



Original Articles

Tumor exposed-lymphatic endothelial cells promote primary tumor growth via IL6

Maureen Van de Velde^{a,1}, Marie Ebroin^{a,1}, Tania Durré^a, Marc Joiret^b, Lionel Gillot^a, Silvia Blacher^a, Liesbet Geris^b, Frédéric Kridelka^{a,c}, Agnès Noel^{a,*}

^a Laboratory of Tumor and Development Biology, GIGA-Cancer, Liege University, B23, Avenue Hippocrate 13, Sart-Tilman, B-4000, Liege, Belgium

^b Biomechanics Research Unit, GIGA-In Silico Medicine, Liege University, B34, Sart-Tilman, 4000, Liège, Belgium

^c Department of Obstetrics and Gynecology, CHU Liege, Sart-Tilman, 4000, Liege, Belgium



ARTICLE INFO

Keywords:

Lymphatic endothelial cells
Lymphangiogenesis
Endothelial cell plasticity
Tumor microenvironment
CAF

ABSTRACT

Solid tumors are composed of tumor cells and stromal cells including lymphatic endothelial cells (LEC), which are mainly viewed as cells forming lymphatic vessels involved in the transport of metastatic and immune cells. We here reveal a new mechanism by which tumor exposed-LEC (teLEC) exert mitogenic effects on tumor cells. Our conclusions are supported by morphological and molecular changes induced in teLEC that in turn enhance cancer cell invasion in 3D cultures and tumor cell proliferation *in vivo*. The characterization of teLEC secretome by RNA-Sequencing and cytokine array revealed that interleukine-6 (IL6) is one of the most modulated molecules in teLEC, whose production was negligible in unexposed LEC. Notably, neutralizing anti-human IL6 antibody abrogated teLEC-mediated mitogenic effects *in vivo*, when LEC were mixed with tumor cells in the ear sponge assay. We here assign a novel function to teLEC that is beyond their role of lymphatic vessel formation. This work highlights a new paradigm, in which teLEC exert “fibroblast-like properties”, contribute in a paracrine manner to the control of tumor cell properties and are worth considering as key stromal determinant in future studies.

1. Introduction

The tumor microenvironment (TME) is a complex ecosystem consisting of cancer cells, extracellular matrix and non-cancer stromal cells (immune, inflammatory, endothelial cells and fibroblasts) [1,2]. It is now well recognized that the complex crosstalk established between cancer cells and stromal cells actively contributes to tumor progression and metastatic dissemination that can occur through blood and/or lymphatic [3–5]. Lymphatic endothelial cells (LEC) lining initial capillaries are supported by a discontinuous basement membrane [6] and connected by “button-like” inter-endothelial adherens junctions formed, at least by homotopic interactions of vascular endothelial cadherin (VE-Cad). These specific discontinuous junctions could facilitate the uptake of interstitial fluid and (immune and cancer) cells, two key features of initial lymphatic vessels (LV) [7].

Within the TME, LEC respond to growth factors (mainly vascular endothelial growth factors: VEGF-A/C) and contribute to an important LV remodeling and the formation of new LV from pre-existing ones [8].

This process of lymphangiogenesis correlates with lymph node (LN) metastasis and poorer clinical outcome [8–10]. After a long debate in the scientific community, experimental mouse model studies demonstrated that metastatic cells in sentinel LN can further spread to distant organs by accessing specialized high endothelial venules (HEV) [11,12]. In addition to providing a route for tumor cell spreading, LV also influence anti-tumor immunity in primary tumor and in draining LN [13–15]. The complex interplay between LEC and the immune system in elaborating an immunosuppressive TME has recently emerged [14,16,17]. Advances in this field have highlighted LEC implication in immune cell recruitment/trafficking and in immunosuppression through different mechanisms that include, the production of PDL-1 promoting CD8⁺ T cell tolerization and indoleamine 2,3-dioxygenase (IDO) leading to tryptophane deletion and inhibition of T cell functions [13,15,18]. LEC appear increasingly as a heterogeneous cell population in terms of molecular and structural features, which display adaptive capacities [19,20]. The implication of LEC plasticity in the TME and how it could be involved during cancer progression and metastatic dissemination

* Corresponding author. Avenue Hippocrate, 13 Tour de Pathologie, B23/+4, route 688 Sart Tilman, B-4000, Liège, Belgium.

E-mail address: agnes.noel@uliege.be (A. Noel).

¹ The authors contributed equally to this work.

<https://doi.org/10.1016/j.canlet.2020.10.020>

Received 23 April 2020; Received in revised form 14 October 2020; Accepted 14 October 2020

Available online 17 October 2020

0304-3835/© 2020 The Authors.

Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license

(<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

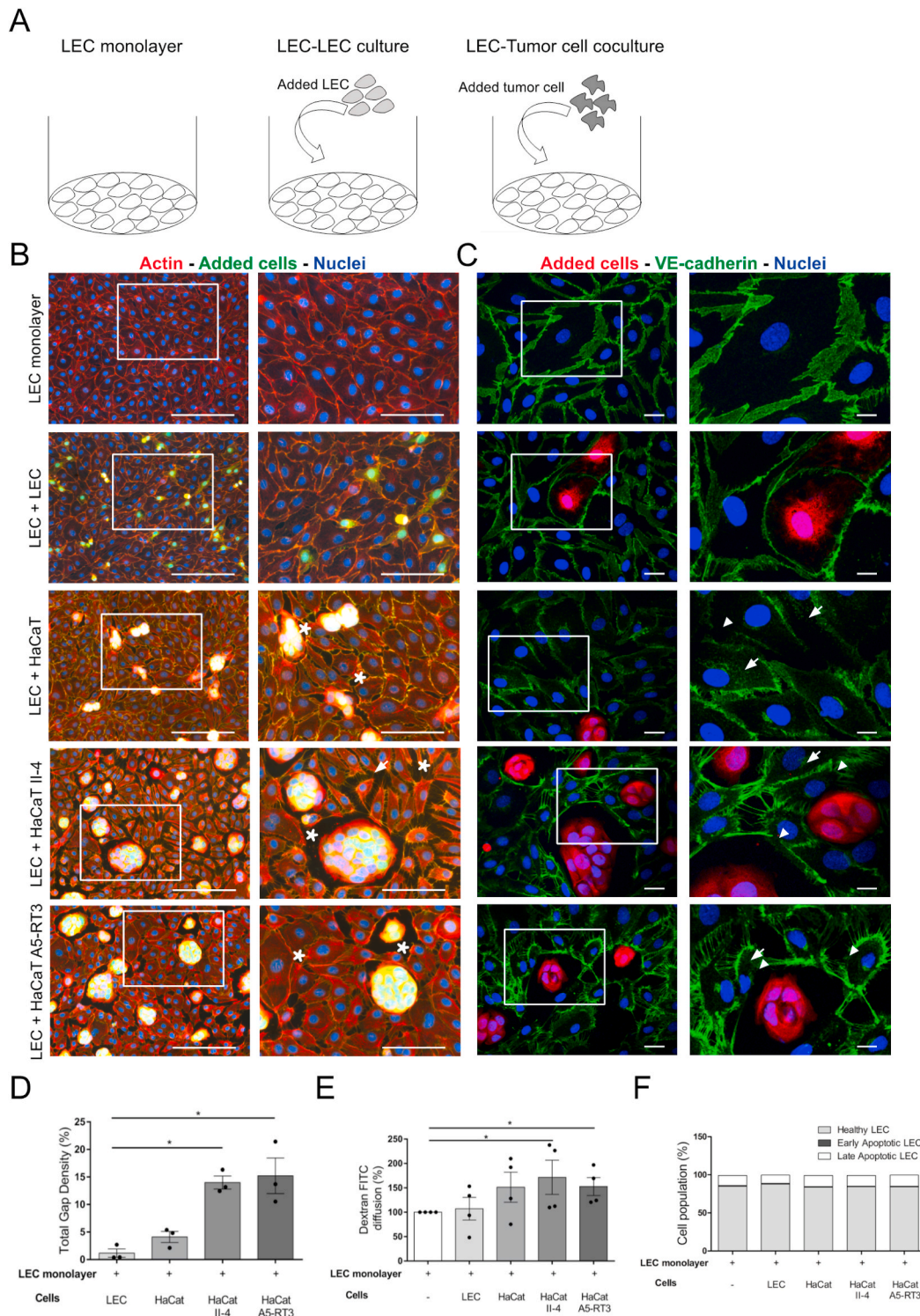


Fig. 1. Direct contacts between LEC and tumor cells induce a disorganization of LEC monolayer (A) Schematic representation of LEC-tumor cell co-cultures. LEC monolayer were exposed or not to LEC (LEC-LEC culture) or to tumor cells (HaCaT series as indicated) (LEC-Tumor cell coculture). (B-C) Actin (B) and VE-cadherin (C) immunostainings of LEC monolayer co-cultured or not with LEC (LEC + LEC) or tumor cells (as indicated). Added cells were labeled either with a green (B) or a red (C) cell tracker. Nuclei were counterstained with DAPI (blue). (B) White asterisks correspond to gaps between cells. Bars = 200 μ m and 100 μ m in the left and right (higher magnification of the insert) images, respectively. (C) Arrow heads delineate the discontinuous staining of VE-cadherin at the LEC surface. White arrows delineate VE-cadherin internalization. Bars = 50 μ m in the left panel and 25 μ m in the right panel (higher magnification of the insert). (D) Quantification of gap density. The histogram is expressed as percentage (surface occupied by gaps divided by the total image surface). (E) Quantification of Dextran-FITC diffusion (in percentage) through a LEC monolayer challenged with LEC or tumor cells. (F) Analysis of LEC apoptosis by flow cytometry. The histogram shows the percentages of each LEC populations: healthy LEC (light grey), early apoptotic LEC (dark grey) and late apoptotic LEC (white). Results are expressed as mean \pm SEM, and statistical analyses were performed using a Wilcoxon-Mann-Whitney test (* p < 0.05). Results are representative of at least three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

