Macrophages define dermal lymphatic vessel calibre during development by regulating lymphatic endothelial cell proliferation

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There were some errors published in Development 137, 3899-3910.

On p. 3900 and p. 3904, the references to Dai et al. (2002) were incorrect. The correct reference is Li et al. (2006).


On p. 3909, ‘We thank E. Aranda for the provision of Csflr⁻/⁻ embryos.’ was omitted from the acknowledgements.

The authors apologise to readers for this mistake.
Macrophages define dermal lymphatic vessel calibre during development by regulating lymphatic endothelial cell proliferation

Emma J. Gordon1,2, Sujata Rao3, Jeffrey W. Pollard4, Stephen L. Nutt5, Richard A. Lang3 and Natasha L. Harvey1,2,*

SUMMARY
Macrophages have been suggested to stimulate neo-lymphangiogenesis in settings of inflammation via two potential mechanisms: (1) acting as a source of lymphatic endothelial progenitor cells via the ability to transdifferentiate into lymphatic endothelial cells and be incorporated into growing lymphatic vessels; and (2) providing a crucial source of pro-lymphangiogenic growth factors and proteases. We set out to establish whether cells of the myeloid lineage are important for development of the lymphatic vasculature through either of these mechanisms. Here, we provide lineage tracing evidence to demonstrate that lymphatic endothelial cells arise independently of the myeloid lineage during both embryogenesis and tumour-stimulated lymphangiogenesis in the mouse, thus excluding macrophages as a source of lymphatic endothelial progenitor cells in these settings. In addition, we demonstrate that the dermal lymphatic vasculature of PU.1−/− and Csf1r−/− macrophage-deficient mouse embryos is hyperplastic owing to elevated lymphatic endothelial cell proliferation, suggesting that cells of the myeloid lineage provide signals that act to restrain lymphatic vessel calibre in the skin during development. In contrast to what has been demonstrated in settings of inflammation, macrophages do not comprise the principal source of pro-lymphangiogenic growth factors, including VEGF-C and VEGF-D, in the embryonic dermal microenvironment, illustrating that the sources of patterning and proliferative signals driving embryonic and disease-stimulated lymphangiogenesis are likely to be distinct.

KEY WORDS: Lymphangiogenesis, Lymphatic vessels, Macrophages, Vascular development, Mouse

INTRODUCTION
Lymphatic vessels play crucial roles during embryonic development and adult homeostasis. These specialised vessels uptake, transport and return interstitial fluid and protein to the bloodstream, traffic cells of the immune system and absorb and transport lipids from the digestive tract. Aberrant lymphatic vessel growth and development (lymphangiogenesis) is associated with the pathogenesis of human disorders including lymphoedema, tumour metastasis and inflammatory conditions such as asthma, psoriasis and rheumatoid arthritis (Alitalo et al., 2005; Tammela et al., 2007; Schoppmann et al., 2002).

We set out to definitively establish whether cells of the myeloid lineage constitute a source of lymphatic endothelial progenitor cells, either during embryogenesis or in the tumour microenvironment, using genetic lineage tracing studies. Here, we present evidence to illustrate that lymphatic endothelial cells arise independently of the myeloid lineage during embryogenesis and in the tumour microenvironment, therefore excluding macrophages as a source of lymphatic endothelial progenitor cells in these settings. In addition, we demonstrate that the dermal lymphatic vessels of...
embryonic macrophage-deficient mice are hyperplastic, suggesting that macrophages normally act to restrain the calibre of dermal lymphatic vessels during development. In contrast to what has been demonstrated in settings of inflammation in the adult, macrophages do not comprise the principal source of VEGFC and VEGFD in the embryonic dermal microenvironment, suggesting that the sources of patterning and proliferative signals driving embryonic and disease-stimulated lymphangiogenesis are likely to be distinct.

**MATERIALS AND METHODS**

**Mouse experiments**

All experiments using animals were approved and conducted in accordance with the SA Pathology Animal Ethics Committee, Walter and Eliza Hall Institute (WEHI) Animal Ethics Committee and University of Cincinnati Institutional Animal Care and Use Committee Regulations. *LysMcCre* mice (Clausen et al., 1999) were obtained from Andrew Roberts (WEHI, Melbourne, Australia) and *ROSAT2R* Cre reporter mice (Soriano, 1999) were obtained from Richard Harvey (The Victor Chang Cardiac Research Institute, Sydney, Australia). *LysMcCre*<sup>+/−</sup>_Rosa26<sup>R+/−</sup> mice were generated by breeding *LysMcCre*<sup>+/−</sup> with *Rosa26<sup>R+/−</sup> mice and progeny were genotyped according to established protocols (Clausen et al., 1999; Soriano, 1999). *PU.1<sup>−/−</sup>* (Dakic et al., 2005), *Rag2<sup>−/−</sup>* (Shinkai et al., 1992), *Wnt7b<sup>−/−</sup>* (Lobov et al., 2005), *Wnt7b<sup>fl<sup>−</sup>fl</sup>* (Rajagopal et al., 2008), *Ang<sub>1</sub>Δ<sub>302</sub>* (Gale et al., 2002), *c-fmsEGFP* (Sasmono et al., 2003), *ZEG* (Novak et al., 2000), *Csf1r<sup>−/−</sup>Cre* (Deng et al., 2010), and *Csf1r<sup>−/−</sup>* (Dai et al., 2002) mice were previously described.

