the flow cytometric data and the functional data might indicate the presence of soluble IL6 family receptors. IL6Rα [95], LIFR [96] and OSMR [97] have been shown to have soluble forms. But only soluble IL6Rα was thought

to be able to work as a substitute to membrane bound IL6Rα, which means soluble IL6Rα could function as a membrane bound IL6Rα to bind firstly with IL6 and then with IL6ST homodimer to trigger the signal pathway, whereas soluble LIFR and soluble OSMR were thought to be antagonists of LIFR and OSMR, respectively, binding with LIF or OSM to prevent their being recognized by functional membrane bound LIFR and OSMR [98]. To test this, ELISA could be performed to exam the possible secretion of soluble IL6 family receptors by LECs. In addition, IL6ST or some other receptor(s) might be able to recognize IL6, LIF and OSM, and stimulate the transport of the related IL6 family receptors from the cytoplasm to the cell surface, although no evidence has been found that other than viral IL6 [65], secreted by HHV-8, IL6ST can directly recognize other ligands. To test this hypothesis, IL6 family ligand treatment followed by flow cytometric analysis on the cell surface and intracellular expression of IL6 family receptors could be performed to track the possible transport of these receptors from cytoplasm to plasma membrane. Additional possibilities include the possibility of experimental error, such as with cell surface staining that did not work properly.

IL6 and IL6ST take important roles in the story between Kaposi's sarcoma-associated herpesvirus (KSHV) or human herpesvirus 8 (HHV-8) and ECs. HHV-8 expresses viral IL-6 (vIL6), which is a homolog of human IL-6 because of their sequence and structural similarities [99, 100]. vIL6 is secreted by HHV-8 infected cells at low level during latency but at high level during the lytic cycle [101]. vIL6 were shown to mainly gather at the endoplasmic reticulum of infected cells and function through autocrine singling pathway by binding to intracellular IL6ST [102]. Interestingly, vIL6 gene transfection induced BECs' differentiation into LECs [103]. In addition to intracellular vIL6, serum vIL6 was detectable in KS patients [101]. Isolated vIL6 was shown to directly function through IL6ST without the need for IL6Rα [65], and induce the

expression of VEGFC as well as angiogenesis and hematopoiesis in mice [104]. As one of the major players that are utilized by cancer cells, including HHV-8 infected cells, to achieve metastasis [12], LECs were confirmed in this thesis to express IL6ST and possible IL6Rα (Figure 2, Figure 4), strongly supporting the potential of LECs to respond to vIL6. Poly I:C increased IL6ST expression (Figure 1) in LECs, supporting the idea that infections by viruses such as HHV-8 itself or co-infection with HIV-1 might aggravate Kaposi's sarcomas because of an increased response of LECs to vIL6. Also, although not shown as a necessary unit, the possible expression of either membrane bound or soluble IL6Rα by LECs might be able to enhance vIL6 signaling and function. Obtaining basic knowledge of the interactions between LECs and vIL6 could help with designing new therapies for Kaposi's sarcoma targeting the interaction between vIL6 and IL6ST (possibly, also between vIL6 and IL6Rα) of LECs.

Importantly the observations in Figure 5 provided direct evidence that LECs can respond to IL6 ligand without the adding of soluble IL6Rα. Since 1997, when two reports showed that human umbilical veins ECs could only respond to IL6 in the presence of sIL6Rα [81, 105], the idea that ECs have no expression of IL6Rα so cannot respond to IL6 without sIL6Rα has been widely accepted and frequently cited during the past 20 year [83, 106-109]. But our observations in Figure 5, together with another report showing IL6 alone could increase the expression of VEGF-C in murine LECs (SV-LEC) [69], could break the paradigm of the knowledge and the experiment methods for further study targeting on the interaction between IL6 and LECs.

In addition to our pervious observation that OSM affected the transcriptional expression of CCL20, CCL21, CXCL10, CXCL12, ICAM-1, VCAM-1 in HMVEC-dLy, HMVEC-LLy and hTERT-HDLEC [19], the amount of researches showing the interactions between LECs and OSM or between LECs and LIF are very limited. Figure 5, which directly showed LECs are able to

respond to these two kinds of IL6 family cytokines, also provided strong foundations for further study about the interactions between LECs and these two IL6 family receptors.

