TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Blockade of Lymphangiogenesis Shapes Tumor-Promoting Adipose Tissue Inflammation

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Tumor-associated lymphangiogenesis correlates with lymph node metastasis and poor outcome in several human malignancies. In addition, the presence of functional lymphatic vessels regulates the formation of tumor inflammatory and immune microenvironments. Although lymphatic structures are often found deeply integrated into the fabric of adipose tissue, the impact of lymphangiogenesis on tumor-associated adipose tissue (AT) has not yet been investigated. Using K14-VEGFR3-Ig mice that constitutively express soluble vascular endothelial growth factor receptor (VEGFR) 3—Ig in the skin, scavenging VEGF-C and VEGF-D, the role of lymphangiogenesis in the generation of an inflammatory response within tumor-associated AT was studied. Macrophages expressing lymphatic vessel endothelial hyaluronan receptor-1 were found within peritumoral adipose tissue from melanoma-bearing K14-VEGFR3-Ig mice, which were further enriched with alternatively activated macrophages based on surface marker CD301/C-type lectin domain family 10 member A expression. The blockade of lymphangiogenesis also resulted in accumulation of the cytokine IL-6, which correlated with enhanced macrophage proliferation of the alternatively activated phenotype. Furthermore, melanomas co-implanted with freshly isolated adipose tissue macrophages grew more robustly than melanomas growing alone. In human cutaneous melanomas, adipocyte-selective *FABP4* transcripts closely correlated with gene signatures of *CLEC10A* and were associated with poor overall survival. These data suggest that the blockade of pathways regulating lymphatic vessel formation shapes an inflammatory response within tumor-associated AT by facilitating accumulation of tumor-promoting alternatively activated macrophages. (*Am J Pathol* 2019, 189: 2102–2114; <https://doi.org/10.1016/j.ajpath.2019.06.010>)

Tumor-associated lymphangiogenesis mediated by the lymphangiogenic factors vascular endothelial growth factor (VEGF)-C and VEGF-D through VEGF receptor (VEGFR)-3 activation contributes to malignant progression, at least in part, by facilitating metastatic spread to regional lymph nodes.^{1,2} However, lymphatic vessels and their drainage function also regulate local inflammation and antitumor host immune responses, thus serving multiple and intricate roles in tumor progression.^{1,3–5}

Lymphatic structures such as collecting lymphatic vessels and lymph nodes are often found embedded in adipose tissue (AT).⁶ In addition to lipid-engorged adipocytes, AT harbors the stromal vascular fraction of cells, including preadipocytes, fibroblasts, vascular endothelial cells, and various immune cells with macrophages comprising the largest group.^{7,8} Evidence suggests that adipocytes together

with classically activated macrophages secrete bioactive molecules, including cytokines that could be related to the development of obesity-associated low-grade systemic inflammation and insulin resistance.^{9–14} It has also been revealed that the blockage of VEGF-C and VEGF-D protects from insulin resistance and hepatic lipid accumulation through the accumulation of alternatively activated macrophages in subcutaneous AT from K14-VEGFR3-Ig mice that constitutively express soluble VEGFR3-Ig in the skin to prevent local VEGFR-3 signaling.¹⁵

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Despite the fact that some tumors grow directly within AT or metastasize to the lymph nodes, AT is perhaps the most frequently overlooked stromal compartment in solid tumors.⁷ The progression of cutaneous melanomas, for example, is often noted when malignant cells enter a vertical growth phase and grow into subcutaneous tissue typically enriched with adipocytes and vascular endothelium. With the use of melanoma as a model, peritumoral AT was found to act as a proximate source of new blood vessels incorporated by the growing tumor mass and inflammatory cells, in particular macrophages.^{16,17} Whether lymphangiogenesis contributes to the generation of an inflammatory response within tumor-associated AT has not yet been assessed.

In this report, with the use of K14-VEGFR3-Ig mice, we unraveled the role of lymphangiogenesis in the generation of tumor-related inflammatory response within tumor-associated AT.^{15,18} In a melanoma model, AT juxtaposed to the growing tumors in K14-VEGFR3-Ig mice exhibited the presence of macrophages expressing the hyaluronan receptor lymphatic vessel endothelial hyaluronan receptor (LYVE)-1 and dense infiltration of alternatively activated macrophages based on surface marker CD301/C-type lectin domain family 10 member A (CLEC10A) expression. This observation was confirmed using mouse C3HBA adenocarcinoma cells implanted in the mammary fat pad of CHY mice, which carry a heterozygous inactivating mutation of VEGFR-3 resulting in an impaired dermal lymphangiogenesis.¹⁹ Furthermore, the blockade of lymphangiogenesis resulted in elevated levels of the cytokine IL-6 within tumor-associated AT from K14-VEGFR3-Ig mice and correlated with enhanced macrophage proliferation of the alternatively activated phenotype. Of importance, melanomas implanted in one of the subcutaneous AT depots of K14-VEGFR3-Ig mice together with freshly isolated AT macrophages grew faster, more robustly, and were more vascularized than those implanted alone. In human cutaneous melanomas, adipocyte-selective *FABP4* transcripts closely correlated with *CLEC10A* and were associated with poor overall survival.

These data suggest that the blockade of pathways regulating lymphatic vessel formation shapes tumor-promoting inflammation within tumor-associated AT by facilitating accumulation of alternatively activated macrophages.