**Immunostaining**

Embryonic day E14.5, E16.5 and E18.5 embryos were collected and processed for whole-mount staining or cryosections (10 μm and 60 μm) as previously described (Gordon et al., 2008). The following primary antibodies were used: rabbit anti-mouse LYVE1 (Angiobio), rabbit anti-mouse PROX1 (Angiobio), goat anti-human PROX1 (R&D Systems), rat anti-mouse CD31 (Biologend), Cy3-conjugated mouse anti-α-smooth muscle actin (Sigma), goat anti-rat neuropilin 2 (R&D Systems), rat anti-mouse F4/80 (EMR1 – Mouse Genome Informatics) (Invitrogen), rabbit IgG fraction to β-galactosidase (MP Biomedicalal), rat anti-mouse GR1 (LY6G – Mouse Genome Informatics) (eBioscience), Alexa-Fluor-488-conjugated rabbit anti-green fluorescent protein (Invitrogen), rabbit anti-phospho-histone H3 (Millipore), goat anti-mouse PU.1 (Santa Cruz Biotechnology), goat anti-mouse VEGFC (A-18, C-20) (Santa Cruz Biotechnology) and Alexa-Fluor-647-conjugated rat anti-mouse F4/80 (Invitrogen). Alexa-Fluor-488/555/647-conjugated secondary antibodies (Invitrogen) were used for visualization. Cells were mounted in Prolong (Invitrogen). Alexa-Fluor-488/555/647-conjugated secondary antibodies (Invitrogen) were used for visualization. Cells were mounted in Prolong (Invitrogen). Alexa-Fluor-647-conjugated rat anti-mouse F4/80 (Invitrogen), goat anti-mouse VEGFC (A-18, C-20) (Santa Cruz Biotechnology) and Alexa-Fluor-647-conjugated rat anti-mouse F4/80 (Invitrogen).

**Cell proliferation assays**

Proliferation of siRNA-transfected cells was assessed using the Cell Titer Aqueous One Solution Proliferation Assay (Promega) according to the manufacturer’s instructions and quantified using an EL808 Ultra Microplate Reader (Bio-tek Instruments, Inc.). To assess the effect of blocking VEGFR3, freshly isolated LEC were plated at 8000 cells per well in fibronectin-coated 96-well plates and cultured in EGM-2 for 24 hours. Conditioned media was added at 50% with EGM-2, with or without recombinant mouse VEGFR3/Fc chimera (500 ng/ml; R&D Systems). Cell proliferation was assessed using the Cell Titer Aqueous One Solution Proliferation Assay following 24 and 48 hours.

For co-culture experiments, E16.5 LEC were isolated and stained with CellVue Claret Far Red Fluorescent Cell Linker (Sigma) according to manufacturer’s instructions, then resuspended in EGM-2. LEC at 5 × 10<sup>4</sup> per well were plated in fibronectin-coated 24-well plates, with or without macrophages (2.5 × 10<sup>4</sup> per well). After 3 or 6 days, cells were detached using trypsin (Lonza), blocked using mouse gamma globulin (Jackson ImmunoResearch Laboratories) and labelled with rat anti-mouse CD31 PE-Cy7 (Biologend). Proliferation of CD31<sup>+</sup> CellVue Claret<sup>−</sup> endothelial cells was analysed on a FC-500 flow cytometer (Beckman Coulter). Proliferation index was calculated using the Proliferation Wizard module in ModFit LT (Verity Software House).

**RNA analysis**

Total RNA was isolated using RNeasy (Ambion) or Trizol (Invitrogen) according to the manufacturer’s instructions. cDNA was synthesised using SuperScript III First-Strand Synthesis SuperMix (Invitrogen) using a mix of oligo(dT) and random primers. Real-time RT-PCR was subsequently performed in triplicate using RT<sup>2</sup> Real-Time SYBR Green/ROX PCR Master Mix (Qiagen) and analysed on a Rotorgene 6000 series PCR machine (Qiagen). Amplification was performed using the primers listed in Table 1. Data were normalised against *Actb* as previously described (Livak and Schmittgen, 2001).

**Microarray analyses**

Total RNA from F4/80<sup>LYVE1</sup><sup>−</sup> and F4/80<sup>LYVE1</sup><sup>+</sup> isolated macrophages was purified using RNeasy (Ambion) according to the manufacturer’s instructions. RNA quality was assessed using a Bioanalyzer picopich (Agilent Technologies). One hundred nanograms of pooled RNA from three independent samples was submitted to the Adelaide Microarray Centre for gene expression analysis. Affymetrix Mouse Gene 1.0 ST Arrays were used according to the Genechip Whole-Transcript (WT) Sense Target Labeling Assay (P/N 701880). Microarray data was analysed using...
Partek Genomics Suite (Partek). Differential gene expression was assessed by ANOVA with the P-value adjusted using step-up multiple test correction (Benjamini and Hochberg, 1995) to control the false discovery rate.

Microarray data are available from Gene Expression Omnibus with accession GSE24492.

**EL4 and LLC solid tumour models**

EL4 lymphoma or LLC carcinoma cells (obtained from C. Bonder, SA Pathology, Adelaide, Australia) were grown in RPMI (Gibco) with 10% fetal bovine serum and 2 mM glutamine at 37°C in 5% CO2 until 90% confluent, then washed and resuspended at 10^7 cells/ml.