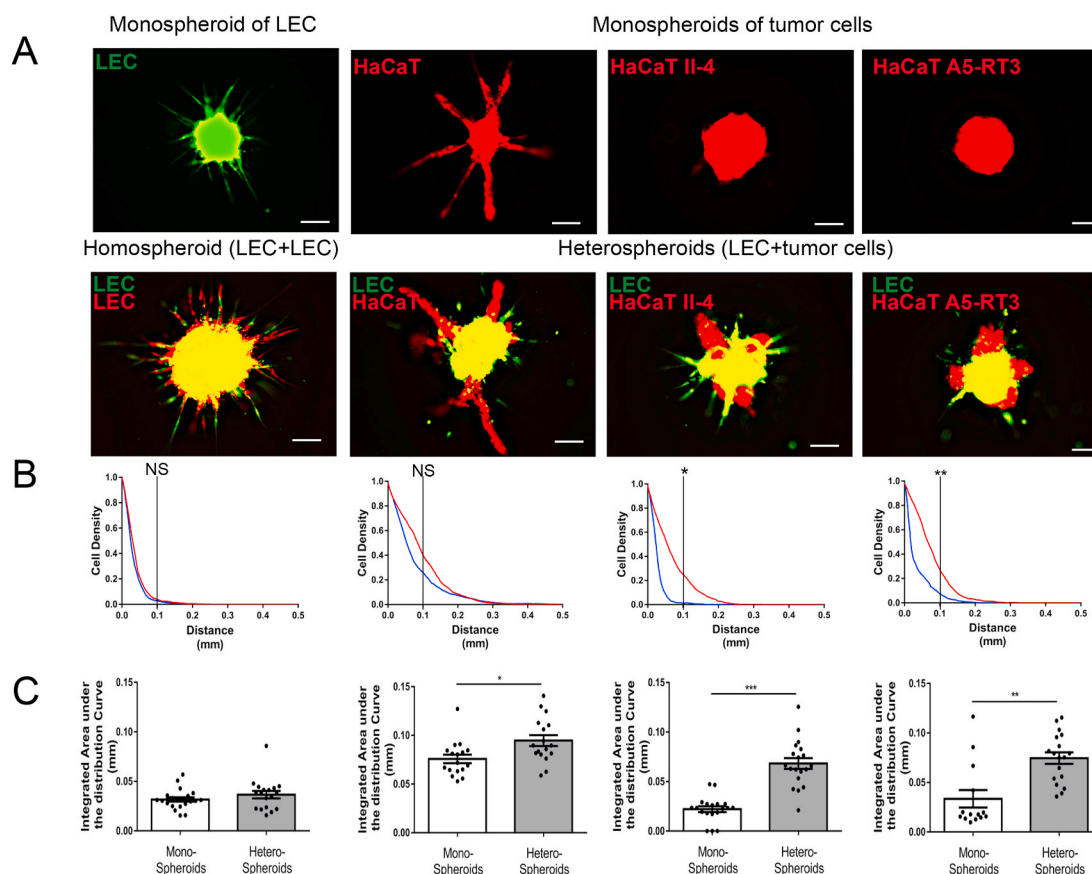


Fig. 2. LEC promotes tumor cell invasion in 3D cocultures. (A) Illustration of mono- and hetero-spheroids visualized by epifluorescence microscopy. LEC and tumor cells (HaCaT series as indicated) were stained by red and green cell trackers, respectively. Spheroids composed of green and red LEC were used as control. Bars = 100 μ m. (B–C) Computerized quantifications of cell invasion represented as cell density (B) in a function of distance from the spheroid border (in mm), in which the blue curve corresponds to monospheroids while the red curve refers to heterospheroids, and the area under the curve (C). All results are representative of two independent experiments (spheroid number/experiment \geq 14). Results are expressed as mean \pm SEM, and statistical analyses were performed using a Wilcoxon-Mann-Whitney test (* p < 0.05; ** p < 0.01; *** p < 0.001). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

remains poorly documented [21,22]. In primary tumors, peritumoral LV are often enlarged and considered as the major route for dissemination. In the opposite, intratumoral LV appear collapsed due to the tumor pressure and are viewed as unfunctional and inconsequential in the TME [9,23–25]. These observations raise questions on LEC involvement in the evolution of TME during cancer progression.

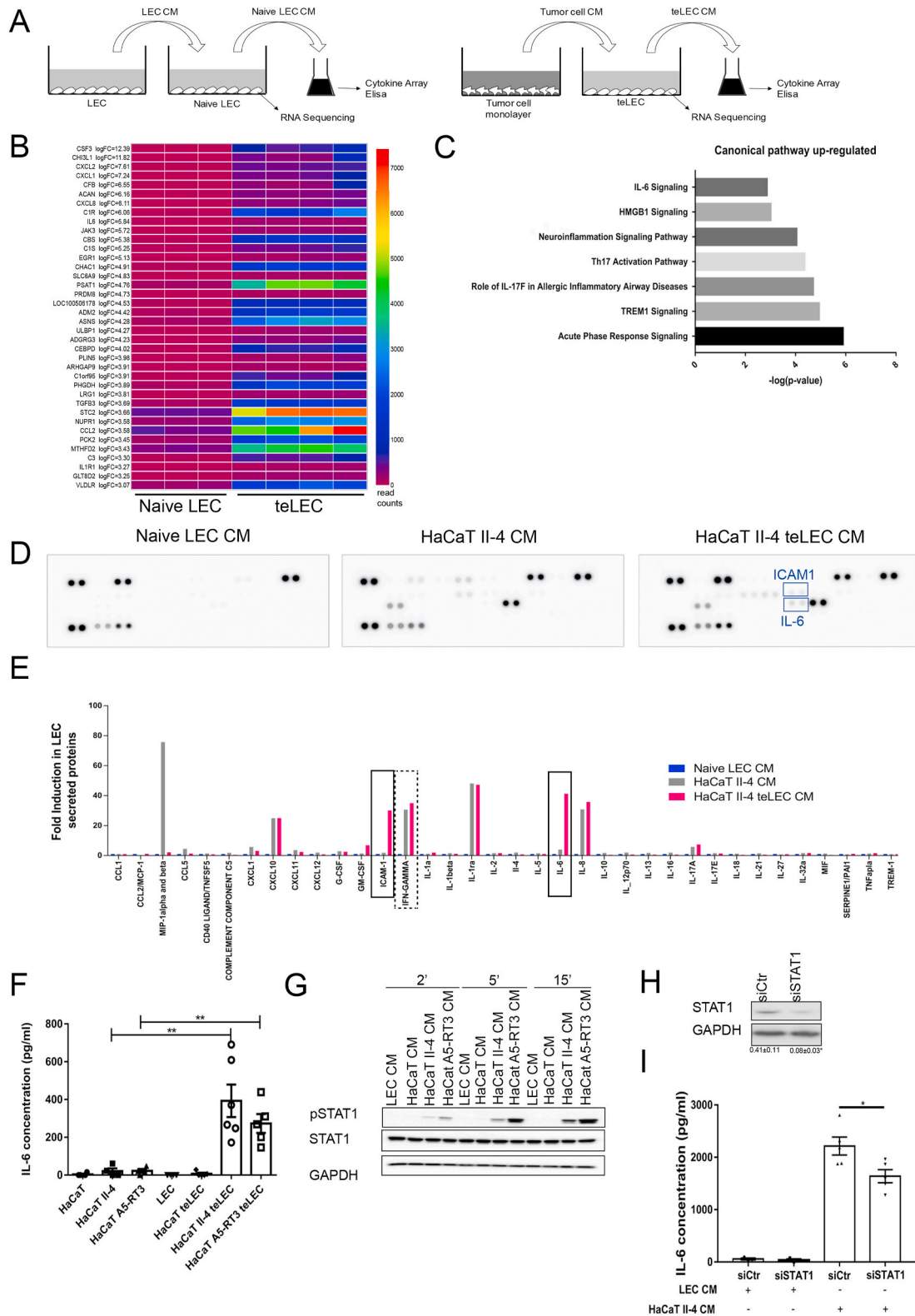
During the last decades, lymphatic research has mainly focused on molecular mechanisms driving lymphangiogenesis and how LV contribute to tumor and immune cell trafficking leading to an immunosuppressive TME and metastatic colonization in LN in distant organs. LEC-derived chemokines drive tumor cell migration towards LV [26,27] and direct tumor cell-LEC contacts promote melanoma cell intravasation and invasion [28]. Tumor cells can also disrupt intercellular interactions between LEC forming gaps in the lymphatic wall that serve as entry sites for tumor cells in lymphatic vessels to reach lymph nodes [29–31]. This “vascular centric view” is neglecting putative roles of LEC in the TME that are far beyond their LV lining functions and pro-metastatic effects. We here speculate that LEC could exert other effects on tumor cells and tumor progression that are independent on their capacity to form a vascular wall. We hypothesized that LEC exposed to tumor cells (teLEC) can be stimulated to produce pro-tumorigenic factors and therefore be active stromal players in the complex TME. To address this issue, we used the HaCaT model of skin squamous cell carcinoma displaying features of tumor progression from benign (HaCaT cells) to low grade malignant (HaCaT-II-4 cells) and metastatic (HaCaT-A5-RT3 cells) tumors [32,33]. The sensitivity of these cells to factors derived from

activated stromal cells (fibroblasts) is well documented [34]. We demonstrate that tumor cells disrupt LEC monolayer integrity and induce a phenotypic switch in teLEC, which in turn promotes cancer cell proliferation and migration. Mechanistically, we provide *in vivo* evidence that IL6-derived from teLEC promotes cancer cell proliferation index that is blocked by neutralizing *anti*-IL6 antibody. We are assigning a novel function to teLEC, which serve as a novel stromal source of regulators of tumor cell proliferation in the TME.