Materials and Methods

Mice

Breeding, maintenance, and genotyping of K14-VEGFR3-Ig and CHY mice was performed as previously described.^{18,20} K14-VEGFR3-Ig male mice, a generous gift from Prof. Kari Alitalo (University of Helsinki, Finland), were on a C57BL/6 background and crossbred with wild-type (WT) C57BL/6 female mice. Ten- to 11-week-old K14-VEGFR3-Ig male mice and WT male littermates were used in

experiments. Female CHY mice were on a C3H background, and the breeding generated either CHY mice (*VEGFR3*^{+/Chy}) or WT littermates (*VEGFR3*^{+/+}). Female mice of fertile age were used for C3HBA breast adenocarcinoma experiments. Animal experiments were performed according to the regulations of the Norwegian State Commission for Laboratory Animals that agreed with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes and Council of Europe (ETS number 123). Experiments were conducted according to the approval from the Association for Assessment and Accreditation of Laboratory Animal Care International accredited Animal Care and Use Program at the University of Bergen (License number 7302 FOTS).

Tumor Cells

B16F10 murine melanoma cells were purchased from ATCC (Manassas, VA). C3HBA breast adenocarcinoma cells were obtained from the National Cancer Institute Division of Cancer Treatment and Diagnosis Tumor Repository (Frederick, MD). Tumor cells were incubated at 37°C in 5% CO₂ in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, St. Louis, MO), supplemented with 10% fetal bovine serum, nonessential amino acids, 100 U/mL penicillin, 100 µg/mL streptomycin, 4 mmol/L L-glutamine (all from Lonza, Basel, Switzerland), and plasmocin (InvivoGEN, San Diego, CA). Cells were detached and dissociated using trypsin-EDTA (Sigma-Aldrich) and passaged every other day.

Implantation of Tumor Cells and Collection of Peritumoral AT

For studies of tumor growth, B16F10 cells (1×10^6 cells) were resuspended in 100 µL of phosphate-buffered saline (PBS) and implanted in the anterior, subcutaneous AT depot or intradermally along the dorsal midline of syngeneic K14-VEGFR3-Ig mice or their WT littermates. C3HBA cells (5×10^6 cells) were implanted in the abdominal mammary fat pad. For IL-6 neutralization, K14-VEGFR3-Ig mice were administered daily an anti-IL-6 (0.5 µg in 100 µL of PBS) antibody (BioXCell, West Lebanon, NH) or PBS intraperitoneally. Tumor size was measured daily with the use of a vernier caliper. Tumor volume was measured with calipers and calculated with the formula $a^2b \times (\pi/6)$, where a and b stand for the shorter and longer diameter of the tumors, respectively. Animals were euthanized by CO₂ inhalation, and tumor samples were collected. For AT studies, peritumoral AT was microdissected away from the tumor mass with the use of a stereoscopic microscope. Control AT from the counterpart depot was collected from age- and sex-matched animals. Tumor and AT samples were weighed and then fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 4-µm thick sections.

Morphometric Analysis of Adipocytes

Morphometric analysis of adipocytes ($n > 250$) from AT samples from three different animals per group was performed with NIS-elements AR software version 4.11 (Nikon, Tokyo, Japan).

Immunohistochemistry and Immunofluorescence

For immunohistochemical staining, tissue sections were deparaffinized and rehydrated before antigen retrieval at 98°C in water bath for 20 to 60 minutes in 0.01 mol/L citrate buffer (pH 6.0). For F4/80 staining, treatment with Proteinase K (Dako, Carpinteria, CA) was used according to the manufacturer's instructions provided. After being blocked with diluted serum from the secondary antibody host for 30 minutes, the sections were incubated with the primary antibody overnight at 4°C. Blocking of endogenous peroxidase was performed with 3% H₂O₂ (Sigma-Aldrich) for 20 minutes. Subsequently, the sections were incubated with a biotinylated anti-rat, anti-rabbit, or anti-goat secondary antibody (Vector Laboratories, Burlingame, CA) for 45 minutes at room temperature. Avidin-biotin-peroxidase was used to augment the antigen-antibody complex reaction for 45 minutes according to the manufacturer's instructions provided (Vectastain ABC Kit; Vector Laboratories) and stained for 1 to 10 minutes with diaminobenzidine tetrahydrochloride (Vector Laboratories). The sections were counterstained with hematoxylin (Thermo Fisher Scientific, Waltham, MA), dehydrated, and mounted with the use of Entellan (Merck Millipore, Darmstadt, Germany). To ensure the specificity of the immunoreactions, parallel sections were run for all of the experiments with the primary antibody omitted. The following primary antibodies were used: rabbit anti-Perilipin-1 (dilution 1:200; Cell Signaling Technology, Danvers, MA), rabbit anti-LYVE1 (dilution 1:100; Abcam, Cambridge, UK), rat anti-F4/80 (dilution 1:50; Abcam), rat anti-CD301 (dilution 1:50; Bio-Rad AbD Serotec, Puchheim, Germany), rabbit anti-IL-4 receptor (IL-4R; dilution 1:250; Abcam), and goat anti-CD31 (dilution 1:100; Santa Cruz Biotechnology, Dallas, TX). For immunofluorescence staining, the sections were incubated with the primary antibody overnight at 4°C. A mixture of the corresponding secondary antibodies was applied for 45 minutes at room temperature thereafter. Finally, the slides were mounted with the use of DAPI mounting medium (Vector Laboratories). The following primary antibodies were used: rabbit anti-LYVE1 (dilution 1:100; Abcam), rat anti-LYVE1 (dilution 1:100; R&D Systems, Minneapolis, MN), rat anti-F4/80 (dilution 1:50; Abcam), rabbit anti-CD11b (dilution 1:200; Abcam), rat anti-CD301 (dilution 1:50; Bio-Rad AbD Serotec), rabbit anti-IL-4R (dilution 1:250; Abcam), rabbit anti-Ki67 (dilution 1:100; Abcam), and rat anti-bromodeoxyuridine [BrdU; BU1/75(ICR1); dilution 1:100; Bio-Rad AbD Serotec]. The secondary antibodies used were: cyanine 2 anti-rat IgG (dilution 1:200; Jackson ImmunoResearch, West Grove, PA), cyanine 3 anti-rabbit IgG (dilution 1:200; Jackson ImmunoResearch), Alexa Fluor 488 anti-rat IgG