**RESULTS**

A population of LYVE1-positive macrophages is intimately associated with the developing lymphatic vasculature

Using c-fmsEGFP transgenic mice that express GFP in cells of the myeloid lineage (Sasmono et al., 2003), we determined that a discrete population of embryonic macrophages coexpressed the hyaluronan receptor LYVE1, also expressed by lymphatic endothelial cells (Banerji et al., 1999). Intriguingly, this LYVE1-positive macrophage population was intimately localised with the developing embryonic lymphatic vasculature (Fig. 1). We discovered that, in some cases, LYVE1-positive, GFP-positive macrophages appeared to be integrated into jugular lymph sacs (Fig. 1B,C) and dermal lymphatic vessels (Fig. 1E-G). In order to establish whether these LYVE1-positive macrophages coexpressed markers of lymphatic endothelial cell identity, expression of the PROX1 homeobox transcription factor, a definitive marker of lymphatic endothelial cell identity (Wigle and Oliver, 1999), was analysed. LYVE1-positive, GFP-positive macrophages closely associated with jugular lymph sacs (Fig. 1K) and dermal lymphatic vessels (Fig. 1M-O) did not express PROX1 (Fig. 1O), indicating that this population of cells was unlikely to be undergoing transdifferentiation to lymphatic endothelial cells.

**Lymphatic endothelial progenitor cells arise independently of the myeloid cell lineage during embryogenesis**

In order to circumvent the possibility that expression of the c-fmsEGFP transgene might be extinguished in macrophages undergoing a transdifferentiation event, and thereby to definitively determine whether cells of the macrophage lineage comprise a pool of lymphatic endothelial progenitor cells, lineage tracing experiments were performed. For these experiments, Csf1r-iCre mice (Deng et al., 2010), expressing Cre recombinase under control of the Csf1r-iCre promoter, expressed selectively in cells of the myeloid lineage, were crossed with Z/EG Cre reporter mice (Novak et al., 2000). The expression of GFP, LYVE1 and PROX1 was then assessed in embryonic macrophages using fluorescent immunostaining and confocal microscopy. In concordance with our observations described above, PROX1 expression was not detected in LYVE1-positive, GFP-positive cells derived from the myeloid lineage that were intimately associated with jugular lymph sacs or dermal lymphatic vessels (Fig. 2B,C,E,F). Similar results were obtained using a second lineage tracing approach in which LysMCre mice (Clausen et al., 1999) were crossed with Rosa26R Cre reporter mice (Soriano, 1999), although the labelling of embryonic macrophages was much more efficient using the Csf1r-iCre line (76% of F4/80-positive cells were positive for reporter gene expression compared with approximately 40% in LysMCre/+;Rosa26R/+ embryos at E14.5). Improved efficiency
of the Csf1r-iCre line for embryonic lineage tracing studies is probably due to the initiation of Csf1r expression at an earlier stage of myeloid development than LysM (Lichanska et al., 1999). Taken together, our lineage tracing data provide strong evidence to exclude cells of the myeloid lineage as a source of lymphatic endothelial progenitor cells during embryogenesis.

**Lymphatic endothelial progenitor cells arise independently of the myeloid cell lineage in the tumour microenvironment**

To trace the fate of cells of the myeloid lineage during tumour lymphangiogenesis, adult LysMCre+/-;ROSA26R+/- mice were implanted subcutaneously with Lewis lung carcinoma (LLC) or EL4 lymphoma cells. In accordance with previously published data (Clausen et al., 1999), Cre excision efficiency in LysMCre+/-;ROSA26R+/- mice was substantially higher in adulthood (94.5% of F4/80-positive cells were positive for β-galactosidase) than in the embryo. Following 10-14 days of tumour growth, peri- and intra-tumoural lymphangiogenesis was examined and the localisation of β-galactosidase-positive cells was investigated with respect to both pre-existing and newly formed lymphatic vasculature. β-galactosidase-positive cells that appeared to be integrated within lymphatic vessels did not express PROX1 (Fig. 3C,D,G,H), demonstrating that cells of the myeloid lineage did not transdifferentiate to generate lymphatic endothelial cells. These data provide strong evidence for the role of the myeloid lineage in contributing to lymphatic vessels during embryogenesis.

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**Fig. 1.** LYVE1-positive macrophages are intimately associated with lymphatic vessels but do not express PROX1. Confocal z-stack images of 60 μm sections demonstrating the localisation of LYVE1-positive, GFP-positive macrophages in close association with, and apparently integrated within (arrowheads), the jugular lymph sac (JLS; A-C) and dermal lymphatic vessel (DLV, D-G) endothelium of E14.5 c-fmsEGFP embryos. G illustrates a 90° rotation of F in order to view the lymphatic vessel in cross-section. Blue and white arrowheads denote two macrophages that appear integrated within this DLV, both of which express GFP, LYVE1 and F4/80. LYVE1-positive macrophages that are apparently integrated within the JLS or DLV endothelium do not express PROX1 (H-O). White circles in K and O illustrate the location of macrophages (arrows) in J and N. K and O illustrate single-channel PROX1 images of J and N, respectively. Data are representative of at least four independent experiments. Scale bars: 150 μm in A; 100 μm in D,H,I; 50 μm in B,E,J,M; 25 μm in C,F,J,K,N,O.

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**Fig. 2.** Lineage tracing in Csf1r-iCre;Z/EG embryos reveals that cells of the myeloid lineage do not comprise a pool of lymphatic endothelial progenitor cells during development. Confocal z-stack images of 60 μm sections demonstrating the localisation of LYVE1-positive, EGF-positive macrophages in close association with, and apparently integrated within (arrows), the JLS (A-C) and DLV (D-F) endothelium of E14.5 Csf1r-iCre;Z/EG embryos. LYVE1-positive macrophages within the JLS or DLV endothelium do not express PROX1 (C,F). White circles illustrate the location of macrophages (arrows) in B and E. C and F illustrate single-channel PROX1 images of B and E, respectively. Data are representative of at least three independent experiments. Scale bars: 100 μm in A,D; 25 μm in B,C,E,F.
to illustrate that lymphatic endothelial cells arise independently of the macrophage lineage during tumour-stimulated lymphangiogenesis.