2. Materials and methods

2.1. Cells and reagents

LEC are human dermal lymphatic microvascular endothelial cells purchased from Lonza (HMVEC-dLyAd, CC-2810, Verviers, Belgium). They were cultured in EGM2-MV medium (herein referred as complete medium) (CC-3202, Lonza) until confluence. Tumor cell lines used were derived from human skin carcinomas: (i) spontaneously immortalized human keratinocyte HaCaT cells, (ii) malignant keratinocyte HaCaT-II-4 cells, and (iii) metastatic keratinocyte HaCaT-A5-RT3 cells [32,33,35]. Tumor cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, 10938-025) complemented with 10% fetal bovine serum (10270-106), 1% glutamine (25030-123) and 1% penicillin–streptomycin (15140-122) (all from ThermoFisher, MA). Serum-starved tumor cells were treated with recombinant human IL6 (I1395, Sigma, Belgium) and a neutralizing antibody against IL6 (2.5



(caption on next page)

Fig. 3. LEC activated by tumor cells (teLEC) shift their transcriptomic and cytokinic profile. (A) Schematic representation of the experimental conditions. LEC were stimulated with conditioned medium (CM) of LEC themselves (Naive LEC) or of tumor cells (teLEC). Their RNA and conditioned media were subjected to RNA sequencing (B, C) and cytokine array (D, E). (B) Heatmap of the 38 most upregulated transcripts. The rows are ranked by \log_2FC decreasing order and the columns are the sample replicates. Each rectangle is scale-colored according to the read counts of the mapped gene (row) for the particular replicate (column). (C) Canonical pathways up-regulated (Ingenuity Pathway Analysis) with a positive z-score. (D) Cytokine array performed on LEC CM, HaCaT-II-4 CM, and teLEC CM. Rectangles represent cytokines enriched in teLEC CM. (E) Quantification of cytokine array spots representing levels of each cytokine in naive LEC CM (blue), HaCaT-II-4 CM (grey) and HaCaT-II-4 teLEC CM (pink). Graph is expressed as fold induction of secreted proteins (dot intensity compared to internal positive control). Rectangles represent cytokines enriched in teLEC CM and the dotted rectangle indicates the cytokine secreted by HaCaT-II-4 cells. (F) IL6 ELISA performed on CM of LEC or tumor cells in monoculture and on CM of teLEC (LEC stimulated by tumor cells as indicated). Graph represents the concentration (pg/ml) of human IL6. (G) LEC were stimulated by LEC CM or HaCaT series CM for the indicated time (minutes). STAT1 phosphorylation was evaluated by Western blot. (H) LEC were transfected with a siRNA targeting STAT1 or with a control siRNA (Ctr). Western blot analyses of STAT1 and GAPDH expression. (I) IL6 ELISA performed on CM of LEC or HaCaT-II-4 CM after siRNA transfection. Results are expressed as mean \pm SEM, and statistical analyses were performed using a Wilcoxon-Mann-Whitney test (* $p < 0.05$, ** $p < 0.01$). All the results are representative of three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

$\mu\text{g/ml}$, MAB 2061, R&D Systems, MN). LEC transfections with siRNAs targeting STAT1 (ON-TARGETplus Human STAT1 (6772), L-003543-00-0005, Dharmacon, Co, USA) or control siRNAs (ON-TARGETplus Non-Targeting Pool, D-00180-10-05, Dharmacon) were performed using Interferin (409-50, Polyplus). All cells used were negative for mycoplasma contamination.

2.2. Confrontation of LEC monolayer to tumor cells

The detailed protocol is available in the supplementary methods.

2.3. Preparation of cell conditioned medium

The detailed protocol is available in the supplementary methods.

2.4. Measurement of apoptosis by Annexin V-violet/Propidium iodide analysis

The detailed protocol is available in the supplementary methods.

2.5. Cell proliferation assay

The detailed protocol is available in the supplementary methods.

2.6. Spheroid assay

The detailed protocol is available in the supplementary methods.

2.7. Immunofluorescence

The detailed protocol is available in the supplementary methods.

2.8. Permeability assay

The detailed protocol is available in the supplementary methods.

2.9. Western blotting

The detailed protocol is available in the supplementary methods.

2.10. RNA-sequencing and statistical analysis

After RNA extraction (High Pure RNA Isolation Kit, 11828665001, Roche), bulk RNA-Sequencing was performed on an Illumina Next-Seq500. The read length was 76 bps. The number of mapped reads per sample was between 19 and 23 million. The average coverage (sequencing depth) was estimated to be 10.8.

The detailed protocol is available in the supplementary methods.

2.11. Cytokine array and ELISA

The detailed protocol is available in the supplementary methods.

2.12. Mice

Six-to seven-week-old Swiss Nude mice (620SWISSNUDE, Charles River, Ecully, France) were used throughout this study. The animals were maintained under a 12-h light-dark cycle with free access to food and water. Animal experiments were performed in compliance with the Animal Ethical Committee of the Liege University (Liege, Belgium) after the approval of the local Animal Ethical Committee.

2.13. Ear sponge assay

Gelatin sponges (Gelfoam, Pfizer, Ixelles, Belgium) were cut into small pieces (5 mm^3) and incubated with cells as previously described [36]. Briefly, HaCaT-A5-RT3 or HaCaT-II-4 cells (2×10^6 cells per sponge) were added to gelatin sponge in the absence or presence of LEC (4×10^6 cell per sponge), in EBM2 serum-free medium. Human neutralizing anti-IL6 antibody ($15 \mu\text{g/ml}$, MAB2061, R&D Systems) or irrelevant mouse IgG2b antibody ($15 \mu\text{g/ml}$, MAB004, R&D Systems) (as negative control) was added to cell suspension. After 30 min of incubation, sponges were embedded in interstitial type I collagen gel (1.5 mg/ml , 41.254.02, Serva). Sponges were subcutaneously inserted into the ears of nude mice and left for 7 days [36,37]. Anti-IL6 or IgG2b antibody was injected directly in the sponge each day with a Hamilton syringe. At the end of the experiment, sponges were harvested, incubated in 4% formol (11699408, VWR) for 4 h, dehydrated in ethanol and fixed in paraffin (X881.2, Leica).

2.14. Immunohistochemistry

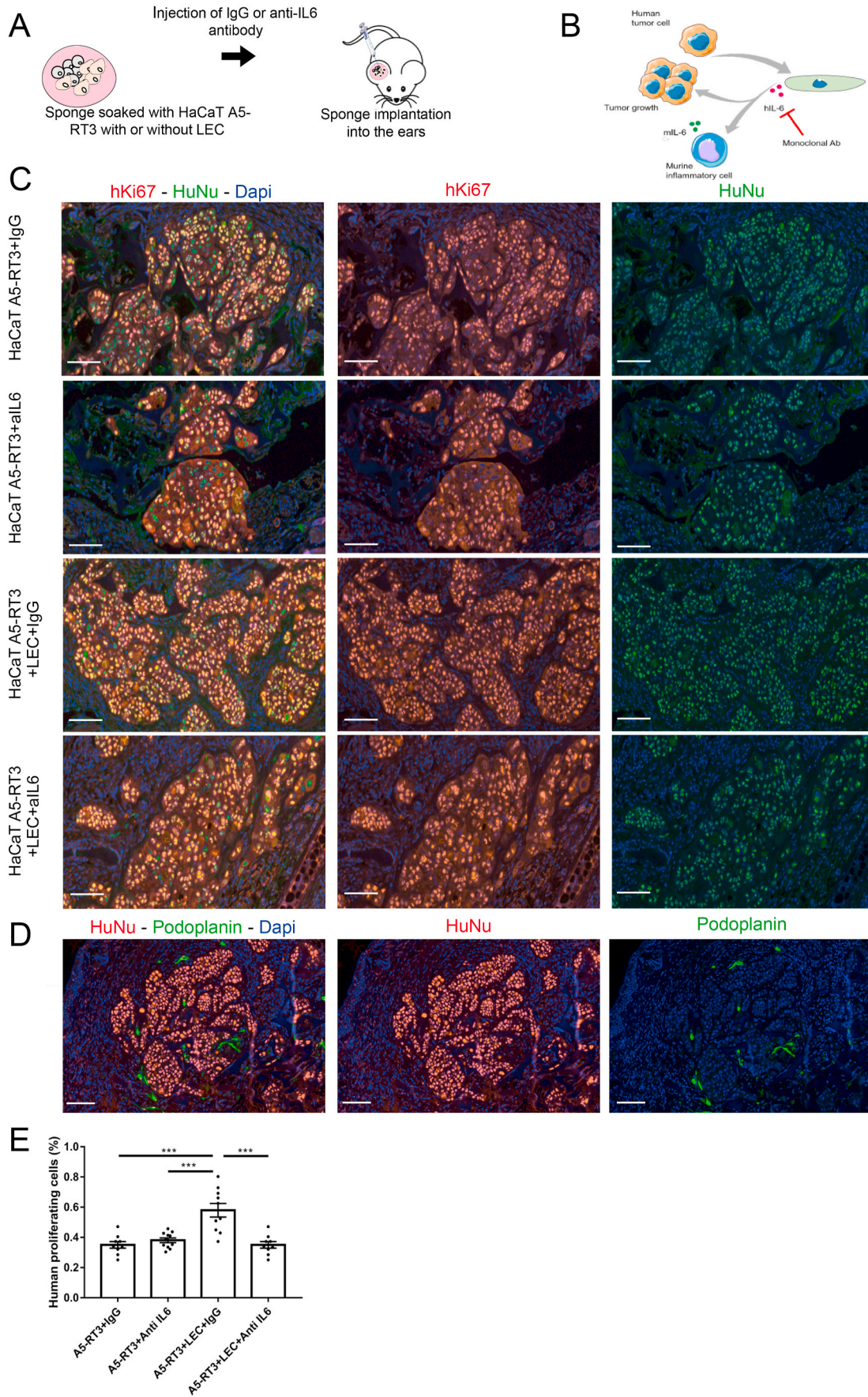
The detailed protocol is available in the supplementary methods.