(H+L) (dilution 1:200; Thermo Fisher Scientific), and Alexa Fluor 568 anti-rabbit IgG (H+L) (dilution 1:200; Thermo Fisher Scientific).

In Vivo BrdU Incorporation

Mice were intraperitoneally administered 200 µL of BrdU solution (10 mg/mL) (Sigma-Aldrich). Incorporation of BrdU into proliferating cells within subcutaneous AT was analyzed 4 hours after injection by immunofluorescence staining.

ELISA

Mouse IL-6 enzyme-linked immunosorbent assay (ELISA) kit was purchased from Sigma-Aldrich. ELISA was performed as recommended by the manufacturer's instructions provided. For the assessment of IL-6, whole AT protein lysates were used. Briefly, peritumoral and control AT samples were homogenized, and cells were sonicated in a total protein lysis buffer (50 mmol/L TRIS-HCl, pH 7.5, 150 mmol/L NaCl, 0.1% SDS, 1% deoxycholate, 1% Triton X-100) supplemented with a protease and phosphatase inhibitors (Roche, Basel, Switzerland). Measurements were done in duplicate.

FACS

Freshly collected subcutaneous AT samples pooled from 10 naive C57BL/6 mice were digested with a mixture of collagenase Type-2 (1 mg/mL; Worthington, Lakewood, NJ), dispase II (2.5 µg/mL; Roche), and DNase (1 mg/mL; Worthington) for 1 hour at 37°C with continuous rotation. The dissociated tissue was centrifuged at $186 \times g$ for 10 minutes. To remove debris, the pelleted stromal vascular fraction was filtered through 70-µm mesh cell strainer (BD Bioscience, San Jose, CA). Contaminating erythrocytes were lysed using red blood cell lysis buffer (BD Bioscience). Cells were resuspended in fluorescence-activated cell sorting (FACS) buffer (0.5% w/v bovine serum albumin, 0.5 mol/L EDTA in sterile PBS) and blocked with FcR-blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany), to prevent nonspecific binding. After incubation for 30 minutes at 4°C with appropriate antibodies, cells were sorted with the use of BD FACS Aria Cell Sorting System with BD FACSDiva software version 8.0 (BD Bioscience). The following antibodies were used: rat anti-CD11b:RPE (Bio-Rad AbD Serotec) and rat anti-F4/80:AF488 (Bio-Rad AbD Serotec). For tumor growth studies, B16F10 cells (2.5×10^5 cells) alone or admixed with CD11b⁺F4/80⁺ cells (2.5×10^5 cells) were injected into the contralateral anterior, subcutaneous AT depots of syngeneic K14-VEGFR3-Ig mice of both sexes.

Database Analysis

The association between expression levels of *FABP4* and survival rates of 458 melanoma patients from the publicly available The Cancer Genome Atlas (TCGA; <http://>

cancergenome.nih.gov, last accessed March 12, 2019) database was determined with the Oncolnc platform (www.oncolnc.org, last accessed March 12, 2019). According to the expression levels of *FABP4*, melanoma samples ($n = 458$) were assigned into two groups of low ($n = 229$) and high ($n = 229$) expression levels of *FABP4*. Expression data were extracted and the Kaplan–Meier estimation curve was plotted with Prism software version 6.0 (GraphPad Software Inc., San Diego, CA).

The gene expression correlation analysis was performed on the Tumour Skin Cutaneous Melanoma - TCGA - 470 - rsem - tcgars data set that contained data from 470 melanoma patients retrieved from the publicly available TCGA

(<http://cancergenome.nih.gov>) database with the use of the microarray analysis and visualization platform R2 (<http://r2.amc.nl>, last accessed February 13, 2019). Pearson's correlation coefficient, r , was calculated with the transform 2log setting. Expression data of the genes of interest were extracted and plotted with Prism software (GraphPad Software Inc.).