The gene expression profile of embryonic LYVE1-positive macrophages resembles that of TIE2-expressing monocytes (TEMs)

Recent work has demonstrated that distinct subpopulations of macrophages play specialised roles in physiological processes including inflammation, angiogenesis and tissue remodelling (Fantin et al., 2010; Pucci et al., 2009; Venneri et al., 2007). Owing to our observation that LYVE1-positive, but not LYVE1-negative, myeloid cells selectively integrated within the embryonic lymphatic vasculature, we sought to characterise the gene expression profiles of embryonic dermal LYVE1-positive and LYVE1-negative macrophages using microarray analysis, in order to further investigate their function. Analysis of the genes selectively enriched in the LYVE1-positive versus LYVE1-negative population, validated by real-time RT-PCR (Table 2), revealed significant overlap with the gene signature of embryonic/foetal TIE2-expressing monocytes (TEMs) and tumour-derived TEMs (Pucci et al., 2009), which have been shown to have a role in developmental (Fantin et al., 2010) and pathological (Pucci et al., 2009; Venneri et al., 2007) angiogenesis. Genes commonly expressed at increased levels in these populations included Lyve1, Mrc1, Cd163, Stab1, Nr1p1, Plxnd1 and Sema6d, whereas genes commonly downregulated in LYVE1-negative and TIE2-negative embryonic macrophages included Sell and Angpt1 (Table 2) (Pucci et al., 2009). Interestingly, our real-time RT-PCR data revealed increased TIE2 expression in LYVE1-positive compared with LYVE1-negative macrophages. Taken together, these data suggest that dermal LYVE1-positive macrophages comprise a population of embryonic macrophages potentially involved in morphogenetic or tissue remodelling events.

Hyperplastic lymphatic vessels in macrophage-deficient mice

Macrophages have been implicated as an important source of pro-angiogenic and pro-lymphangiogenic factors in settings of adult inflammation, including the tumour microenvironment (Baluk et al., 2005; Cursiefen et al., 2004; Jeon et al., 2008; Kataru et al., 2009; Kim et al., 2009; Maruyama et al., 2007; Schoppmann et al., 2002). In order to define whether cells of the myeloid lineage are important for embryonic lymphangiogenesis, we investigated the

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<th>Fold increase by array</th>
<th>Fold increase by RT-PCR</th>
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<th>Fold decrease by array</th>
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<td>Nr1p1</td>
<td>9.49</td>
<td>1.66</td>
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<tr>
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<tr>
<td>Plxnd1</td>
<td>2.71</td>
<td>1.56</td>
<td>0.18</td>
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<tr>
<td>Cd163</td>
<td>3.07</td>
<td>2.47</td>
<td>≤0.1</td>
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Table 2. Genes differentially expressed between F4/80+LYVE1+ and F4/80+LYVE1– macrophages isolated from E15.5 skin

Fold change in the expression level of indicated genes in F4/80+LYVE1+ macrophages versus F4/80+LYVE1– macrophages. Values were determined by microarray analysis and real-time RT-PCR. P-values were calculated using Student’s paired t-test from real-time RT-PCR values. These data are representative of at least three independent cell isolations.
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process of lymphatic vascular development in PU.1−/− embryos. Mice deficient in the ets domain containing transcription factor PU.1 are devoid of macrophages, granulocytes and lymphocytes and die around birth (Back et al., 2004; McKercher et al., 1996; Scott et al., 1994). Assessment of the development and patterning of the lymphatic vasculature in embryonic PU.1−/− skin at E14.5, E16.5 and E18.5 revealed strikingly hyperplastic lymphatic vessels in comparison to wild-type counterparts (Fig. 4A–F). We ascribed this phenotype to a lack of myeloid cells by examining the lymphatic vascular pattern in Rag2−/− embryos that are devoid of mature lymphocytes (Shinkai et al., 1992); Rag2−/− embryos displayed lymphatic vessels of an indistinguishable calibre to wild-type counterparts (Fig. 4G). Immunostaining of wild-type E16.5 skin revealed very few GR1-positive granulocytes compared with the abundance of macrophages, suggesting that macrophage deficiency was responsible for lymphatic vessel hyperplasia in PU.1−/− skin. A cell-autonomous role for PU.1 in endothelial cells was ruled out by examining the expression of PU.1 protein in the embryo; PU.1 was not apparent in blood or lymphatic endothelial cells but was observed in CD45-positive haematopoietic cells and LYVE1-positive macrophages (see Fig. S1 in the supplementary material). Hyperplastic lymphatic vessels were also observed in the dermis of Csf1r−/− embryos that are deficient in the receptor for colony-stimulating factor 1 receptor and have a paucity of myeloid cells (Fig. 4F,G) (Dai et al., 2002). Taken together, these data suggest that macrophages regulate dermal lymphatic vessel calibre during embryonic development.

In order to delineate the mechanism underlying dermal lymphatic vessel hyperplasia in PU.1−/− embryos, branching of lymphatic vessels and proliferation of lymphatic endothelial cells were investigated. Although branching was unaffected (Fig. 5A–C), the mitotic index of lymphatic endothelial cells was significantly increased in PU.1−/− skin (Fig. 5D–F). This suggested that macrophages do not influence dermal lymphatic vessel sprouting during development, but control either the proliferation or apoptosis of lymphatic endothelial cells.