5.2 THE DEVELOPMENT OF 3D TISSUE MODELS CONTAINING LECS

The development of 3D tissue models containing LECs was another goal of this thesis project. It was initiated by using Matrigel™ as a 3D culture material to grow LECs. In accordance with others' observations [13, 15, 23, 24], this thesis project also showed that Matrigel™ supported the forming of networks of LECs (Figure 6 and Figure 7), a process sometimes regarded as a model for lymphangiogenesis [13]. At the same time, doubts exist that the Matrigel™ induced "lymphangiogenesis" of LECs is in fact lymphangiogenesis, in that the networks formed by LECs on Matrigel™ have no lumen and the networks are formed by rearrangement of cells rather than the sprouting of tubes [25]. Different from other's observation that other cell types including fibroblasts and a breast cancer cell line also formed networks in Matrigel™ [25], we found the network formation on Matrigel™ was highly restricted to LECs (Figure 7Figure 8). The contradiction between our observations and others' observations, shows the variability of this 3D culture method with LECs. The complexity of the components in Matrigel™ increases the difficulty in analyzing the data obtained by Matrigel™ involved assays. The limitations of the Matrigel™ assay increased our interest in developing a new 3D culture method for LECs.

By modifying a lung 3D mucosal tissue model developed by our collaborators [21], we have developed a lung 3D mucosal tissue model containing LECs. Thus far, this project only included basic analyses to show some initial advantages of tissue models in the aspects of successfully imitating tissue structures (Figure 11, Figure 14, Figure 15) and supporting the

survival of LECs (Figure 12, Figure 13, Figure 14, Figure 15). In addition, no evidence was provided here that the tissue model supported LEC network formation or lymphangiogenesis.

In this thesis, a 3D lung mucosal tissue model containing LECs has been developed. Further modifications could be attempted to the current model to achieve different research aim. First, we could perform the tissue models with all types of primary cells isolated from animal species under study (e.g., mouse, monkey, or ferret) to replace or augment animal studies. Second, we could include more cell types (e.g., BECs, macrophages, DCs) into the tissue model to increase complexity. Third, we could include Matrigel™ component(s) as a stromal component into the model to attempt to force the vascularization of LECs. Fourth, we can add airborne pathogens (e.g., Influenza virus) at the apical side or basal side of the model to study pathogens' invasion into the tissue or release from the model. In addition, more GeneCopoeia real-time RT-PCR microarrays could be performed on LECs isolated from the tissue models to examine the expression of cytokine receptor mRNAs, extending the cytokine receptor expression study of this thesis with a comparison of 2D and 3D culturing of LECs.

In summary, the thesis provides a strong foundation for further LEC research by providing a database of cytokine receptor expression profiles of LECs as an analytic tool, and an in vitro lung mucosal tissue model containing LECs as an experimental tool.

6.0 PUBLIC HEALTH SIGNIFICANCE

The public health significances of the work represented by this thesis lies mainly in the importance of LECs and lymphatic biology in health. Lymphangiogenesis induced by cancer cells and cancer viruses (HHV-8, for example) is thought to be one important mechanism of cancer metastasis [30]. LECs as the construction unit of lymphatic vessels and LN sinuses also have the potential to regulate the immune system by controlling trafficking of DCs and mediating self-tolerance [1]. Though LECs are meaningful to study, focused research on LECs only started at the turn of the century when LEC-specific markers were found [2]. This thesis has increased the knowledge of LECs with the respect to LEC cytokine receptor expression profiling and development of an in vitro 3D culture method for LECs that will work as a strong experiment tool for further LEC study. Overall the data obtained through this thesis project have implications for public health significance. The study of IL6 family cytokine receptor expression by LECs provided clues that LECs may be candidate targets for IL6-focused therapy say for cancer [110]. Further, we have shown that LECs have receptors or co-receptors for several pathogens including HIV-1, malarial parasite and *Mycobacterium tuberculosis*, indicating the LEC-target therapies could be developed to prevent or treat infectious diseases.