Statistical Analysis

The statistical significance was assessed by unpaired t -test or one-way analysis of variance followed by Tukey's multiple comparisons test, using Prism software (GraphPad Software

Figure 1 Tumor-bearing K14-VEGFR3-Ig mice demonstrate decreased peritumoral adipose tissue (AT) mass and reduced adipocyte size. **A:** Graphical representation of the study design. B16F10 melanoma cells (1×10^6 cells) were implanted in the fat pad of wild-type (WT) or K14-VEGFR3-Ig transgenic (TG) mice. **B:** Body weights of control and tumor-bearing mice of two genotypes. **C** and **D:** Weights of subcutaneous white AT (SWAT; **C**) and epididymal white AT (EWAT; **D**) from control and tumor-bearing mice of two genotypes. Decreased tissue mass reveals atrophy of peritumoral AT from TG mice. **E:** Hematoxylin-eosin (H&E) histologic examination of control and peritumoral AT from WT and TG mice. Immunohistochemical analysis of control and peritumoral AT from WT and TG mice using an antibody against the Perilipin-1, an adipocyte-specific lipid-coated protein. **F:** Adipocyte size quantification demonstrates drastically reduced size of fat cells from peritumoral AT from TG mice. **G:** Immunohistochemical analysis of control and peritumoral AT from WT and TG mice using an antibody against the lymphatic vessel endothelial hyaluronan receptor (LYVE) 1, a lymphatic endothelium-specific hyaluronan receptor. **Arrows** mark LYVE1⁺ cells. **Asterisk** marks LYVE1⁺ vessel. **H:** Quantification of lymphatic vessels reveals presence of LYVE1⁺ structures in peritumoral AT from WT mice only. Data are expressed as means $\pm$ SEM. $n = 3$ to 6 mice per group (**B–D**); $n > 250$ cells per group (**F**); $n = 3$ to 4 mice per group (**H**). *** $P < 0.001$ (statistical analysis with one-way analysis of variance followed by Tukey's multiple comparisons test). Scale bars: 50 μ m (**E**); 25 μ m (**G**). n.d., not detected.

Figure 2 Peritumoral adipose tissue (AT) from K14-VEGFR3-Ig mice shows increased accumulation of macrophages of alternatively activated phenotype based on surface marker CD301 expression. **A** and **B**: Immunofluorescence analysis reveals lymphatic vessel endothelial hyaluronan receptor (LYVE) ¹⁺ cells coexpressing murine macrophage markers F4/80 (**A**) and Cd11b (**B**). **Arrows** mark LYVE¹⁺ macrophages. **C**: Immunohistochemical analysis of control and peritumoral AT from wild-type (WT) and transgenic (TG) mice using an antibody against the F4/80. **D**: Quantification of F4/80⁺ cells demonstrates increased infiltration of macrophages in peritumoral AT from TG mice. **E**: Immunohistochemical analysis of control and peritumoral AT from WT and TG mice using an antibody against the CD301, a marker of alternatively activated macrophages. **F**: Quantification of CD301⁺ cells reveals increased accumulation of alternatively activated macrophages in peritumoral AT from TG mice. Data are expressed as means $\pm$ SEM. $n = 3$ to 4 mice per group (**D** and **F**). *** $P < 0.001$ (statistical analysis with one-way analysis of variance followed by Tukey's multiple comparisons test). Scale bars: 25 μ m (**A** and **B**); 50 μ m (**C** and **E**).

Inc.). The value of $P < 0.05$ was considered statistically significant. Whenever possible, the investigators (M.W. and E.S.S.) were partially blinded for assessing the outcome (eg, immunohistochemistry). To determine the correlation between gene expression levels in skin cutaneous melanoma patients in the TCGA data set, Pearson's correlation coefficient, r , was calculated. To demonstrate the association between gene expression levels and survival rate of skin cutaneous melanoma patients in the TCGA data set, multivariate Cox regression and Kaplan–Meier analysis were performed.

Results

Peritumoral AT from K14-VEGFR3-Ig Mice Is Atrophied and Typified by Decreased Adipocyte Size

In mice there are two subcutaneous white AT depots (or fat pads) located along the scapulae (descending from the neck to the axillae). We took advantage of their anatomic location and implanted B16F10 murine melanoma cells (1×10^6 cells) directly in one of the fat pads of K14-VEGFR3-Ig mice (referred to as TG in the figures) or WT littermates,

to characterize peritumoral AT (Figure 1A). At 14 days after implantation, peritumoral AT was microdissected away from the surrounding tumor mass and compared with control AT. Control and tumor-bearing mice had a comparable body weight for both genotypes (Figure 1B). Although the weight of control AT was similar between WT and K14-VEGFR3-Ig mice, the weight of peritumoral AT from transgenic mice was on average twofold lower than that from WT littermates (Figure 1C). The weight of epididymal white AT between control and tumor-bearing mice for both genotypes was not significantly changed (Figure 1D). Under microscopic examination of hematoxylin and eosin–stained sections, adipocytes from peritumoral AT for both genotypes appeared smaller than their normal counterparts (Figure 1E). This observation was confirmed by staining AT sections with an antibody against the lipid droplet-associated protein, perilipin-1 (Figure 1E). Furthermore, when measured with the use of NIS-elements AR software, adipocytes from peritumoral AT from K14-VEGFR3-Ig mice were approximately 60% smaller than adipocytes from WT mice (Figure 1F). Decreased adipocyte size from control AT from K14-VEGFR3-Ig mice was also observed