Macrophage-derived WNT7B does not initiate apoptosis in dermal lymphatic endothelial cells

Recent work demonstrated that macrophages direct postnatal regression of the hyaloid vasculature via an angiopoietin-2-coordinated and WNT7B-initiated death switch (Lobov et al., 2005; Rao et al., 2007). In order to determine whether macrophage-derived WNT7B delivers a death switch to lymphatic endothelial cells that is responsible for the regulation of lymphatic vessel calibre, we investigated dermal lymphatic vascular patterning in Csf1r-iCre; Wnt7bfl/fl−/− embryos (S.R., J.W.P. and R.A.L., unpublished). No alterations in lymphatic vessel calibre were observed in these embryos, nor in embryos homozygous for a hypomorphic Wnt7b mutation (Wnt7b2/2) (Lobov et al., 2005) (see Fig. S2 in the supplementary material), suggesting that...
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**Angiopoietin 2 is a pro-survival signal in lymphatic endothelial cells**
To determine whether macrophages might orchestrate a death switch influenced by angiopoietin 2 in embryonic lymphatic endothelial cells, removal of which could result in lymphatic vessel hyperplasia, we examined lymphatic vessel patterning in an allelic series of $PU.1^{+/+};Angpt2^{−/−}$ mutant mice. We hypothesised that if angiopoietin 2 was important for coordinating a death switch in lymphatic endothelial cells, we might expect to see exacerbated hyperplasia of lymphatic vessels in $PU.1^{+/+};Angpt2^{−/−}$ and $Angpt2^{−/−}$ mice. By contrast, our data revealed that removal of one allele of $Angpt2$ in a $PU.1^{−/−}$ background rescued the lymphatic hyperplasia phenotype (Fig. 6A-C,E). In accordance with previous data (Dellinger et al., 2008; Gale et al., 2002), the dermal lymphatic vessels of $Angpt2^{−/−}$ mice were strikingly sparse and hypoplastic (Fig. 6D). These observations suggest that in contrast to the phenotype in the skin, the jugular lymph sacs of E14.5 $PU.1^{+/+}$ embryos were hypoplastic compared with wild-type littermates (Fig. 7), suggesting that macrophages might play a pro-lymphangiogenic role in this embryonic microenvironment. No significant differences in lymphatic vessel calibre or number of branch-points were observed in the submucosa of E16.5 or E18.5 $PU.1^{+/+}$ embryos compared with wild-type littermates, nor in lymphatic vessels lining the pleural cavity of E16.5 embryos (see Fig. 5 in the supplementary material). These data reveal that the role played by macrophages during embryonic lymphangiogenesis might be tissue- or context-dependent.

**Expression of lymphangiogenic factors is elevated in $PU.1^{+/+}$ skin**
In order to investigate the mechanism underlying increased proliferation of lymphatic endothelial cells in macrophage-deficient mice, the relative expression of known pro-lymphangiogenic factors was quantified in wild-type and $PU.1^{+/+}$ skin using real-time RT-PCR. The expression of genes characteristic of lymphatic endothelial cells including Proxl and Nrp2 was elevated in $PU.1^{+/−}$ skin compared with $PU.1^{+/+}$ control littermates, presumably due to lack of removal by phagocytic macrophages (Fig. 6G). These data suggest that macrophages do not actively program the apoptosis of lymphatic endothelial cells during development, nor are they likely to be recruited to the developing lymphatic vasculature to engulf apoptotic cell corpses.

**The role of macrophages in the regulation of lymphatic vessel calibre is tissue-specific**
In order to ascertain whether the phenotype of lymphatic vessel hyperplasia in myeloid-cell-deficient mice extended to all embryonic tissues, we investigated lymphatic vascular development and patterning in a range of $PU.1^{−/−}$ organs. In contrast to the phenotype in the skin, the jugular lymph sacs of E14.5 $PU.1^{+/−}$ embryos were hypoplastic compared with wild-type littermates (Fig. 7), suggesting that macrophages might play a pro-lymphangiogenic role in this embryonic microenvironment. No significant differences in lymphatic vessel calibre or number of branch-points were observed in the submucosa of E16.5 or E18.5 $PU.1^{+/−}$ embryos compared with wild-type counterparts, nor in lymphatic vessels lining the pleural cavity of E16.5 embryos (see Fig. 5 in the supplementary material). These data reveal that the role played by macrophages during embryonic lymphangiogenesis might be tissue- or context-dependent.

**Fig. 6. Angiopoietin 2 is a pro-survival signal in lymphatic endothelial cells.** (A-D) Whole-mount immunostaining and confocal microscopy of dorsal skin from an allelic series of E16.5 wild-type (A), $PU.1^{+/+};Ang2^{+/−}$ (B), $PU.1^{−/−};Ang2^{−/−}$ (C) and $PU.1^{+/+};Ang2^{−/−}$ (D) embryos demonstrates rescue of lymphatic vessel hyperplasia in $PU.1^{+/+};Ang2^{−/−}$ skin (C compared with B) and pronounced lymphatic vessel hypoplasia in $PU.1^{−/−};Ang2^{−/−}$ skin (D). The patterning and recruitment of vascular smooth muscle to the large arteries and veins in the dorsal skin of mutant mice (B-D) is indistinguishable from that of wild-type littermates (A). NRP2-positive hair follicles are particularly pronounced in Ang2−/− skin (D). E) Lymphatic vessel diameter was quantified using LVAP and ImageJ software. Data are representative of at least four independent experiments. Data shown represent the mean ± s.e.m. P-values were calculated using Student's paired t-test. * P ≤ 0.05. (F,G) Whole-mount immunostaining and confocal microscopy of wild-type (F) and $PU.1^{+/−}$ (G) dorsal skin stained with antibodies to activated caspase-3 and VEGFR3. (H-J) Immunostaining of transverse sections through c-fmsEGFP jugular lymph sacs stained with LYVE1 and activated caspase-3 antibodies. J is a single-channel image of I illustrating a caspase-3-positive apoptotic cell (arrowhead). Scale bars: 150 μm in A-D; 100 μm in F,G; 100 μm in H; 25 μm in I,J.
skin compared with wild-type littermates, whereas the expression of genes characteristic of blood vascular endothelial cells (Nrp1) or blood vessel-associated smooth muscle cells (Acta2) was not substantially altered (Fig. 8). These data reflect the selective hyperplasia of lymphatic vessels in the skin of PU.1–/– mice; the calibre of blood vessels and patterning of the major arteries and veins appeared normal (Fig. 4). In accordance with the increased proliferation of lymphatic endothelial cells observed in PU.1–/– mice, elevated levels of established pro-lymphangiogenic stimuli including Vegfc (Jeltsch et al., 1997), Vegfd (Stacker et al., 2001), Angpt2 (Gale et al., 2002), Angpt1 (Morisada et al., 2005) and Mmp2 (Bruyere et al., 2008) were observed in PU.1–/– skin (Fig. 8).