7.0 MATERIALS AND METHODS

Cell Lines, Media and Cell culture

The human telomerase reverse transcriptase transfected human dermal lymphatic endothelial cells (hTERT-HDLEC) was a gift from Dr. Michael S. Pepper of University of Pretoria. The cell line was developed as described in [20]. Human dermal microvascular lymphatic endothelial cells (HMVEC-dLy) and human lung microvascular lymphatic endothelial cells (HMVEC-LLy) were purchased from Lonza (MD, USA). Ferret lung isolated LECs (FeLg-LEC) and macaque jejuna isolated LECs (R24J) were prepared as described previously [15]. HMVEC-dLy, HMVEC-LLy, hTERT-HDLEC were cultured in EGM-2MV medium (BulletKit™, Lonza, USA). Ferret and macaque LECs were cultured in EGM-2 medium (BulletKit™, Lonza, USA). All primary LECs were used for experiments before passage 10. Human lung normal fibroblast cell line MRC5 was purchased from ATCC (VA, USA). MRC5 cells were cultured in DMEM media (Lonza, USA) supplemented with 10% FBS (Thermo Fisher Scientific, USA), 1% HEPES (Hyclone), 1% 100X non essential amino acids (Gibco™, Thermo Fisher Scientific, USA), 1% 10,000 units/mL penicillin and 00,000 ug/mL streptomycin mixture (Gibco™, Thermo Fisher Scientific, USA), 1% 200mM L-glutamine (Gibco™, Thermo Fisher Scientific, USA), and 1% 100mM sodium Pyruvate (Gibco™, Thermo Fisher Scientific, USA). 16HBE14o- was a gift from Dr. Michael Yezzi, University of California, San Francisco. 16HBE14o- cells were cultured on fibronectin coated flasks: 1ml (for T25 flasks) or 2ml (for T75 flask) fibronectin coating solution (88% LHC basal medium (Invitrogen™, Thermo Fisher Scientific, USA), 0.1% bovine serum albumin (Sigma-Aldrich, USA), 1% 3mg/ml Vitrogen 100 collagen I (BD Bioscience, USA) and 1% 1mg/ml human fibronectin (BD Bioscience)) were used to coat the flask. MEM media (Invitrogen™,

Thermo Fisher Scientific, USA) supplemented with 10% FBS (Thermo Fisher Scientific, USA), 1% 200mM L-glutamine (Gibco™, Thermo Fisher Scientific, USA) and 1% 10,000 units/mL penicillin and 100,000 ug/mL streptomycin mixture (Gibco™, Thermo Fisher Scientific, USA) were used to culture 16HBE. All cells were cultured at 37 °C and 5% CO2 in humidified air. For all kinds of cells, 80–90% confluent monolayers of cells were regarded as ready for performing passaging or experiments. Confluent cells were washed with either PBS (Lonza, USA) for MRC5 and LECs, or HBSS (Thermo Fisher Scientific, USA) for 16HBE, then treated with either 0.05% trypsin-EDTA (Gibco™, Thermo Fisher Scientific, USA) for MRC5, or PET™ (Athena Environmental Sciences, USA) for 16HBE, or accutase (Sigma-Aldrich, USA) for LECs for 3-5 min to dissociate attached cells, treatment were stopped by adding cell type specific medium. Dissociated cells were then centrifuged at 1200rpm and resuspended in type specific medium for further passaging or experiments.

Lung Mucosal Tissue Models

The setup of the basic 3D tissue models has been described [9] by Dr. Mattias Svensson's laboratory at The Karolinska Institutet, and with whom we collaborated to learn this approach. The mucosal lung tissue models were generated on 3μm Falcon™ Cell Culture Inserts for use with 6-well plates (Corning, USA) through a 3 week culturing process. In brief, an acellular layer mixture of type1 fetal bovine collagen (3.1 mg/ml, Advanced Biomatrix, USA) and DMEM was seeded on the top of the inserts set on 6-well plates. Then a 30min incubation was performed at 37°C to achieve gel formation. Then another collagen and DMEM mixture containing MRC5 fibroblast cells inside ($2.3X10^5$ cells for each model) were added as a cellular layer on the top of the acellular layer. 2h 37°C incubation was then done and followed by adding 2ml DMEM media into the bottom of the wells (outside the inserts). Models were then incubated at 37°C for 7 days