Figure 3 Peritumoral adipose tissue (AT) from CHY mice shows reduced adipocyte size and increased accumulation of macrophages of alternatively activated phenotype based on surface marker CD301 expression. **A** and **B**: Hematoxylin-eosin (H&E) histologic examination (**A**) and quantification (**B**) of adipocyte size from control and peritumoral AT from wild-type (WT) and CHY mice. **C** and **D**: Immunohistochemical analysis of control and peritumoral AT from WT and CHY mice using an antibody against the CD301 (**C**) and quantification of CD301⁺ cells (**D**). Data are expressed as means $\pm$ SEM. $n > 300$ cells per group (**A** and **B**); $n = 3$ mice per group (**C** and **D**). *** $P < 0.001$ (statistical analysis with one-way analysis of variance followed by Tukey's multiple comparisons test). Scale bars: 50 μ m (**A** and **C**).

compared with WT littermates. The mean sizes were $1421 \pm 67 \mu\text{m}^2$ and $865 \pm 34 \mu\text{m}^2$ for adipocytes from control AT from WT and K14-VEGFR3-Ig mice, respectively ($n > 250$ cells per group). For adipocytes from peritumoral AT the mean sizes were $371 \pm 11 \mu\text{m}^2$ for WT mice and $129 \pm 4 \mu\text{m}^2$ for transgenic mice ($n > 250$ cells per group) (Figure 1F). Similarly, the number of adipocytes was reduced in peritumoral AT from transgenic mice compared with WT littermates (data not shown).

The lymphatic vasculature in the AT from K14-VEGFR3-Ig and WT mice was next investigated (Figure 1G). Immunohistochemical analysis of control and peritumoral AT revealed the existence of lymphatic vessels within peritumoral AT from WT mice only as judged by the presence of LYVE1⁺ structures (Figure 1H). However, peritumoral AT from K14-VEGFR3-Ig mice was infiltrated by what appeared to be a population of variably shaped LYVE1⁺ single cells rather than lymphatic endothelial cells forming tube-like structures (Figure 1G).

Peritumoral AT from K14-VEGFR3-Ig Mice Is Characterized by Local Proliferation of Alternatively Polarized Macrophages

Because macrophages are known to express LYVE1, the colocalization of LYVE1 was examined with a murine macrophage and myeloid cell markers, F4/80 and CD11b,

respectively. Indeed, immunofluorescence analysis revealed the expression of LYVE1 by F4/80⁺ and CD11b⁺ cells (Figure 2, A and B). Next, macrophages in control and peritumoral AT were quantitatively and qualitatively characterized from both genotypes.^{21–24} Immunohistochemical analysis revealed abundance of F4/80⁺ macrophages in peritumoral versus control AT with a significant increase observed in K14-VEGFR3-Ig mice compared with WT mice (Figure 2, C and D). Further analysis of the peritumoral AT from transgenic mice demonstrated an increased number of macrophages expressing the C-type lectin 10A (CLEC10A/CD301), a marker of alternative polarization compared with peritumoral AT from WT littermates (Figure 2, E and F). No significant difference was observed in the number of CD8⁺ T cells in peritumoral AT from transgenic versus WT mice (data not shown).

The observed phenotype was confirmed with another model of lymphatic insufficiency, CHY mice, which carry a heterozygous inactivating mutation of VEGFR-3. Syngeneic C3HBA adenocarcinoma cells were implanted in the mammary fat pad of CHY mice and WT littermates. At 16 days after implantation, adipocytes were found from peritumoral AT from CHY mice approximately 60% smaller compared with WT mice under microscopic examination of hematoxylin and eosin–stained sections (Figure 3A). In contrast, the size of adipocytes from control AT from CHY mice was found to be significantly increased compared with

Figure 4 Increased levels of IL-6 characterize peritumoral adipose tissue (AT) from K14-VEGFR3-Ig mice. **A:** IL-6 levels in control and peritumoral AT from wild-type (WT) and transgenic (TG) mice measured by enzyme-linked immunosorbent assay. **B:** Immunohistochemical analysis of control and peritumoral AT from WT and TG mice using an antibody against the IL-4 receptor. **C:** Quantification reveals increased accumulation of IL-4R⁺ cells in peritumoral AT from TG mice. **D:** Tumor growth profiles in TG mice treated every day with anti-IL-6 monoclonal antibody [mAb; 0.5 μ g in 100 μ L of phosphate-buffered saline (PBS)] or PBS (Control). **E** and **F:** Immunohistochemical analysis of peritumoral AT from TG mice using an antibody against the IL-4 receptor (IL-4R; **E**) and quantification of IL-4R⁺ cells (**F**). **G:** Immunofluorescence analysis reveals F4/80⁺ macrophages coexpressing IL-4 receptor. **H:** The mitotic figures in F4/80⁺ IL-4R⁺ macrophages are occasionally observed in peritumoral AT from TG mice. Data are expressed as means $\pm$ SEM. $n = 4$ to 6 mice per group (**A**); $n = 3$ mice per group (**C**); $n = 5$ to 6 mice per group (**D**); $n = 4$ mice per group (**E** and **F**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ (statistical analysis with one-way analysis of variance followed by Tukey's multiple comparisons test). Scale bars: 100 μ m (**B**); 50 μ m (**E**); 25 μ m (**G**); 10 μ m (**H**).