2.15. Statistics

Statistical analysis was performed using Prism 7 software (Graphpad Software, San Diego, CA, USA). Results were analyzed using the non-parametric Mann-Whitney test with two-tailed p value. $P < 0.05$ was considered as statistically significant. In histograms, mean \pm SEM were represented. For proliferation assay, results are expressed as mean \pm SEM and Anova two-way significance test was used (* $p < 0.05$, *** $p < 0.001$).

3. Results

To study the reciprocal cross-talk between tumor cells and LEC, we used the HaCaT cell series characterized by a progression from benign to malignant and invasive tumor: immortalized keratinocytes (HaCaT), low-grade malignant keratinocytes (HaCaT-II-4) or high grade



(caption on next page)

Fig. 4. teLEC-secreted IL6 enhanced HaCaT-A5-RT3 proliferation *in vivo*. (A) Schematic representation of the ear sponge assay using gelatin sponges soaked with human HaCaT-A5-RT3 with or without human LEC. (B) Human IL6 was targeted in the sponge by using a neutralizing antibody against human IL6, but not murine IL6. (C) Tumor cell proliferation was evaluated by HuNu (green) and hKi67 (red) co-immunostainings. Bars = 100 μ m. (D) The presence of human LEC was assessed by double HuNu (red) and human podoplanin (green) staining. Blue corresponds to Dapi staining. Bars = 100 μ m. (E) Histogram represents tumor cell proliferation (hKi67/HuNu density) assessed by a computer-assisted method ($n \geq 9$). Results are expressed as mean \pm SEM, and statistical analyses were performed using a Wilcoxon-Mann-Whitney test ($***p < 0.001$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

metastatic keratinocytes (HaCaT-A5-RT3) [35,38,39]. Human primary LEC (HMVEC-dLy) cultured with tumor cells or their conditioned medium (CM) are referred herein as tumor exposed LEC: “teLEC”. We first determined the cellular changes induced by tumor cells on LEC properties (morphological changes in LEC monolayer), and *vice versa* by LEC on tumor cells (*in vitro* invasion).

3.1. Tumor cells induce morphological changes in LEC and affect LEC monolayer integrity

Since some tumor cells can induce LEC retraction forming gaps between cells [30,31], we first examined the impact of human skin carcinoma cells on LEC monolayer integrity (Fig. 1). LEC were cultured alone (LEC monolayer) or co-cultured with tumor cells labeled with a cell tracker (co-culture) (Fig. 1A). LEC addition to a LEC monolayer (LEC-LEC culture) was used as control to assess the specificity of the observed effects (Fig. 1A). The added LEC integrated into the monolayer without affecting its integrity (Fig. 1B and C). In sharp contrast, the HaCaT cell series induced gaps between LEC (Fig. 1B) and a discontinuity in the endothelial-specific junctional protein VE-cadherin staining at the cell surface (Fig. 1C). A computerized method was set up to quantify gaps generated in LEC monolayer. The global gap density was 3-fold higher when LEC were confronted to malignant HaCaT-II-4 cells and metastatic HaCaT-A5-RT3 cells than to immortalized HaCaT cells, which exerted a faint effect on LEC integrity (Fig. 1D). The tumor cell-induced disruption of LEC monolayer was further confirmed in a permeability assay (Fig. 1E). Dextran-FITC diffusion was enhanced by the addition of tumor cells, but not LEC on top of the endothelial barrier. FACS analyses revealed that LEC apoptosis was similar in all experimental conditions (Fig. 1F). These data demonstrate that malignant keratinocytes induced the dissociation of LEC-LEC interactions without affecting cell viability. LEC are thus sensitive to malignant HaCaT cells that drastically impact their inter-cellular interactions.

3.2. LEC promote tumor cell invasion *in vitro*

A putative reciprocal effect of LEC on tumor cell properties was next investigated in spheroids embedded in a 3D collagen matrix (Fig. 2A–C). Spheroids were formed with a single cell type (monospheroids) or with two cell types stained with different cell trackers (green for LEC and red for tumor cells) (heterospheroids) (Fig. 2A). As control, spheroids formed of red and green stained LEC were used (homospheroids) to check that cell trackers did not influence cell behavior. In mono- and homo-spheroids, LEC displayed a capacity to sprout out and invade the collagen matrix (Fig. 2A). The distribution of tumor cell density around the spheroids was determined by using a computerized method of image analysis (Fig. 2B and C). When malignant (HaCaT-II-4) and metastatic (HaCaT-A5-RT3) tumor cells were mixed with LEC in heterospheroids, cancer cell migration was 2 to 3-fold enhanced as compared to that observed in monospheroids of tumor cells (Fig. 2A–C) ($p < 0.01$). In contrast, LEC only slightly affected the behavior of immortalized HaCaT cells (Fig. 2A–C) ($p < 0.05$). The apparently high migratory capacity of those cells could rely on their proliferative capacity. Tumor cells failed to affect LEC migration (SFig.1). These data point to the capacity of LEC to promote the invasiveness of malignant and metastatic tumor cells in a collagen matrix. They clearly provide evidence that these tumor cells are sensitive to factors produced by LEC.

3.3. Tumor cells induce secretome changes in LEC

To investigate the molecular mechanisms through which LEC influence tumor cell properties, we carried out a transcriptomic analysis through RNA-Sequencing on LEC cultured with control medium (“naive LEC”) or CM of tumor HaCaT-II-4 cells (“teLEC”) (Fig. 3A). This global transcriptomic analysis revealed 994 gene tags differentially expressed, with a $\log_2FC \geq 1$, i.e. 491 upregulated and 503 downregulated in teLEC. The heatmap represents the 38 most up-regulated genes and ranked by decreasing \log_2FC (Fig. 2B). Interleukin-6 (IL6) belongs to the ten most upregulated genes and shows an average fold change of 57.3 ($\log_2FC = 5.84$) (Fig. 3B). Gene ontology and enrichment analysis was done with IPA and revealed up-regulated canonical pathways enriched in teLEC. Interestingly most of the pathways are associated to inflammatory response (Fig. 3C).

CM of naive LEC, HaCaT-II-4 tumor cells or teLEC were next screened for a panel of cytokines/chemokines by using a cytokine array (Fig. 3D). In most cases, the resulting levels of secreted proteins reflected the production of tumor cells cultured alone. Interestingly, we noticed a huge production of IL6 in teLEC, while it was not detected in naive LEC and faintly produced by tumor cells (Fig. 3D and E). Specific ELISA were used to determine the exact protein concentrations in each experimental condition (Fig. 3F). The different HaCaT tumor cell CM secreted low amounts of IL6 (below 60 pg/ml). In contrast, a huge production of IL6 was detected in teLEC activated by HaCaT-II-4 (392.8 ± 85.91 pg/ml) and HaCaT-A5-RT3 (273.2 ± 50.06 pg/ml) as compared to those activated by HaCaT cells (6.83 ± 5.49 pg/ml) or naive LEC (below detection threshold) (Fig. 3F). These data revealed a 5- to 6-fold enhancement of IL6 content in teLEC CM as compared to the sum of protein amounts in naive LEC CM and tumor cell CM. ICAM-1 production was also increased in teLEC activated by HaCaT-II-4 (1440 ± 211.4 pg/ml) and HaCaT-A5-RT3 (1225 ± 336 pg/ml), although to a lesser extent since ICAM-1 was produced both by naive LEC (395.8 ± 109.5 pg/ml) and tumor cells (145 ± 15.65 pg/ml for HaCaT-II-4 and 209.1 ± 24.76 pg/ml for HaCaT-A5-RT3) under basal conditions. The cytokine array analyses revealed the production of putative IL6 inducers in tumor cells: IFN γ , IL8, IL17, GM-CSF and MIP-1 α/β , while TNF α , IL1 and IL4 were almost not detected at a protein level (Fig. 3e). To assess the putative role of tumor cell-derived cytokines on IL6 secretion, we evaluated, in LEC cultures, the phosphorylation status of STAT1 (Tyr 701), a downstream mediator of cytokines (Fig. 3g). When LEC were stimulated with CM of tumor cells, STAT1 phosphorylation was increased, with a pic at 15 min, suggesting its role in IL6 stimulation. In HaCaT-II-4 cells, STAT1 down-regulation by siRNA (Fig. 3h) partially reduced IL6 production (Fig. 3i). These data indicate that tumor-derived factors contributes, at least partially, to IL6 production by LEC via STAT1 activation. Several factors are likely implicated in the stimulation of IL6 production by LEC.