to allow the forming of the stroma layer of the model with media inside and outside inserts changed for every second day. After 7 days, media inside the inserts were removed and media outside the inserts were replaced with 1.5 ml DMEM media. Then epithelial cells were seeded at the amount of 5 $X10^4$ cells in 50 µl complete DMEM to each insert. And 2h incubation followed by adding 1.5 complete DMEM into the inserts was done as describe above. Submerged cultures were incubated for an additional 3 days in 5% CO2 at 37°C to allow the epithelial cells to form a confluent monolayer. Finally the air exposure was done by removing the media from the inserts and changing the 1.5ml DMEM media outside the insert every second day. This process lasted for 4-10 days. At the 7th day of air exposure, tissue models ready to be analyzed.

For models containing LECs between the stroma layer (MRC5 layer) and epithelial layer (16HBE): After culturing the stroma layer (MRC5 layer) for 7 days, media inside the inserts were removed and media outside the inserts were replaced with 1.5 ml DMEM media or "EGM" media (1:1 mixture of DMEM and EGM2-MV). Then $1X10^5$ hTERT-LECs in 50ul media (DMEM or "EGM") were then added on the top of the 7-days models. Following 2h 37°C incubation, 1.5 media (DMEM or "EGM") was added into the inserts. 3-4 days 37°C incubation was then done to let LECs' survival. Then 16HBE seeding, 3-days culturing and 7-days air exposure are done as described in the "basic model" part, except for the media used for the models containing LECs are either DMEM or "EGM".

For models containing LECs inside the stroma layer (MRC5 layer): after the 30min incubation of an acellular layer mixture of type1 fetal bovine collagen (3.1 mg/ml, Advanced Biomatrix, USA) and DMEM, another collagen and DMEM mixture containing MRC5 cells and hTERT-HDLECs inside ($2.3X10^5$ of MRC5 cells and $1X10^5$ hTERT-HDLECs for each model) were added as a cellular layer on the top of the acellular layer. Then 7-days incubation, 16HBE

seeding, 3-days culturing and 7-days air exposure are done as described in the "basic model" part, except for the media used for the models containing LECs are either DMEM or "EGM".

Pre-staining LECs with CM-dil

Confluent hTERT-HDLECs on T75 flask were washed one time with PBS, then treated with 3ml accutase (Sigma-Aldrich, USA) for 3min at 37°C to detach from culture flasks, followed by adding 2ml EGM-2MV to stop accutase digestion. Cells were centrifuged at 1200rpm for 5 min, resuspened in 1ml EGM media without serum and counted for cell amount. Then 5µl CellTracker™ CM-DiI Dye (Molecular Probes™, Thermo Fisher Scientific, USA) were added for every $1X10^6$ cells, incubated at 37°C for 20min. Then cells were washed by being centrifuged at 1500rpm for 5min and then resuspended in 1ml EGM media (Lonza, USA) without serum. The washes were totally repeated 2 more times. Then the cells are ready to be added into the tissue model or plated on the Matrigel™ Matrix (Corning, USA).

Immunohistochemistry

The fixation, freezing, cryosectioning and immunohistochemistry of 3D tissue models and control macaque tissues was performed as described [21]. Antibodies used for IHC were specific for the following targets: podolanin (D2-40, Dako, USA), vimentin (RV202, Abcam, Germany), and mouse IgG1 (DK-2600, Dako, Glostrup, Denmark).

LEC Network Formation Assay on Matrigel™

The network forming assay is modified from [6]. Matrigel™ Matrix (general type or growth factor reduced, Corning, USA) were plated in 24-well plate (180ul for each well) and the plates were then put in 37℃ for 30min to achieve gelation. LECs within EGM-2MV media were then added into the coated wells (50,000 cells/well) top and incubated for 18-40h. Cells were observed under an inverted microscope Nikon TS100 and pictures were taken after 2, 16, 18, 24 and/or 40h.