By contrast, the expression of Vegfa was not substantially altered (Fig. 8). PU.1–/– skin also contained elevated expression of Mmp14. MMP14 is a membrane-bound matrix metalloproteinase that activates pro-MMP2 (Sato et al., 1992), illustrating a mechanism by which the processing and activity of MMP2, and potentially additional pro-lymphangiogenic proteins, could be further enhanced in macrophage-deficient skin. These data suggest that the increased proliferation of lymphatic endothelial cells in PU.1–/– skin might occur as a direct result of the elevated expression and potentially increased activity of lymphangiogenic signals in the dermal microenvironment including Vegfc, Vegfd, Angpt2, Angpt1 and Mmp2.

Macrophages are not the major source of lymphangiogenic growth factors in the embryonic dermis

Macrophages have been hypothesised to drive inflammation-stimulated lymphangiogenesis by producing pro-lymphangiogenic signals including VEGFC and VEGFD. Our data demonstrated that the expression levels of pro-lymphangiogenic factors including Vegfc, Vegfd and Mmp2 were increased in the skin of macrophage-deficient mice, suggesting that macrophages do not constitute the major source of VEGFC and VEGFD required for development and patterning of the embryonic lymphatic vasculature. In order to investigate the relative expression levels of Vegfc, Vegfd and additional pro-lymphangiogenic stimuli in macrophages compared with other cell populations in the embryonic skin, F4/80-positive macrophages were isolated from single-cell suspensions of embryonic dermis and the expression of candidate genes was quantified in the macrophage population versus macrophage-depleted dermal cells using real-time RT-PCR. In accordance with the above data, the levels of Vegfc, Vegfd and Mmp2 expressed by macrophages constituted less than 10% of the total levels of these pro-lymphangiogenic genes expressed in the embryonic dermis (see Fig. S4 in the supplementary material). By contrast, the expression of macrophage-specific genes including Emr1 (encoding F4/80) and Mmp8 was restricted to the F4/80-positive population (see Fig. S4 in the supplementary material). Further investigation revealed that lymphatic endothelial cells express the majority of Angpt2 and Tiel1 in wild-type dermis, whereas Angpt1 and Vegfc were expressed at substantially higher levels in dermal cells depleted of endothelial cells and macrophages (see Fig. S5 in the supplementary material). These data suggest that Angpt1 and Vegfc expression is increased in the dermal environment as a result of macrophage deficiency, although the precise cell types responsible for their production remain uncharacterised. Taken together, our data illustrate that in contrast to the scenario of inflammation-stimulated neo-lymphangiogenesis, macrophages do not provide the principal source of lymphangiogenic signals during embryonic dermal lymphatic vascular development. Instead, our
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Fig. 8. Expression of pro-lymphangiogenic factors is elevated in the skin of PU.1−/− mice. Semi-quantitative RT-PCR was used to determine the relative level of gene expression in PU.1−/− (n=4) compared with wild-type (n=4) E16.5 skin samples. Data are depicted as the percentage change in mRNA expression in PU.1−/− compared with wild-type skin and represent the mean ± s.e.m. No change in expression between PU.1−/− and wild-type skin is represented by 0%. P-values were calculated using Student’s paired t-test. *, P<0.05.

Data suggest that macrophages in the embryonic dermis normally restrain the expression of pro-lymphangiogenic growth factors and proteases produced by additional cell populations resident in the dermal microenvironment.

Macrophages regulate lymphatic endothelial cell proliferation in vitro

In order to investigate the mechanism by which embryonic dermal macrophages regulate the proliferation of lymphatic endothelial cells, in vitro primary embryonic cell culture assays were established. Lymphatic endothelial cells isolated from the dermis of E16.5 mouse embryos were cultured together with E16.5 dermal macrophages or conditioned media harvested from cultured embryonic macrophages, whole dermal cell suspensions or dermal cell suspensions depleted of macrophages. These assays demonstrated that co-culture of macrophages with lymphatic endothelial cells stimulated lymphatic endothelial cell proliferation, indicating that embryonic macrophages are able to provide pro-proliferative signals to lymphatic endothelial cells in vitro (Fig. 9A). By contrast, the addition of macrophage- or dermal-conditioned media to embryonic lymphatic endothelial cells did not result in significantly elevated LEC proliferation (Fig. 9B). These data suggest that negative regulation of lymphatic endothelial cell proliferation by macrophages during development in vivo could be mediated via an indirect mechanism that involves additional cell types in the embryonic dermis and/or that pro-lymphangiogenic signals potentially require direct cell-cell contact between macrophages and LEC.