3.4. LEC stimulate tumor cell growth *in vivo*

IL6 is a pleiotropic cytokine that promote inflammation and tumor cell the proliferation. In line with a previous report [35], we confirmed that HaCaT-A5-RT3 proliferation was stimulated by recombinant IL6 and blocked by a neutralizing anti-human IL6 (SFig.2). To determine teLEC impact on tumor cell growth *in vivo*, we confronted metastatic skin carcinoma cells (HaCaT-A5-RT3) and LEC in the ear sponge assay [36,37]. Gelatin sponges soaked with HaCaT-A5-RT3 with or without LEC were implanted in ears of nude mice (Fig. 4A). During 7 days,

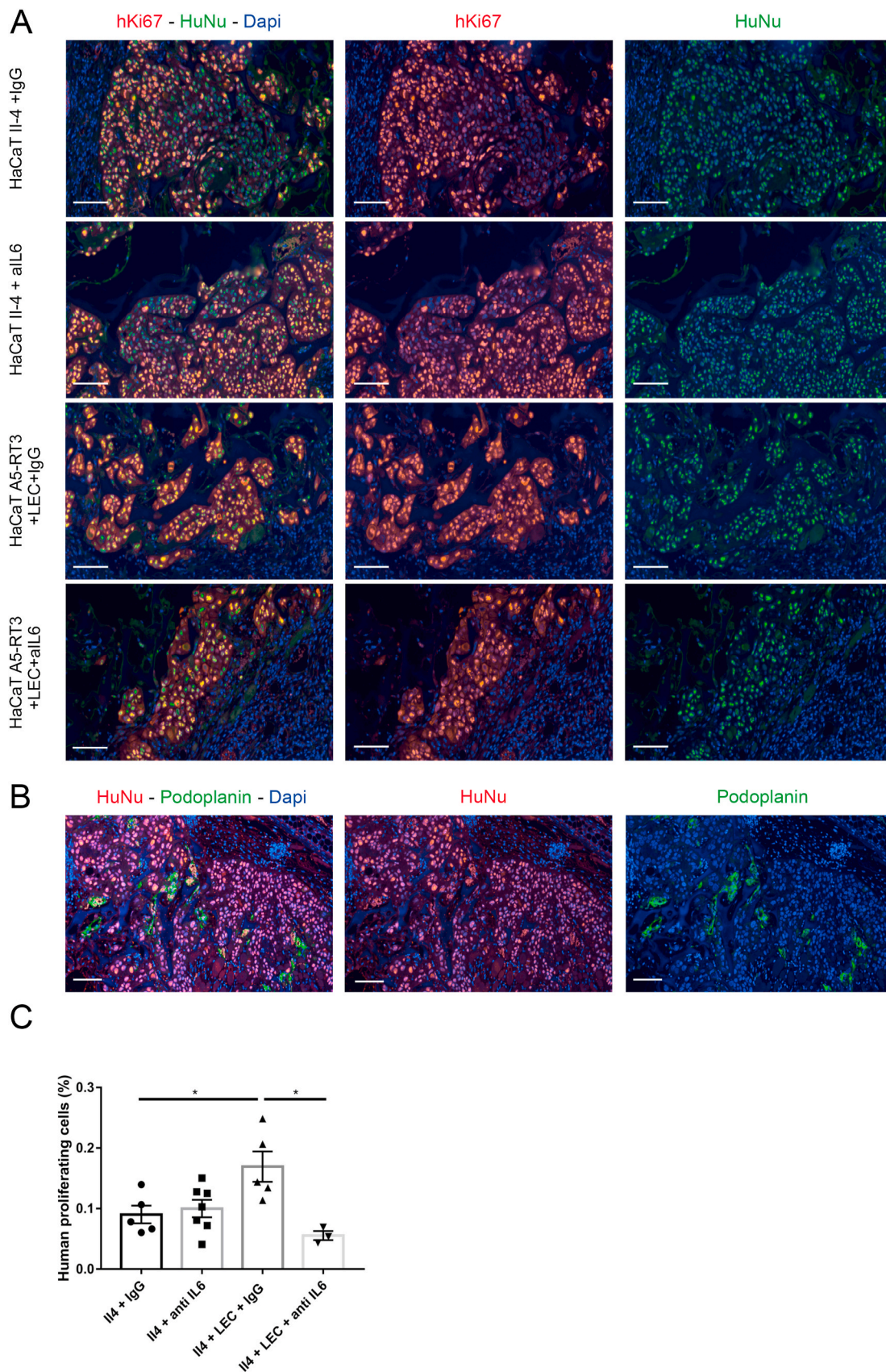


Fig. 5. teLEC-derived IL6 enhanced HaCaT-II-4 proliferation *in vivo*. (A) Tumor cell proliferation was determined as in Fig. 4. Bars = 100 μ m. (B) The presence of human LEC was assessed by double HuNu (red) and human podoplanin (green) stainings. Blue corresponds to Dapi staining. Bars = 100 μ m. (C) Histogram represents tumor cell proliferation (hKi67/HuNu density) ($n \geq 3$). Results are expressed as mean \pm SEM, and statistical analyses were performed using a Wilcoxon-Mann-Whitney test (* $p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

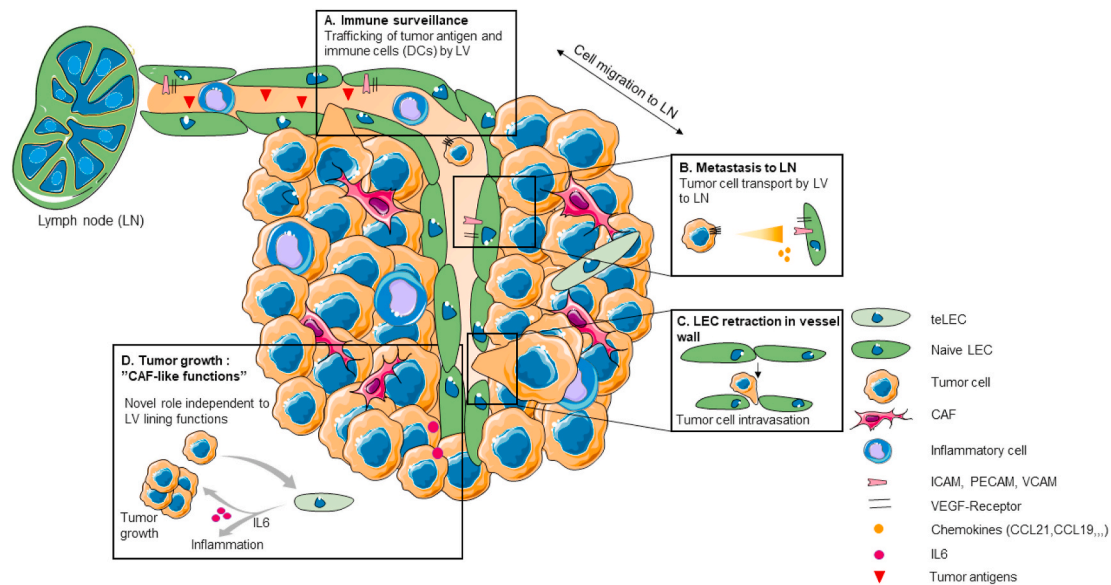


Fig. 6. Graphical abstract of teLEC role in regulating tumor growth. LEC contribute to cancer progression as a structural component of lymphatic vessels (LV) that i) transport tumor antigens and immune cells (such as dendritic cells: DC) for immune surveillance (A); and ii) secrete cytokines (CCL19, CCL21 ...) and cell adhesion molecules (ICAM, VCAM ...) to attract and guide tumor cells towards LV for metastatic dissemination to lymph node (LN) (B). LEC exposed to tumor cells (teLEC) display morphological changes associated to cell retraction leading to gap formation in the lympho-endothelial wall that can offer entry site for tumor cells (C). In addition, teLEC produce pro-tumorigenic factors including at least IL6 that in turn promotes tumor growth (D). Such a reciprocal paracrine cross-talk between tumor cells and LEC highlight the capacities of LEC to exert "fibroblastic-like" (CAF-like) functions in the tumor microenvironment (D) that warrant to revisit the "vascular centric view" of LEC (A-C).

sponges were daily injected with a control IgG antibody or a neutralizing antibody targeting human, but not murine IL6 (Fig. 4B). Tumor cell proliferation was assessed by double immunostaining using antibodies against human nuclei (HuNu) and human Ki67 (Fig. 4C). The presence of human LEC was checked by a double staining using anti-human podoplanin and anti-HuNu (Figs. 4D and 5B). Notably, tumor cell exposition to LEC led to increased tumor cell proliferation, which was abrogated by anti-human IL6 (Fig. 4E). In line with IL6 pro-inflammatory effect, the neutralizing IL6 antibody reduced the inflammatory response as assessed by F4/80 macrophage staining (SFig. 3). Altogether, our data argue that LEC-derived IL6 promotes tumor growth. We next verified that the *in vivo* effects of LEC on tumor cell proliferation are not specific to metastatic HaCaT-A5-RT3 cells. Gelatin sponges soaked with HaCaT-II-4 cells with or without LEC were implanted in ears of nude mice (Fig. 5A). Interestingly, a LEC-derived IL6 stimulation of tumor cell proliferation was again observed and blocked by anti-IL6 antibody (Fig. 5C).

