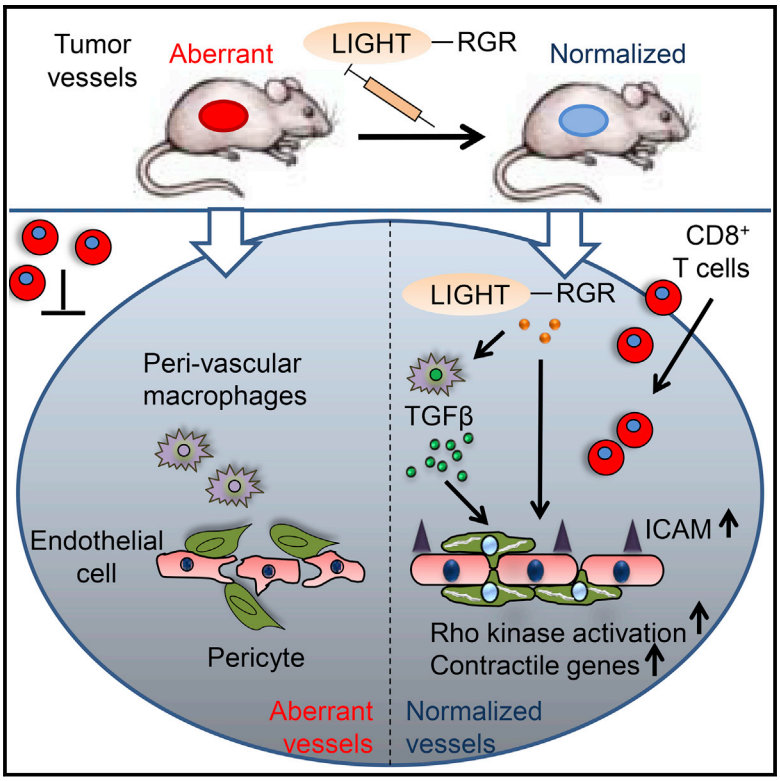
Article

Cell Reports

Intratumoral LIGHT Restores Pericyte Contractile Properties and Vessel Integrity

Graphical Abstract



Authors

Anna Johansson-Percival, Zhi-Jie Li, Devina D. Lakhiani, Bo He, Xiao Wang, Juliana Hamzah, Ruth Ganss

Correspondence

ganss@perkins.uwa.edu.au

In Brief

Pericytes are unique support cells of the microvasculature that are able to contract. However, this capacity is altered in leaky tumor vessels. Johansson-Percival et al. developed a cytokine therapy that restores contractility of intratumoral pericytes and vessel integrity. These effects are specific to the vascular bed and enhance anti-tumor therapies.

Highlights

- LIGHT targeting into tumors normalizes the vasculature and improves therapy
- Vascular integrity is restored by inducing pericyte contractility
- LIGHT triggers a peri-vascular signaling cascade involving macrophages and TGF-β
- LIGHT-RGR effects are Rho kinase-dependent and restricted to the vascular bed

Johansson-Percival et al., 2015, Cell Reports *13*, 2687–2698
December 29, 2015 ©2015 The Authors
http://dx.doi.org/10.1016/j.celrep.2015.12.004

Cell Reports
Article

Intratumoral LIGHT Restores Pericyte Contractile Properties and Vessel Integrity

Anna Johansson-Percival,[1] Zhi-Jie Li,[1] Devina D. Lakhiani,[1] Bo He,[1] Xiao Wang,[2] Juliana Hamzah,[2] and Ruth Ganss[1,*]
[1]Vascular Biology and Stromal Targeting
[2]Targeted Drug Delivery, Imaging, and Therapy
Harry Perkins Institute of Medical Research, The University of Western Australia, Centre for Medical Research, Nedlands, Western Australia 6009, Australia
*Correspondence: ganss@perkins.uwa.edu.au
http://dx.doi.org/10.1016/j.celrep.2015.12.004