TIE1 regulates embryonic LEC proliferation in vitro

Given the rescue of dermal lymphatic vessel hyperplasia in PU.1−/−; Angpt2−/− embryos and the phenotype of lymphatic vessel hypoplasia in Angpt2−/− embryos, we investigated whether signalling via the angiopoietin-TIE axis regulated lymphatic endothelial cell proliferation in vitro. To this effect, siRNA against Tie1, Tie2(Tek) or Angpt2 was introduced to primary embryonic LEC in culture and the resultant effect on LEC proliferation was measured. Reduction of Tie1, but not Tie2 or Angpt2, expression in LEC resulted in a reduction of proliferation of approximately 20% (Fig. 9C). These data illustrate that signalling via the TIE1 receptor mediates pro-proliferative events in LEC in vitro, supporting recently published data demonstrating a crucial role for TIE1 activity during lymphatic vascular development (D’Amico et al., 2010; Qu et al., 2010).

DISCUSSION

The focus of this study was to define whether cells of the myeloid lineage contribute to lymphatic vascular development by either: (1) Constituting a pool of lymphatic endothelial progenitor cells; or (2) Providing a crucial source of pro-lymphangiogenic growth factors. Here, we provide extensive lineage tracing evidence to illustrate that the lymphatic vasculature of both the mouse embryo and the tumour microenvironment arises independently of the myeloid cell lineage, thereby excluding macrophages as a reservoir of lymphatic endothelial progenitor cells in these settings. Furthermore, our data illustrate the novel finding that dermal macrophages are not a principal source of pro-lymphangiogenic signals during embryogenesis, but act instead to restrain the calibre of dermal lymphatic vessels. Our data also highlight important pro-lymphangiogenic roles of angiopoietin 2 and TIE1.

The embryonic origin of the lymphatic vasculature has been debated for over 100 years. Work in the mouse embryo provided evidence to validate Florence Sabin’s hypothesis that lymphatic vessels in the early vertebrate embryo originate from the veins (Srinivasan et al., 2007), whereas studies in model systems including the avian (Wilting et al., 2006; Wilting et al., 2000) and Xenopus embryos (Ny et al., 2005), provided evidence to support a dual origin from venous and mesenchymal progenitor cell components. Our lineage tracing data in the mouse embryo provide strong evidence to exclude cells of the macrophage lineage as a source of lymphatic endothelial progenitor cells during embryogenesis, in agreement with the data of Srinivasan and colleagues (Srinivasan et al., 2007). In addition, our lineage tracing data in two models of tumour-stimulated lymphangiogenesis also excluded cells of the macrophage lineage as a source of lymphatic endothelial progenitors in the tumour microenvironment. This finding is in contrast to the suggestions of others, mostly generated using in vitro assays, that, in settings of inflammation-stimulated neo-lymphangiogenesis, macrophages possess the ability to transdifferentiate to lymphatic endothelial cells. Although we observed the apparent integration of myeloid-derived LYVE1-positive cells into lymphatic vessels, lineage-tracing analyses revealed that none of these cells expressed PROX1, a crucial marker of lymphatic endothelial cell identity. Other groups claiming that macrophages or haematopoietic-derived endothelial progenitor cells integrate to lymphatic vessels during neo-lymphangiogenesis did not examine PROX1 expression (Jiang et al., 2008; Maruyama et al., 2007; Religa et al., 2005), but instead analysed expression of cell surface markers including LYVE1 and VEGFR3, which have also been reported to be expressed on cells of the macrophage lineage (Schledzewski et al., 2006; Schoppmann et al., 2002; Skobe et al., 2001). Our results suggest that when evaluating haematopoietic cell contribution to developing endothelium, defined markers specific for endothelial cell identity should be employed. Interestingly, while this paper was in preparation, Zumsteg and colleagues (Zumsteg et al., 2009) published a study incorporating lineage tracing of myeloid cells using CD11bCre; Z/EG mice, into which prostate adenocarcinoma TRAMP-C1 cells were implanted. In contrast to our data, rare GFP-positive, LYVE1-positive, PROX1-positive cells were observed integrated into tumour lymphatic vessels (Zumsteg et al., 2009). This difference might potentially be explained by variations in tumour type or Cre lines used for lineage tracing.
Nonetheless, our extensive lineage tracing data in LysMCre+/–;ROSA26R+/– mice using two mouse tumour models found no evidence for the transdifferentiation of myeloid cells into lymphatic endothelial cells within the peri-tumoural or intra-tumoural lymphatic vasculature. Our data are in accordance with He and colleagues (He et al., 2004), who found that transplanted bone marrow cells did not contribute to the generation of LLC tumour-stimulated lymphatic endothelium.

Why are macrophages observed in such intimate association with lymphatic vessels? We hypothesise that this association might reflect transient macrophage migration across the lymphatic endothelium (into or out of vessels) or, alternatively, a place of residence important for fulfilling an immune surveillance role (i.e. by surveying the passing contents of lymph). Discriminating between these possibilities will rely on the use of intravital microscopy to analyse macrophage migration in real time. Our gene profiling analyses of LYVE1-positive versus LYVE1-negative macrophages isolated from the embryonic dermis illustrated that LYVE1-positive macrophages share a gene signature closely related to TIE2-expressing monocytes (TEMs) (Pucci et al., 2009). TEMs in the tumour environment have been shown to promote angiogenesis (Venneri et al., 2007) and, in the embryo, promote angiogenesis by facilitating the anastomosis of sprouting blood vessels (Fantin et al., 2010). Our data suggest that LYVE1-positive macrophages fulfil a role in lymphangiogenesis that is distinct from either of these angiogenic functions. Nonetheless, the intimate association of LYVE1-positive macrophages within lymphatic vessels might reflect a role of these cells in lymphatic vascular morphogenesis and/or remodeling.