4. Discussion

It is now well recognized that LEC contribute to cancer progression as the structural component of lymphatic vessels [22]. Our investigation of the cross-talk established between cancer cells and LEC led to uncover a novel mechanism through which LEC exposed to tumor cells (teLEC) can directly regulate tumor growth. Mechanistically, we identify a reciprocal paracrine signaling pathway between tumor cells and LEC, in which IL6 production by teLEC exerts a protumorigenic effect.

The innovative concept that LEC can exert pro-tumorigenic effects independent to their LV lining functions is supported by *in vitro* and *in vivo* data (Fig. 6). We provide evidence that *in vitro*, in a 3D-spheroid assay, LEC enhanced the invasion of malignant (HaCaT-II-4) and metastatic (HaCaT-A5-RT3) cells, but not that of immortalized HaCaT cells. We next took advantage of the original ear sponge assay [36] to expose HaCaT-A5-RT3 cells and HaCaT-II-4 to LEC *in vivo*. In this system, LEC and tumor cells were confined in a gelatin sponge surrounded by a collagen matrix to avoid cell scattering. We provide *in vivo* evidence that

the direct exposition of LEC to tumor cells promotes tumor growth. Notably, the LEC-mediated mitogenic effect on tumor cells, was abrogated by neutralizing anti-IL6 antibody demonstrating a novel paracrine pathway between tumor cells and LEC. These original data underline the impact of LEC exposition to tumor cells, as well as the importance of the reciprocal cross-talk and cooperative interaction between LEC and tumor cells in cancer progression.

IL6 is one of the major inflammatory interleukins that has been linked to cancer progression [35,40,41]. Through a cytokine array, IL6 appeared as the most modulated soluble factor in teLEC. Interestingly, IL6 was produced at very low levels by tumor cells and almost not detected in CM of naive LEC. Significantly elevated IL6 concentration (>6-fold protein amounts) was detected in teLEC as compared to naive LEC. Importantly, IL6 contributes to a complex, reciprocally regulated cytokine network including IFN γ , IL8, GM-CSF, IL17 and MCP-1 [35]. We provide evidence that Stat1 activation contributes, at least partially to IL6 secretion by teLEC. It is likely that a cocktail of tumor-derived factors is involved in IL6 stimulation. The modulation of pro-inflammatory cytokine secretion observed in teLEC is in line with a recent study reporting a strong inflammatory gene expression signature in LEC isolated from murine mammary 4T1 tumors [21]. IL6 is released by various cells in the TME including cancer-associated fibroblasts (CAF). In pancreatic cancer, two distinct CAF subtypes have been characterized by either their myfibroblastic or inflammatory (IL6 producer) phenotype [42,43]. The originality of our finding relies on the demonstration that LEC can serve as a key source of IL6 with pro-tumorigenic functions and reveals a new level of complexity to be considered in the TME. In the present study, we focused on IL6, being one of the most modulated molecules in teLEC, as a proof-of-principle. Given the importance of cancer-associated stromal cells, it is now worth revisiting how LEC contribute to cancer progression. This is of particular importance, as single cell sequencing data from different stromal cell types including endothelial cells [44] and CAF [42] are currently emerging and those analyses should not neglect LEC as a putative key contributor of TME and producer of pro-tumorigenic and pro-metastatic factors. Furthermore, the finding of pro-inflammatory

factor secretion by LEC in response to tumor cell-derived factors is of utmost interest since pro-inflammatory factors are able to enhance cancer progression and to favor resistance to treatment [45].

LEC have been reported to contribute to cancer progression through several mechanisms including lymphangiogenesis and the chemoattraction of tumor cells through the secretion of chemokines that guide tumor cells towards lymphatic vessels (Fig. 6). Tumor cells can induce LEC retraction and generate gaps in the lymphendothelial wall that serve as entry gates to the lymphatic vasculature [29]. These data are in line with the morphological changes (weakened intercellular junctions) observed in LEC monolayer exposed to the HaCaT cell series used here. The modulation of cell adhesion molecules in LEC exposed to tumor cells such as ICAM-1 [46,47] and VCAM-1 [21] can also contribute to tumor cell intravasation [21,48] (Fig. 6). Accordingly, we found enhanced ICAM-1 production in teLEC. The increased expression of VCAM-1 in cancer-associated lymphatic vessels has been recently reported to promote lymphatic permeability by weakening lymphatic junctions [21, 49]. In addition to those effects on tumor cell intravasation into lymphatics, LEC have attracted substantial attention in their capacity to modulate the immune response [16,50,51] (Fig. 6). Our exciting discovery that teLEC can directly influence proliferative and invasive capabilities of cancer cells reveals a new dimension of the reciprocal cross-talk between tumor cells and LEC. We show that the role of LEC in cancer extends beyond the formation of lymphatic vessels providing a route for metastatic dissemination and contributing to the immune response.

Although the diversity in the developmental origins of lymphatic vessels together with as well as heterogeneity and multifunctional features of LEC are emerging in physiological conditions [19,20], LEC plasticity in pathological conditions is less documented. Our work highlights a new paradigm, in which LEC exposed to tumor cells are subjected to morphological and molecular changes that directly influence tumor cell behavior and contribute to cancer progression. We propose that LEC should be considered as important stromal cells in the TME that can have additional roles independent of their LV lining functions. They can act as “CAF-like cells” by secreting tumor-promoting factors and are worth in-depth investigating in future research.

Ethics

Animal experiments were performed in compliance with the Animal Ethical Committee of the Liege University (Liege, Belgium) after the approval of the local Animal Ethical Committee.

Authors' contributions

Van de Velde Maureen: Conceptualization, Investigation, Methodology, Visualization, Writing. **Ebroin Marie:** Conceptualization, Investigation, Methodology, Visualization, Writing. **Durré Tania:** Conceptualization, Investigation, Writing. **Gillot Lionel:** Investigation, Visualization. **Joiret Marc:** Investigation, Software. **Blacher Silvia:** Investigation, Formal analysis. **Geris Liesbet:** Funding acquisition. **Kridelka Frédéric:** Funding acquisition, Conceptualization. **Noël Agnès:** Funding acquisition, Conceptualization, Visualization, Writing, Supervision.

Declaration of competing interest

The authors declare that no competing interests exist.

Acknowledgements

This work was supported by: grants from the Fonds de la Recherche Scientifique - FNRS (F.R.S.-FNRS, Belgium), the Fondation contre le Cancer (foundation of public interest, Belgium), the Fonds spéciaux de la Recherche (University of Liège), the Fondation Hospitalo Universitaire

Léon Fredericq (FHULF, University of Liège), the “Direction Générale Opérationnelle de l’Economie, de l’Emploi et de la Recherche” from the Service Public de Wallonie (SPW, Belgium), the Walloon Region through the FRFS-WELBIO strategic research programme, the Wallonia-Brussels Federation (grant for Concerted Research Actions, A.R.C. 19/23-21 « INovLYMPHATIC). LG is supported by the European Research Council under the European Union’s Horizon 2020 research and innovation programme (FP/2014–2020)/ERC (Grant Agreement n. 772418). We thank the GIGA (Groupe Interdisciplinaire de Génoprotéomique Appliquée, University of Liege, Belgium) for the access to the various platforms: (1) GIGA-Genomics platform (2) GIGA-Proteomics platform (3) GIGA-Imaging and Flow Cytometry platform, (4) GIGA-Bioinformatics platform, and (5) GIGA-Mouse facility and Transgenics platform. We acknowledge the technical support of Emilie Feyereisen. M.V.D., M.E., L.G., are supported by a F.R.S.-FNRS-Télévie grant. T.D. is supported by the Walloon Region grant “Walinnov Immucan – 1610119”.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.canlet.2020.10.020>.