WT counterparts (Figure 3A). The mean sizes were $2934 \pm 69 \mu\text{m}^2$ and $3258 \pm 73 \mu\text{m}^2$ for adipocytes from control AT from WT and CHY mice, respectively ($n > 300$ cells per group) (Figure 3B). For adipocytes from peritumoral AT the mean sizes were $1174 \pm 44 \mu\text{m}^2$ for WT mice and $457 \pm 19 \mu\text{m}^2$ for mutated mice ($n > 300$ cells per group) (Figure 3B). In addition, immunohistochemical analysis revealed a 2.5-fold increase in the number of macrophages expressing CD301 in peritumoral AT from mutated mice (Figure 3, C and D). No difference was observed in the number of CD301⁺ macrophages in control AT from mutated versus WT mice (Figure 3, C and D).

The expression of IL-6, compared with control AT, was strikingly up-regulated in peritumoral AT from C57BL/6 mice.¹⁶ In addition, it was recently revealed that IL-6 signaling

promoted alternative macrophage polarization through the induction of IL-4R expression.^{25–27} Thus, it was speculated that peritumoral AT-derived IL-6 might regulate macrophage polarization in this model. It was first confirmed that IL-6 was expressed in peritumoral AT from both WT and K14-VEGFR3-Ig mice. IL-6 expression was approximately 1.7-fold higher in peritumoral AT from K14-VEGFR3-Ig mice than from WT littermates (Figure 4A). Next, an increase abundance of cells expressing IL-4R was found in peritumoral AT from K14-VEGFR3-Ig mice as revealed by immunohistochemical analysis (Figure 4, B and C). Of note, neutralization of IL-6 by *in vivo* treatment with anti-IL-6 antibody significantly inhibited the growth of melanomas (Figure 4D) and decreased the number of IL-4R⁺ cells within the peritumoral AT from K14-VEGFR3-Ig mice compared with controls (Figure 4, E

Figure 5 Peritumoral adipose tissue (AT) from transgenic (TG) mice demonstrates increased accumulation of proliferating macrophages. **A** and **B**: Immunofluorescence analysis (**A**) and quantification (**B**) of bromodeoxyuridine (BrdU)⁺CD11b⁺ cells in control and peritumoral AT from wild-type (WT) and TG mice. The **inset** shows CD11b⁺BrdU⁺ cells in peritumoral AT from TG mice. **C** and **D**: Immunofluorescence analysis (**C**) and quantification (**D**) of CD301⁺Ki67⁺ cells in control and peritumoral AT from WT and TG mice. Data are expressed as means ± SEM. $n = 3$ mice per group (**A–D**). $^{**}P < 0.01$, $^{***}P < 0.001$ (statistical analysis with one-way analysis of variance followed by Tukey's multiple comparisons test). Scale bars: 100 μ m (**A**, **main image**, and **C**). Original magnification, $\times 63$ (**A**, **inset**).

and F). Finally, the colocalization of IL-4R with F4/80 confirmed IL-4R expression by macrophages (Figure 4G). On occasion, mitotic figures were also detected within peritumoral AT from K14-VEGFR3-Ig mice (Figure 4H).

To better assess the extent of macrophage proliferation in peritumoral AT, cell proliferation was analyzed *in vivo* by administering BrdU. Incorporation of BrdU was measured by cells within AT 4 hours after BrdU injection. Almost twice as many BrdU⁺CD11b⁺ cells were detected in peritumoral AT from K14-VEGFR3-Ig mice compared with WT littermates (Figure 5A and B). Peritumoral AT was also found from K14-VEGFR3-Ig mice highly infiltrated with Ki-67⁺ macrophages expressing CD301, indicative of their alternative form of activation (Figure 5, C and D).²⁸

Macrophages within Peritumoral AT Increase Tumor Growth and Vascularization

To assess tumor growth and vascularization, B16F10 murine melanoma cells (1×10^6 cells) were directly implanted in one of the fat pads or in the dorsal dermis (far from the fat

pads) of K14-VEGFR3-Ig or WT mice (Figure 6A). Melanomas implanted in the fat pad of K14-VEGFR3-Ig mice grew faster and more robustly than with WT littermates (Figure 6B). The lack of dermal lymphatic vessels, however, did not affect the growth of tumors at a site distant from the AT depots (Figure 6B). At 14 days after implantation, tumors growing in the fat pads weighed almost twice and thrice as much as melanomas implanted in the dorsal dermis in K14-VEGFR3-Ig (2.5 ± 0.3 g versus 0.7 ± 0.1 g; $n = 6$; $P < 0.001$) and WT (1.7 ± 0.2 g versus 0.8 ± 0.2 g; $n = 6$; $P < 0.05$) mice, respectively (Figure 6C). Immunohistochemical staining revealed increased blood vessel density, as analyzed by the numbers of CD31⁺ structures per area, within tumors growing in AT in both K14-VEGFR3-Ig and WT mice (Figure 6, D and E). Of importance, however, melanomas implanted in the fat pad of K14-VEGFR3-Ig mice were significantly more vascularized than with WT littermates (Figure 6, D and E).