Stimulation of LECs with poly I:C and RNA isolation

LECs that formed 80–90% confluent monolayers in flasks were treated with 25μg/ml poly I:C (InvivoGen, USA) parallel with mock treatment for 24h. Total RNA extractions were performed using Trizol (Life Technologies, USA) and RNeasy® Mini Kit (Qiagen, Germany) according to the manufacturer's recommendation. The same total RNA extraction protocol was used to extract RNAs from the 3D organotypic culture models.

Taqman™ Real-time RT-PCR

Confluent monolayer of HMVEC-dLy, HMVEC-LLy or hTERT-HDLEC cells that had been treated with or without poly I:C for 24h or established and mature 3D mucosal tissue model were treated with 900ul TRIzol (Thermo Fisher Scientific, USA) (tissue models were then homogenized in TRIzol by a power homogenizer). Then RNA extraction, template preparation and real-time RT-PCR for host mRNAs using a 2-step protocol were performed as described before [22]. Probes and Primers of human VEGFA (Hs0090055_m1), VEGFC (Hs01099203_m1), CCR6 (Hs00171121), DARC (Hs01011079), IL2RG (Hs00953624_m1), IL7R (Hs00902338_g1), TLR3 (Hs01551078_m1), IFNAR1 (Hs01066116), EPOR (Hs00959427), PDGFRA (Hs00998018), IL6ST (Rh01006738), IL6Ra (Hs01075666_m1), CNTFR (Hs00181798_m1), LIFR (Hs01123581_m1), OSMR (Hs00384278_m1), IL11Ra (Hs00234415_m1) and β-GUS (Hs99999908_m1) were purchased from Applied Biosystem (Taqman® gene expression assay, USA) for real-time real-time RT-PCR. Then the real-time RT-PCR (comparative Ct method) was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, USA) as described in [15]. All samples were assayed in duplicate and with concurrent controls that lack reverse transcriptase in the cDNA synthesis reaction.

GeneCopoeia™ real-time RT-PCR Array

The ExProfile™ Human Cytokine Receptor Related Gene qPCR Array (GeneCopoeia, USA) was performed by Dr. Stella Berendam following the manufacturer's suggestion.

Cell isolation form 3D tissue model

Established and mature tissue models were removed from the membrane of inserts with a scalpel, washed twice with RPMI-1640 (Sigma-Aldrich, USA) supplemented with 1% 200mM L-glutamine (Gibco™, Thermo Fisher Scientific, USA) and 1% 10,000 units/mL penicillin and 100,000 ug/mL streptomycin mixture (Gibco™, Thermo Fisher Scientific, USA), cut into small pieces before a 30min incubation in 25ul/ml collagenase A (Roche Life Science, USA) and 20ul/ml DNase I (Sigma-Aldrich, USA) at 37 °C with stirring. Enzyme treatments were stopped by adding RPMI-1640 (Sigma-Aldrich, USA) supplemented with 10% FBS (Thermo Fisher Scientific, USA), 1% 200mM L-glutamine (Gibco™, Thermo Fisher Scientific, USA) and 1% 10,000 units/mL penicillin and 100,000 ug/mL streptomycin mixture (Gibco™, Thermo Fisher Scientific, USA). Then the solution containing enzymatically digested tissues were mashed though 70um cell strainers (Corning, USA) to isolate single cells. Isolated cells were then wash twice with PBS by resuspending cells with 10ml PBS, spinning at 1200rpm and discard the supernatant. Finally, tissue isolated cells were resuspended in PBS and ready for further experiments.