References

- [1] J.A. Joyce, J.W. Pollard, Microenvironmental regulation of metastasis, *Nat. Rev. Canc.* 9 (2009) 239–252, <https://doi.org/10.1038/nrc2618>.
- [2] D. Hanahan, R.A. Weinberg, Hallmarks of cancer: the next generation, *Cell* 144 (2011) 646–674, <https://doi.org/10.1016/j.cell.2011.02.013>.
- [3] A. Alitalo, M. Detmar, Interaction of tumor cells and lymphatic vessels in cancer progression, *Oncogene* 31 (2012) 4499–4508, <https://doi.org/10.1038/onc.2011.602>.
- [4] Y. Kim, M.A. Stolarska, H.G. Othmer, The role of the microenvironment in tumor growth and invasion, *Prog. Biophys. Mol. Biol.* 106 (2011) 353–379, <https://doi.org/10.1016/j.pbiomolbio.2011.06.006>.
- [5] Q. Ma, L.C. Dieterich, M. Detmar, Multiple roles of lymphatic vessels in tumor progression, *Curr. Opin. Immunol.* 53 (2018) 7–12, <https://doi.org/10.1016/j.coi.2018.03.018>.
- [6] Paupert, et al., Lymphangiogenesis in post-natal tissue remodeling: lymphatic endothelial cell connection with its environment, *Mol. Aspect. Med.* 32 (2011) 146–158, <https://doi.org/10.1016/j.mam.2011.04.002>.
- [7] J.P. Sleeman, W. Thiele, Tumor metastasis and the lymphatic vasculature, *Int. J. Canc.* 125 (2009) 2747–2756, <https://doi.org/10.1002/ijc.24702>.
- [8] W. Zheng, A. Aspelund, K. Alitalo, Lymphangiogenic factors, mechanisms, and applications, *J. Clin. Invest.* 124 (2014) 878–887, <https://doi.org/10.1172/JCI71603>.
- [9] S. Karaman, M. Detmar, S. Karaman, M. Detmar, Mechanisms of lymphatic metastasis, *J. Clin. Invest.* 124 (2014) 922–928, <https://doi.org/10.1172/JCI71606.922>.
- [10] S. Zhang, D. Zhang, S. Yi, M. Gong, C. Lu, Y. Cai, X. Tang, L. Zou, The relationship of lymphatic vessel density, lymphovascular invasion, and lymph node metastasis in breast cancer: a systematic review and meta-analysis, *Oncotarget* 8 (2017) 2863–2873, <https://doi.org/10.18632/oncotarget.13752>.
- [11] M. Brown, F.P. Assen, A. Leithner, J. Abe, H. Schachner, G. Asfour, Z. Bago-Horvath, J.V. Stein, P. Uhrin, M. Sixt, D. Kerjaschki, Lymph node blood vessels provide exit routes for metastatic tumor cell dissemination in mice, *Science* 359 (2018) 1408–1411, <https://doi.org/10.1126/science.aal3662>, 80-.
- [12] E.R. Pereira, D. Kedrin, G. Seano, O. Gautier, E.F.J. Meijer, D. Jones, S.M. Chin, S. Kitahara, E.M. Bouta, J. Chang, E. Beech, H.S. Jeong, M.C. Carroll, A.G. Taghian, T.P. Padera, Lymph node metastases can invade local blood vessels, exit the node, and colonize distant organs in mice, *Science* 359 (2018) 1403–1407, <https://doi.org/10.1126/science.aal3622>, 80-.
- [13] R.H. Farnsworth, M.G. Achen, S.A. Stackner, The evolving role of lymphatics in cancer metastasis, *Curr. Opin. Immunol.* 53 (2018) 64–73, <https://doi.org/10.1016/j.coi.2018.04.008>.
- [14] T.P. Padera, E.F.J. Meijer, L.L. Munn, The lymphatic system in disease processes and cancer progression, *Annu. Rev. Biomed. Eng.* 18 (2016) 125–158, <https://doi.org/10.1146/annurev-bioeng-112315-031200>.
- [15] A.W. Lund, M. Wagner, M. Fankhauser, E.S. Steinskog, M.A. Broggi, S. Spranger, T. F. Gajewski, K. Alitalo, H.P. Eikesdal, H. Wiig, M.A. Swartz, Lymphatic vessels regulate immune microenvironments in human and murine melanoma, *J. Clin. Invest.* 126 (2016) 3389–3402, <https://doi.org/10.1172/JCI79434>.
- [16] C.M. Card, S.S. Yu, M.A. Swartz, Emerging roles of lymphatic endothelium in regulating adaptive immunity, *J. Clin. Invest.* 124 (2014) 943–952, <https://doi.org/10.1172/JCI73316>.
- [17] T. Tammela, K. Alitalo, Lymphangiogenesis: molecular mechanisms and future promise, *Cell* 140 (2010) 460–476, <https://doi.org/10.1016/j.cell.2010.01.045>.