It was therefore speculated that the increased growth of melanomas implanted in K14-VEGFR3-Ig mice was related to an elevated number of macrophages within

Figure 6 Blockade of lymphangiogenesis accelerates the growth of tumors implanted in the fat pad by affecting their vascularization. **A:** Graphical representation of the study design. B16F10 melanoma cells (1×10^6 cells) were implanted either in the fat pad or dorsal dermis (no fat pad) of wild-type (WT) or transgenic (TG) mice. **B:** Tumor growth profiles in WT and TG mice. **C:** Quantification of tumor weights. **D:** Immunohistochemical analysis of tumors implanted either in the fat pad or dorsal dermis (no fat pad) of WT or TG mice using an antibody against the CD31, the pan-endothelial cell marker. **E:** Blood vessel density quantification. Data are expressed as means $\pm$ SEM. $n = 6$ mice per group (**B** and **C**); $n = 3$ mice per group (**E**). * $P < 0.05$, *** $P < 0.001$ (statistical analysis with one-way analysis of variance followed by Tukey's multiple comparisons test). Scale bar = 200 μ m (**D**).

tumor-associated AT as previously observed.¹⁶ For that reason, B16F10 melanomas (2.5×10^5 cells) alone or together with freshly isolated F4/80⁺CD11b⁺ AT macrophages (2.5×10^5 cells) were implanted in contralateral fat pads of K14-VEGFR3-Ig mice (Figure 7A). Indeed, the presence of AT macrophages resulted in an accelerated growth of B16F10 melanomas (Figure 7B) characterized by an increased number of macrophages (Figure 7, C and D) and blood vessel density (Figure 7, E and F) as revealed by immunohistochemical analysis.

Because AT strongly accelerates mouse melanoma growth, OncoLnc was used to conduct overall survival analysis for *FABP4*, which encoded the fatty acid binding protein found in adipocytes, in 458 human skin cutaneous melanoma samples from TCGA database. The high expression of *FABP4* was associated with poor overall survival ($P < 0.0362$) in cutaneous melanoma patients (Figure 8A). Spurred by this observation, it was determined whether correlation could be found between the expression levels of *FABP4* with genes associated with macrophage infiltration after an analysis of 470 human skin cutaneous melanoma samples from TCGA database. First, a strong correlation was found among the gene expression levels of *FABP4* and perilipin-1 (*PLIN1*). Of importance, the gene expression levels of *FABP4* also correlated with *IL6* as well as a marker of alternative macrophage polarization *CLEC10A* but not a pan-macrophage marker *CD68* (Figure 8B). We therefore hypothesized that the expression of IL-6 might contribute to the presence of

alternatively activated macrophages within tumor-associated AT in human melanomas. Indeed, the gene expression levels of *IL6* strongly correlated with both *CLEC10A* and *IL4R*. Conversely, no correlation was found between *IL6* and *NOS2* expressed by classically activated macrophages (Figure 8C). Instead, a strong correlation was observed between *FABP4* and *FLT4/VEGFR3* and lymphangiogenic growth factors such as *VEGFC* and, to a lower extent, *VEGFD*. In addition, a correlation was observed between *FLT4* and *NOS2*, implying a possible relationship between the presence of lymphatic vessels and classically activated macrophages (Figure 8D).

Discussion

To the best of our knowledge this is the first experimental study to address the role of lymphangiogenesis in the regulation of inflammatory response within tumor-associated AT. Here, we show that the blockade of VEGFR-3 activation renders AT primed or permissive for tumor growth through the accumulation of tumor-promoting macrophages.

Although intimately associated with the microenvironment of many tumors, AT is perhaps the most frequently overlooked stromal compartment.^{17,29,30} Nevertheless, it has recently been reported that AT is not a passive bystander in tumorigenesis. For example, tumor cells can influence adjacent adipocytes to acquire an activated phenotype

Figure 7 Adipose tissue macrophages (ATMs) stimulate the growth of tumors implanted in the fat pad. **A:** Graphical representation of the study design. B16F10 melanoma cells (2.5×10^5 cells) without or with freshly isolated CD11b⁺F4/80⁺ ATMs (2.5×10^5 cells) were implanted in the fat pad of transgenic (TG) mice. Tumors were harvested 14 days after implantation. **B:** Growth profiles of tumors implanted without or with ATMs in the fat pad of TG mice. **C and D:** Immunohistochemical analysis (**C**) and quantification (**D**) of CD301⁺ cells in peritumoral AT from tumors implanted without or with ATMs in the fat pad of TG mice. **E and F:** Immunohistochemical analysis (**E**) and quantification (**F**) of CD31⁺ vessels in peritumoral AT from tumors implanted without or with ATMs in the fat pad of TG mice. Data are expressed as means $\pm$ SEM. $n = 6$ mice per group (**A** and **B**); $n = 3$ mice per group (**C–F**); * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ (statistical analysis with *t*-test). Scale bars: 50 μ m (**C**); 200 μ m (**E**).

characterized by reduced size and sustained lipolysis.³¹ In turn, tumor-associated adipocytes can provide a source of free fatty acids.³¹ The β -oxidation of free fatty acids can, therefore, fulfill the energy requirements of rapidly proliferating malignant cells.^{7,31} Adipocyte lipolysis might also contribute, at least in part, to an accelerated growth of tumors implanted in the fat pad of K14-VEGFR3-Ig mice, because the size of tumor-associated adipocytes was strikingly reduced. This phenotype was confirmed with the use of another model of lymphatic vessel dysfunction, CHY mice. Of note, the size of adipocytes from control AT decreased in K14-VEGFR3-Ig mice compared with adipocytes from WT counterparts. In CHY mice, however, the size of adipocytes from control AT increased relative to adipocytes from WT mice. This finding is consistent with the previous observation that the skin from CHY mice is characterized by an increased lipid accumulation compared with WT counterparts.³² Nevertheless, lipid droplets released from tumor-associated adipocytes in both K14-VEGFR3-Ig and CHY mice might promote macrophage recruitment and might augment an inflammatory response, resolution of which might depend on the lymphatic drainage function.³³