Flow cytometric analysis

Confluent monolayer DLY, LLY or hTERT-HDLEC were detached from culture flasks by accutase (Sigma-Aldrich, USA) for 3–4 min, immediately washed with EGM2-MV. Detached Cells from 2D culture were stained with either APC-conjugated mAbs against human OSMR (R&D systems, USA, FAB4389A, 0.01µg/µl), IL27R (R&D systems, USA, FAB14791A, 0.025µg/µl), LIFR (R&D systems, USA, FAB249A, 0.01µg/µl), or BV421-conjugated mAbs against human CD130 (BD Bioscience, USA, 564153, 0.2µg/µl), or BB515-conjugated mAbs

against human CD126 (BD Bioscience, USA, 564623, 0.2μg/μl), or PE-conjugated mAbs against human Podoplanin (Angibio, USA, 11-009OE, 0.05μg/μl) for surface expression. Isolated cells from 3D model were stained with PerCP-Cy5.5-conjugated mAbs against human EpCAM (BD Bioscience, USA, 347199, 0.002μg/μl) and PE-Cy7-conjugated mAbs against human Podoplanin (Angibio, USA, 337013, 0.01μg/μl). Stained cells were analyzed using a BD LSRFortessa™ flow cytometer (BD Bioscience, USA). Staining with isotype control Abs was performed in parallel in all studies. Flow cytometry data were analyzed using the FlowJo (FlowJo, USA).

Statistical Analyses

Statistical analyses were performed using the Prism software package (GraphPad, USA). A P-value <0.05 was considered significant.

APPENDIX: SUPPLEMENTARY FIGURES

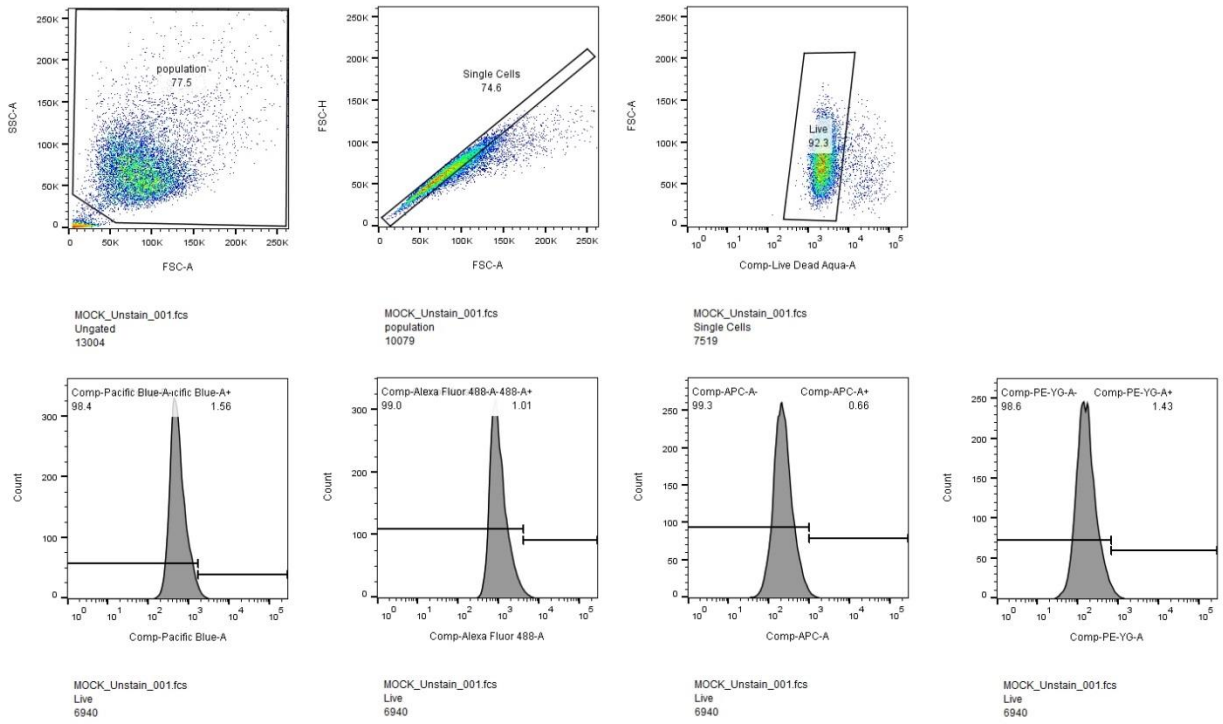

Figure 16. Representative gating strategy used for flow cytometric analyses of LEC cell surface cytokine receptor levels.