- [18] N. Bordry, M.A.S. Broggi, K. de Jonge, K. Schaeuble, P.O. Gannon, P.G. Foukas, E. Danenberg, E. Romano, P. Baumgaertner, M. Fankhauser, N. Wald, L. Cagnon, S. Abed-Maillard, H. Maby-El Hajjami, T. Murray, K. Ioannidou, I. Letovanec, P. Yan, O. Michielin, M. Matter, M.A. Swartz, D.E. Speiser, Lymphatic vessel density is associated with CD8+ T cell infiltration and immunosuppressive factors in human melanoma, *Oncimmunology* 7 (2018), e1462878, <https://doi.org/10.1080/2162402X.2018.1462878>.
- [19] T.V. Petrova, G.Y. Koh, Organ-specific lymphatic vasculature: from development to pathophysiology, *J. Exp. Med.* 215 (2018) 35–49, <https://doi.org/10.1084/jem.20171868>.
- [20] M.H. Ullmar, T. Mäkinen, Heterogeneity in the lymphatic vascular system and its origin, *Cardiovasc. Res.* 111 (2016) 310–321, <https://doi.org/10.1093/cvr/cvw175>.
- [21] L.C. Dieterich, K. Kapaklikaya, T. Cetintas, S.T. Proulx, C.D. Commerford, K. Ikenberg, S.B. Bachmann, J. Scholl, M. Detmar, Transcriptional profiling of breast cancer-associated lymphatic vessels reveals VCAM-1 as regulator of lymphatic invasion and permeability, *Int. J. Canc.* 145 (2019) 2804–2815, <https://doi.org/10.1002/ijc.32594>.
- [22] S.A. Stackner, S.P. Williams, T. Karnezis, R. Shayan, S.B. Fox, M.G. Achen, Lymphangiogenesis and lymphatic vessel remodelling in cancer, *Nat. Rev. Canc.* 14 (2014) 159–172, <https://doi.org/10.1038/nrc3677>.
- [23] K.H. Pak, A. Jo, H.J. Choi, Y. Choi, H. Kim, J.H. Cheong, The different role of intratumoral and peritumoral lymphangiogenesis in gastric cancer progression and prognosis, *BMC Canc.* 15 (2015) 1–8, <https://doi.org/10.1186/s12885-015-1501-9>.
- [24] L.C. Dieterich, M. Detmar, Tumor lymphangiogenesis and new drug development, *Adv. Drug Deliv. Rev.* 99 (2016) 148–160, <https://doi.org/10.1016/j.advdr.2015.12.011>.
- [25] T.P. Padera, A. Kadambi, E. Di Tomaso, C. Mouta Carreira, E.B. Brown, Y. Boucher, N.C. Choi, D. Mathisen, J. Wain, E.J. Mark, L.L. Munn, R.K. Jain, Lymphatic metastasis in the absence of functional intratumor lymphatics, *Science* 296 (2002) 1883–1886, <https://doi.org/10.1126/science.1071420>, 80-.
- [26] S. Das, E. Sarrou, S. Podgrabinska, M. Cassella, S.K. Mungamuri, N. Feirt, R. Gordon, C.S. Nagi, Y. Wang, D. Entenberg, J. Condeelis, M. Skobe, Tumor cell entry into the lymph node is controlled by CCL1 chemokine expressed by lymph node lymphatic sinuses, *J. Exp. Med.* 210 (2013) 1509–1528, <https://doi.org/10.1084/jem.20111627>.
- [27] J.D. Shields, M.S. Emmett, D.B.A. Dunn, K.D. Joory, L.M. Sage, H. Rigby, P. S. Mortimer, A. Orlando, J.R. Levick, D.O. Bates, Chemokine-mediated migration of melanoma cells towards lymphatics - a mechanism contributing to metastasis, *Oncogene* 26 (2007) 2997–3005, <https://doi.org/10.1038/sj.onc.1210114>.
- [28] P. Pekkonen, S. Alve, G. Balistreri, S. Gramolelli, O. Tatti-Bugaeva, I. Paatero, O. Niiranen, K. Tuohinto, N. Perälä, A. Taiwo, N. Zinovkina, P. Repo, K. Icaý, J. Ivaska, P. Saharinen, S. Hautaniemi, K. Lehti, P.M. Ojala, Lymphatic endothelium stimulates melanoma metastasis and invasion via MMP14-dependent Notch3 and β 1-integrin activation, *Elife* 7 (2018) 1–28, <https://doi.org/10.7554/eLife.32490>.
- [29] K. Uchida, M. Sakon, H. Ariyoshi, S. Nakamori, M. Tokunaga, M. Monden, Cancer cells cause vascular endothelial cell (vEC) retraction via 12(S)HETE secretion: the possible role of cancer cell derived microparticle, *Ann. Surg. Oncol.* 14 (2007) 862–868, <https://doi.org/10.1245/s10434-006-9225-3>.
- [30] C. Vonach, K. Viola, B. Giessrigl, N. Huttary, I. Raab, R. Kalt, S. Krieger, T.P.N. Vo, S. Madlener, S. Bauer, B. Marian, M. Hämmerle, N. Kretschy, M. Teichmann, B. Hantusch, S. Stary, C. Unger, M. Seelinger, A. Eger, R. Mader, W. Jäger, W. Schmidt, M. Grusch, H. Dolznig, W. Mikulits, G. Krupitza, NF- κ B mediates the 12(S)-HETE-induced endothelial to mesenchymal transition of lymphendothelial cells during the intravasation of breast carcinoma cells, *Br. J. Canc.* 105 (2011) 263–271, <https://doi.org/10.1038/bjc.2011.194>.
- [31] D. Kerjaschki, Z. Bago-Horvath, M. Rudas, V. Sexl, C. Schneckenleithner, S. Wolbank, G. Bartel, S. Krieger, R. Kalt, B. Hantusch, T. Keller, K. Nagy-Bojarszky, N. Huttary, I. Raab, K. Lackner, K. Krautgasser, H. Schachner, K. Kaserer, S. Rezar, S. Madlener, C. Vonach, A. Davidovits, H. Nosaka, M. Hämmerle, K. Viola, H. Dolznig, M. Schreiber, A. Nader, W. Mikulits, M. Gnant, S. Hirakawa, M. Detmar, K. Alitalo, S. Nijman, F. Offner, T.J. Maier, D. Steinhilber, G. Krupitza, Lipoxigenase mediates invasion of intrametastatic lymphatic vessels and propagates lymph node metastasis of human mammary carcinoma xenografts in mouse, *J. Clin. Invest.* 121 (2011) 2000–2012, <https://doi.org/10.1172/JCI44751>.
- [32] P. Boukamp, R.T. Petrussevska, D. Breitkreutz, J. Hornung, A. Markham, N. E. Fusenig, Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line, *J. Cell Biol.* 106 (1988) 761–771, <https://doi.org/10.1083/jcb.106.3.761>.
- [33] M.M. Mueller, W. Peter, M. Mappes, A. Huelsen, H. Steinbauer, P. Boukamp, M. Vaccariello, J. Garlick, N.E. Fusenig, Tumor progression of skin carcinoma cells in vivo promoted by clonal selection, mutagenesis, and autocrine growth regulation by granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor, *Am. J. Pathol.* 159 (2001) 1567–1579, [https://doi.org/10.1016/S0002-9440\(10\)62541-2](https://doi.org/10.1016/S0002-9440(10)62541-2).
- [34] M.M. Mueller, N.E. Fusenig, Tumor-stroma interactions directing phenotype and progression of epithelial skin tumor cells, *Differentiation* 70 (2002) 486–497, <https://doi.org/10.1046/j.1432-0436.2002.700903.x>.
- [35] W. Lederle, S. Depner, S. Schnur, E. Obermueller, N. Catone, A. Just, N.E. Fusenig, M.M. Mueller, IL-6 promotes malignant growth of skin SCCs by regulating a network of autocrine and paracrine cytokines, *Int. J. Canc.* 128 (2011) 2803–2814, <https://doi.org/10.1002/ijc.25621>.
- [36] M. Van de Velde, M. García-Caballero, T. Durré, F. Kridelka, A. Noël, Ear sponge assay: a method to investigate angiogenesis and lymphangiogenesis in mice, *Methods Mol. Biol.* 1731 (2018) 223–233, https://doi.org/10.1007/978-1-4939-7595-2_20.
- [37] M. García-Caballero, M. Van De Velde, S. Blacher, V. Lambert, C. Balsat, C. Ericpim, T. Durré, F. Kridelka, A. Noël, Modeling pre-metastatic lymphovascular niche in the mouse ear sponge assay, *Sci. Rep.* 7 (2017) 1–16, <https://doi.org/10.1038/srep41494>.
- [38] C. Maillard, M. Jost, M.U. Römer, N. Brunner, X. Houard, A. Lejeune, C. Munaut, K. Bajou, L. Melen, K. Dano, P. Carmeliet, N.E. Fusenig, J.M. Foidart, A. Noël, Host plasminogen activator inhibitor-1 promotes human skin carcinoma progression in a stage-dependent manner, *Neoplasia* 7 (2005) 57–66, <https://doi.org/10.1593/neo.04406>.
- [39] S. Depner, W. Lederle, C. Gutschalk, N. Linde, A. Zajonc, M.M. Mueller, Cell type specific interleukin-6 induced responses in tumor keratinocytes and stromal fibroblasts are essential for invasive growth, *Int. J. Canc.* 135 (2014) 551–562, <https://doi.org/10.1002/ijc.27951>.
- [40] A. Masjedi, V. Hashemi, M. Hojjat-Farsangi, G. Ghalamfarsa, G. Azizi, M. Yousefi, F. Jadidi-Niaragh, The significant role of interleukin-6 and its signaling pathway in the immunopathogenesis and treatment of breast cancer, *Biomed. Pharmacother.* 108 (2018) 1415–1424, <https://doi.org/10.1016/j.biopha.2018.09.177>.
- [41] M.Y. Taher, D.M. Davies, J. Maher, The role of the interleukin (IL)-6/IL-6 receptor axis in cancer, *Biochem. Soc. Trans.* 46 (2018) 1449–1462, <https://doi.org/10.1042/BST20180136>.
- [42] G. Biffi, T.E. Oni, B. Spielman, Y. Hao, E. Elyada, Y. Park, J. Preall, D.A. Tuveson, IL1-induced Jak/STAT signaling is antagonized by TGF β to shape CAF heterogeneity in pancreatic ductal adenocarcinoma, *Canc. Discov.* 9 (2019) 282–301, <https://doi.org/10.1158/2159-8290.CD-18-0710>.
- [43] D. Öhlund, A. Handly-Santana, G. Biffi, E. Elyada, A.S. Almeida, M. Ponz-Sarvise, V. Corbo, T.E. Oni, S.A. Hearn, E.J. Lee, I.L.C. Chio, C.-I. Hwang, H. Tiriari, L. A. Baker, D.D. Engle, C. Feig, A. Kultti, M. Egeblad, D.T. Fearon, J.M. Crawford, H. Clevers, Y. Park, D.A. Tuveson, Distinct populations of inflammatory fibroblasts and myofibroblasts in pancreatic cancer, *J. Exp. Med.* 214 (2017) 579–596, <https://doi.org/10.1084/jem.20162024>.
- [44] A. Takeda, M. Hollmén, D. Dermadi, J. Pan, K.F. Brulois, R. Kaukonen, T. Lönnberg, P. Boström, I. Koskivuo, H. Irijala, M. Miyasaka, M. Salmi, E. C. Butcher, S. Jalkanen, Single-cell survey of human lymphatics unveils marked endothelial cell heterogeneity and mechanisms of homing for neutrophils, *Immunity* 51 (2019) 561–572, <https://doi.org/10.1016/j.immuni.2019.06.027>, e5.
- [45] Q. Ma, L.C. Dieterich, M. Detmar, Multiple roles of lymphatic vessels in tumor progression, *Curr. Opin. Immunol.* 53 (2018) 7–12, <https://doi.org/10.1016/j.coi.2018.03.018>.
- [46] D.O. Miteva, J.M. Rutkowski, J.B. Dixon, W. Kilarski, J.D. Shields, M.A. Swartz, Transmural flow modulates cell and fluid transport functions of lymphatic endothelium, *Circ. Res.* 106 (2010) 920–931, <https://doi.org/10.1161/CIRCRESAHA.109.207274>.
- [47] M. Suarez-Carmona, M. Bourcy, J. Lesage, N. Leroi, L. Syne, S. Blacher, P. Hubert, C. Ericpim, J.M. Foidart, P. Delvenne, P. Birembaut, A. Noël, M. Polette, C. Gilles, Soluble factors regulated by epithelial-mesenchymal transition mediate tumour angiogenesis and myeloid cell recruitment, *J. Pathol.* 236 (2015) 491–504, <https://doi.org/10.1002/path.4546>.
- [48] B. Garmy-Susini, C.J. Avraamides, J.S. Desgrosellier, M.C. Schmid, P. Foubert, L. G. Ellies, A.M. Lowy, S.L. Blair, S.R. Vandenberg, B. Datnow, H.-Y. Wang, D. A. Cheresch, J. Varner, PI3K α activates integrin α 4 β 1 to establish a metastatic niche in lymph nodes, *Proc. Natl. Acad. Sci. U. S. A.* 110 (2013) 9042–9047, <https://doi.org/10.1073/pnas.1219603110>.
- [49] W.E. Cromer, S.D. Zawieja, B. Tharakan, E.W. Childs, M.K. Newell, D.C. Zawieja, The effects of inflammatory cytokines on lymphatic endothelial barrier function, *Angiogenesis* 17 (2014) 395–406, <https://doi.org/10.1007/s10456-013-9393-2>.
- [50] E.D. Lucas, B.A.J. Tamburini, Lymph node lymphatic endothelial cell expansion and contraction and the programming of the immune response, *Front. Immunol.* 10 (2019) 36, <https://doi.org/10.3389/fimmu.2019.00036>.
- [51] M. Brown, L.A. Johnson, D.A. Leone, P. Majek, K. Vaahtomeri, D. Senfter, N. Bukosza, H. Schachner, G. Asfour, B. Langer, R. Hauschild, K. Parapatics, Y. K. Hong, K.L. Bennett, R. Kain, M. Detmar, M. Sixt, D.G. Jackson, D. Kerjaschki, Lymphatic exosomes promote dendritic cell migration along guidance cues, *J. Cell Biol.* 217 (2018) 2205–2221, <https://doi.org/10.1083/jcb.201612051>.