Our analyses of lymphatic vascular development in macrophage-deficient PU.1−/− and Csf1r−/− mice revealed pronounced dermal lymphatic vessel hyperplasia in these mutants compared with their wild-type counterparts. These data illustrate that in contrast to what has been proposed in settings of inflammation, macrophages do not provide the principal source of pro-lymphangiogenic signals in the embryonic mouse dermis. Our relative quantification analyses of pro-lymphangiogenic gene expression in macrophages compared with macrophage-depleted dermal cells revealed that cells of the macrophage lineage contribute less than 10% of the total Vegfc, Vegfd and Mmp2 levels expressed in the embryonic dermis. We don’t, however, discount a role for macrophage-derived lymphangiogenic factors based purely on their magnitude of expression as recent work has illustrated that conditional deletion of myeloid cell-derived VEGFA has a profound effect on tumour angiogenesis even in the absence of a detectable change in the amount of total VEGFA expressed in the tumour microenvironment (Stockmann et al., 2008). In addition, owing to the limited availability of reliable commercial reagents, our analyses were restricted to assessing mRNA rather than protein level and activity. Our in vitro co-culture experiments demonstrated that the addition of primary embryonic macrophages to primary embryonic lymphatic endothelial cells resulted in elevated lymphatic endothelial cell proliferation. Addition of VEGFR3/Fc to conditioned media assays did not inhibit lymphatic endothelial cell proliferation. Data shown represent the mean ± s.e.m. of three independent experiments. $P$-values were calculated using Student’s paired t-test. *, $P<0.05$.

![Fig. 9. Regulation of lymphatic endothelial cell proliferation in vitro. (A, B) Co-culture of primary embryonic dermal macrophages (A), but not macrophage or skin-cell-conditioned media (B), with primary embryonic dermal lymphatic endothelial cells resulted in elevated lymphatic endothelial cell proliferation. Addition of VEGFR3/Fc to conditioned media assays did not inhibit lymphatic endothelial cell proliferation. (C) siRNA-mediated knockdown of Tie1 in primary embryonic dermal lymphatic endothelial cells resulted in significantly decreased lymphatic endothelial cell proliferation. Data shown represent the mean ± s.e.m. of three independent experiments. $P$-values were calculated using Student’s paired t-test. *, $P<0.05$.](image-url)
that we observed in \textit{PU.1}^{op/op} embryos. Definitive analysis of the role of macrophage-derived factors in embryonic and inflammation-stimulated lymphangiogenesis will rely on the generation of conditional knockout mice.

Our studies determined that the hyperplasia of lymphatic vessels in the dermis of macrophage-deficient mice occurred not as a result of the absence of a macrophage-initiated endothelial cell death switch, as occurs in the postnatal hyaloid blood vasculature, but instead as a result of lymphatic endothelial cell hyper-proliferation. Lymphatic endothelial cell hyper-proliferation in macrophage-deficient skin is potentially due to increased expression, proteolytic processing and activity of pro-lymphangiogenic signals in the dermal microenvironment including \textit{Vegfc}, \textit{Vegfd} and \textit{Mmp2}. Precisely how macrophages control the expression and activity of these pro-lymphangiogenic signals in the skin remains to be elucidated. Of particular interest was the finding that the hyperplasia observed in dermal lymphatic vessels of \textit{PU.1}^{op/op} mice did not extend to dermal blood vessels. The calibre and the patterning of arteries, veins and capillaries in embryonic \textit{PU.1}^{op/op} skin were indistinguishable from wild-type skin; in concordance with this data, the expression of pro-angiogenic genes, including \textit{Vegfa}, was not altered. These data reveal a selective influence of dermal macrophage-derived factors on the regulation of lymphatic vessel calibre in the skin during embryonic development.

Recent work by Suda and colleagues (Kubota et al., 2009) demonstrated that \textit{op/op} mice harbouring a reduced number of macrophages (Cecchini et al., 1994) exhibited a transient defect in postnatal lymphatic vascular development. In contrast to our work, reduced lymphatic vessel branching was observed in selected \textit{op/op} tissues. As \textit{PU.1}^{op/op} mice die around birth (Back et al., 2004; McKercher et al., 1996; Scott et al., 1994), it was not possible to examine postnatal lymphatic vessel development in this line, and therefore not plausible to determine whether the differences observed in lymphatic vascular phenotypes between \textit{op/op} and \textit{PU.1}^{op/op} mice were due to the tissue type or developmental stage analysed, genetic background of each mouse line or retention of some macrophages in \textit{op/op} mice. Regardless, we observed pronounced dermal lymphatic vessel hyperplasia during embryogenesis in two independently generated lines of \textit{PU.1}^{op/op} mice (Dakic et al., 2005; McKercher et al., 1996), as well as in \textit{CSF1R}^{op/op} mice. Our data suggest that macrophages in various tissue compartments of the embryo might play distinct lymphangiogenic roles.

In conclusion, our data clarify a long-debated question in the field of lymphangiogenesis research by illustrating that cells of the myeloid and macrophage lineage do not comprise a pool of lymphatic endothelial progenitor cells. Furthermore, we have determined that cells of the myeloid lineage provide signals that regulate dermal lymphatic vessel calibre during development by controlling lymphatic endothelial cell proliferation. Defining the precise identity, role and relative contribution of myeloid-cell-derived signals during development and inflammation-stimulated neo-lymphangiogenesis will be the focus of future work. Our data have important implications when considering the targeting of macrophages for anti-lymphangiogenic therapies.

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Competing interests statement

The authors declare no competing financial interests.

Author contributions

N.L.H., E.J.G. and R.A.L. designed the research, N.L.H. and E.J.G. performed the research, S.L.N., S.R. and J.W.P. contributed vital reagents and all authors analysed the data. N.L.H. and E.J.G. wrote the manuscript.

Supplementary material

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