Apart from lipid storage function, AT secretes cytokines such as IL-6.⁷ IL-6 is frequently viewed as a proinflammatory

cytokine owing to its abundance in inflammatory settings such as obesity.³⁴ However, accumulating evidence suggests a broader role for IL-6, including association with the inflammatory response resolution. Recently, it has been found that IL-6 stimulates proliferation as well as primes for IL-4-dependent alternative activation of AT macrophages (ATMs) by inducing expression of IL-4R.^{25–27} Alternatively activated macrophages have been implicated in the suppression of host antitumor immunity, stimulation of angiogenesis, and metastatic dissemination, contrarily to tumor-suppressive classically activated macrophages.^{35,36} It should be noted, however, that owing to their plastic potential the functional polarization of macrophages into only two groups is an oversimplification. Nevertheless, the local proliferation of ATMs could account for enrichment of macrophages expressing CD301/CLEC10A, a marker of alternative activation, in peritumoral AT from K14-VEGFR3-Ig mice. It has also been revealed that the exposure to T helper type 1 cytokines, such as tumor necrosis factor α , inhibits the proliferation of ATMs.²⁷ The development of tumor-associated inflammation and immunity relies on lymphatic vessels and their drainage function.⁴ Tumors implanted in K14-VEGFR3-Ig mice exhibited drastically reduced expression of proinflammatory mediators compared with those implanted in WT littermates.⁴ Accounting for this feature,

Figure 8 The expression of adipocyte marker *FABP4* is associated with poor overall survival in cutaneous melanoma patients. **A:** Overall survival of skin cutaneous melanoma (SKCM) patients generated using OncoLnc platform. The Cancer Genome Atlas (TCGA) data of 458 melanoma patients were assigned into low or high groups according to the expression level of *FABP4* reported as RNASeq values. Patients displaying an *FABP4* expression level ranging from 0 to 15 were considered as low (solid curve) and patients displaying *FABP4* expression level ranging from 15 to 2465 were considered as high (dotted curve). **B–D:** Correlation of gene expression levels using data set acquired from R2 platform containing 470 cutaneous melanoma patients (Tumor Skin Cutaneous Melanoma - TCGA - 470 - rsem - tcgars). **Black lines** denote the linear regression. Pearson's correlation coefficient, r , is shown.

macrophages from tumor-associated AT from K14-VEGFR3-Ig mice might be exposed to the microenvironment facilitating their proliferation.

The importance of ATMs in facilitating tumor growth was demonstrated by the fact that melanomas coimplanted with freshly isolated macrophages grew more rapidly and were significantly more vascularized than those implanted alone. The extent to which ATMs contribute to the disease progression in humans remains to be deciphered. With the use of a cohort of 458 cutaneous skin melanoma patients, it was demonstrated that the expression of *FABP4* is associated with poor overall survival. Of importance, expression of *FABP4* correlated positively with *CLEC10A*. It is therefore tempting to speculate whether the presence of macrophages within tumor-associated AT in human melanomas contributes to the disease progression.

It is now recognized that macrophages express angiogenic factors, including VEGF-A and IL-6.³⁷ Of interest, it was revealed that IL-6 can directly induce angiogenesis in a variety of models with similar potency to VEGF-A.³⁸ This finding together with increased levels of IL-6 observed in peritumoral AT from K14-VEGFR3-Ig mice

suggest that IL-6 might play an important proangiogenic role in our model. The extent to which adipocytes and macrophages separately contribute to this process remains to be assessed.

In several solid tumor types, VEGFR-3 has been found expressed on angiogenic blood vessels.³⁹ In addition, angiogenic sprouting and vascular network formation have been inhibited by blockade of the receptor.⁴⁰ However, the expression of VEGFR-3 has been confined primarily to the vasculature within the tumor tissue. Because tumors expand at their periphery, peritumoral AT might therefore serve as a source of new blood vessels. Indeed, apart from sprouting angiogenesis, several other mechanisms of neo-vascularization have been identified in solid tumors, including vessel co-option.⁴¹ We might only speculate whether this process accounts for an increased number of CD31⁺ structures within tumors implanted into the fat pad of K14-VEGFR3-Ig mice.

Taken together, these results identify a complex role of lymphangiogenesis in tumor progression that requires further investigation. Tumor-associated lymphangiogenesis contributes to malignant progression, at least in part, by

facilitating metastatic spread to regional lymph nodes. These data suggest that the blockade of VEGFR-3 signaling aids in the induction of an inflammatory response within AT associated with accumulation of alternatively activated macrophages that stimulate tumor growth and vascularization. The development of an inducible mouse model with a spatiotemporal regulation of lymphatic vessels in and around the tumor tissue may prove useful in dissecting the dynamics of an inflammatory response. The contribution of lymphatic vessels to the local inflammatory response within tumor-associated AT may be of therapeutic importance for tumors growing within AT or metastases to the lymph nodes.

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