Shown is a representative gating strategy with unstained, untreated HMVEC-LLy cells. The positive staining population was set as 1% of isotype antibody stained cells. These data show one representative experiment out of at least two independent repeats performed with each LEC population.

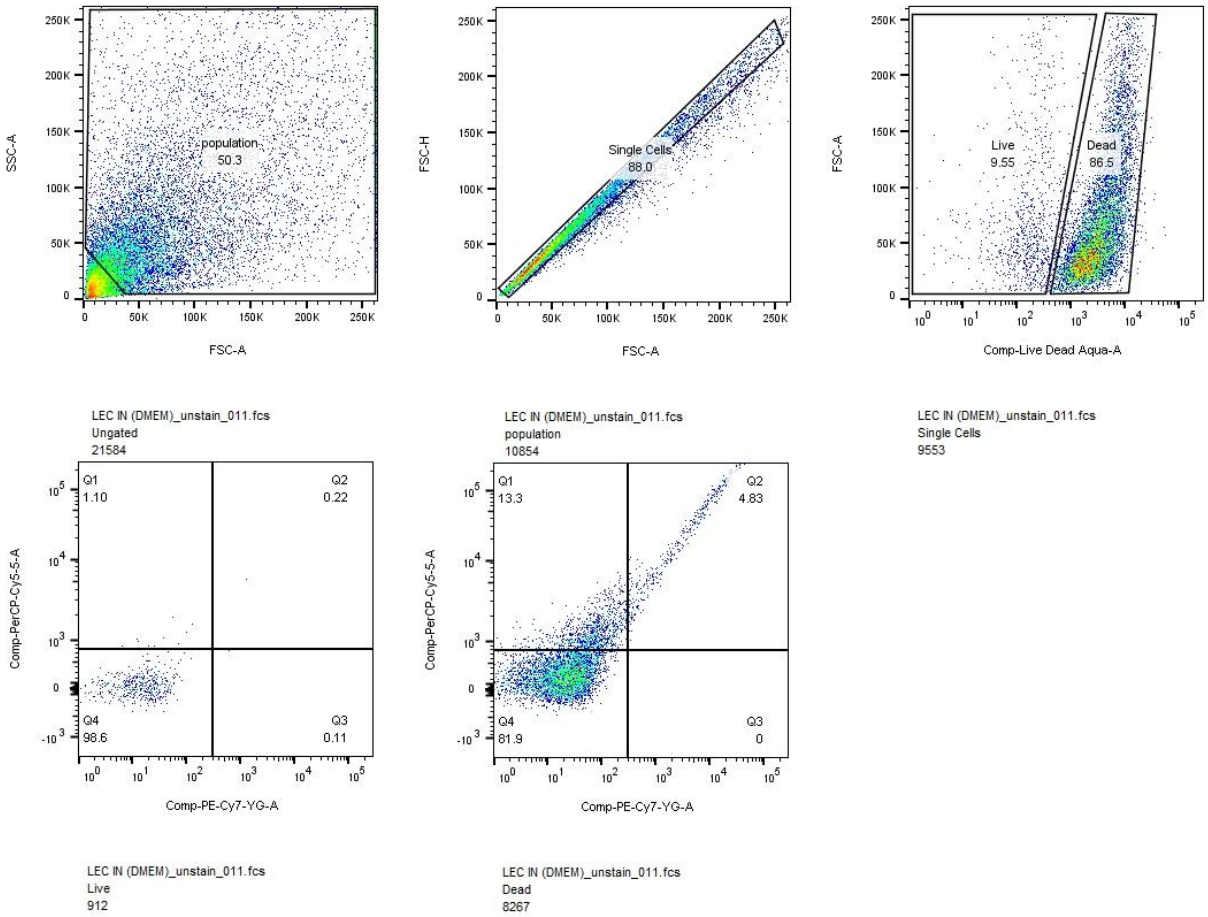

Figure 17. Representative gating strategy used for flow cytometric analyses of cell amount and survival status of cell isolates from tissue models.

Shown is a representative gating strategy with unstained model isolated cells from a model containing LECs (inside) cultured in DMEM. The positive staining population was set as 1% of isotype antibody stained cells. These data show one representative experiment.

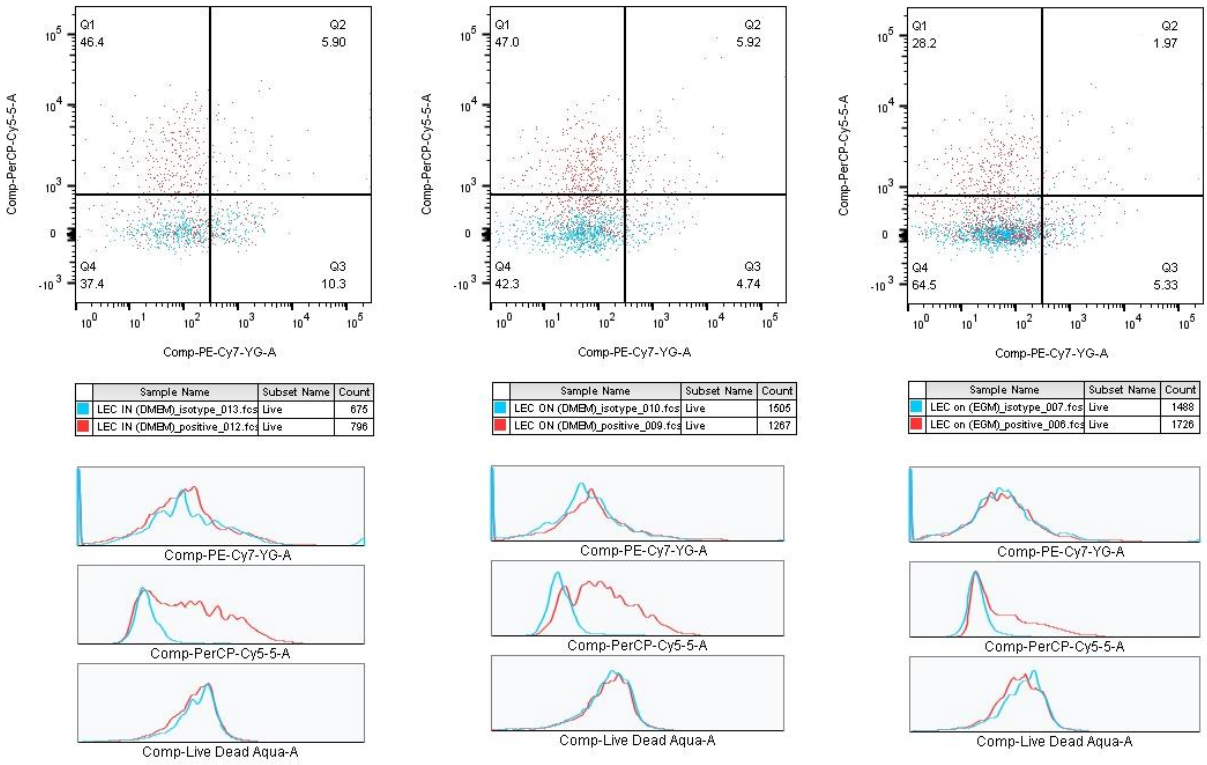

Figure 18. Cell type specific staining on cell isolations from the 3D tissue models.

A model containing LECs (inside) cultured in DMEM medium (left), a model containing LECs (between) cultured in DMEM medium (middle,) and a model containing LECs (between) cultured in EGM medium (right) were disaggregated to generate a single cell population and cell type marker staining was performed followed by flow cytometry. PerCP-Cy5.5 (y-axis) conjugated EpCAM-1 antibody was used as epithelial cell (16HBE here) marker, and PE-Cy7 (x-axis) conjugated Podoplanin was used as LEC marker. Isotype antibodies (ex. PerCP-Cy5.5 conjugated mouse IgG1) staining results were shown in blue, positive antibodies (ex. PerCP-Cy5.5 conjugated anti-human EpCAM-1) staining results were shown in red. EpCAM-1 staining worked well whereas podoplanin staining was not as clean due to strong background provided by the isotype antibody. These data represent one experiment.

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