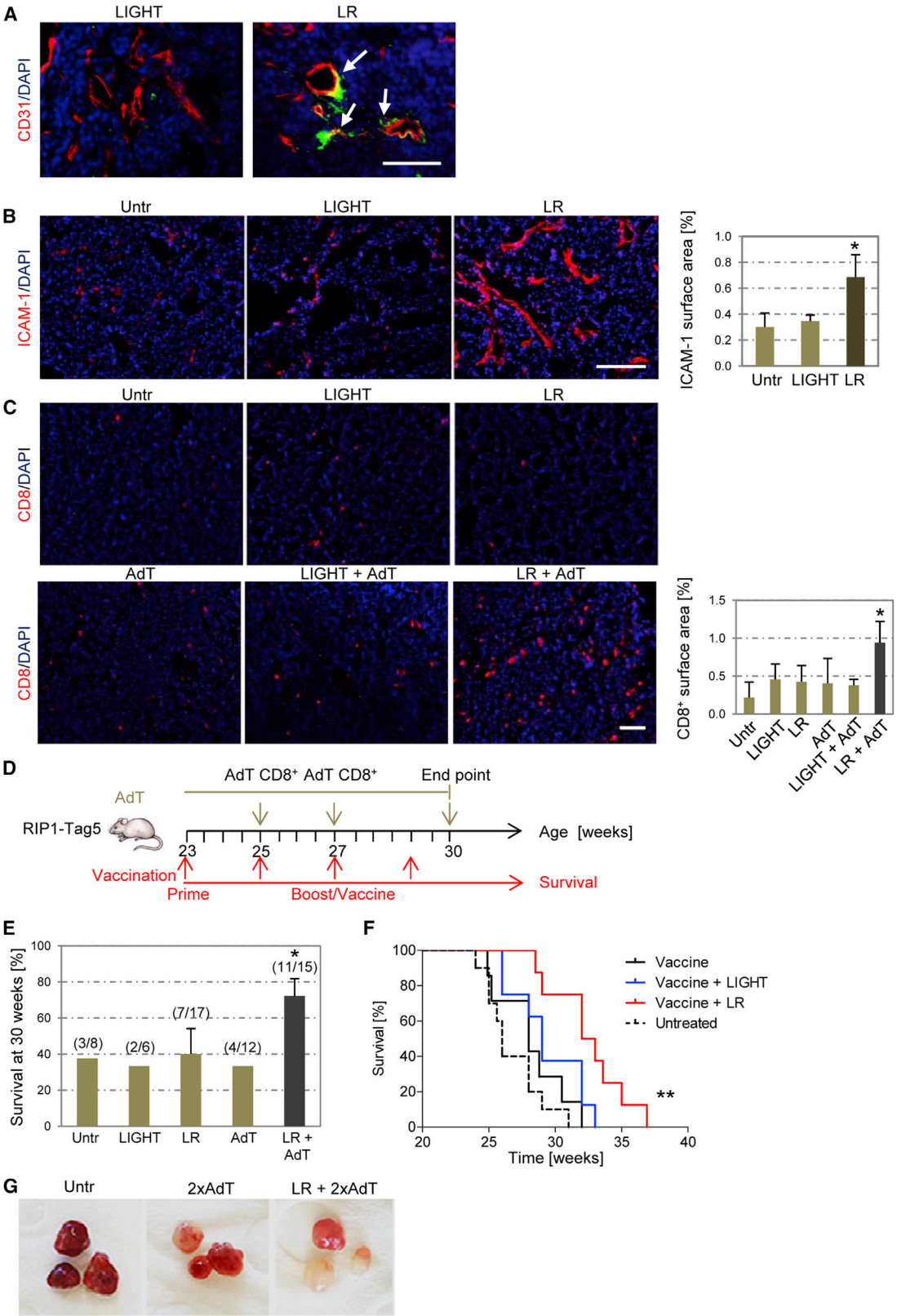
SUMMARY

Normalization of the tumor vasculature is an emerging concept shown to improve anti-cancer therapy. However, there are currently no clinical interventions that effect long-lasting normalization. Here, we have developed a strategy for normalization by specific intratumoral delivery of LIGHT/TNFSF14. Importantly, normalization occurs by induced expression of contractile markers in intratumoral pericytes, which in turn re-establishes tight pericyte-vessel alignment. Restoring vessel integrity improves tumor perfusion and acts as adjuvant to chemo- and immunotherapy. Mechanistically, intratumoral LIGHT induces pericyte differentiation and normalization via Rho kinase signaling. Minute amounts of LIGHT act in a paracrine fashion to trigger an amplifying cascade involving transforming growth factor β (TGF-β) from peri-vascular macrophages. That these effects can be reproduced by adoptive transfer of LIGHT-stimulated macrophages alone demonstrates their central role in regulating pericyte differentiation. Our findings highlight a crucial role of pericyte contractile properties in vascular normalization, effected by macrophage signaling, thus providing so far unexplored anti-cancer opportunities.

INTRODUCTION

The tumor microenvironment harbors a network of stromal cells and growth factors that actively promote cancer progression and immune suppression. Chronic pro-angiogenic signaling between tumor-associated immune cells and the vasculature reduces tumor perfusion and ultimately drug access (Coussens et al., 2013). In contrast, specific modulation or reprogramming of stromal signaling networks can lead to tumor vessel normalization and increased responses to anti-cancer chemo or immune therapies (De Palma et al., 2008; Johansson et al., 2012b; Rolny et al., 2011). Normalized tumor vasculature collectively describes more organized, homogeneous, and better perfused vessels in contrast to the chaotic vasculature of untreated solid tumors (Carmeliet and Jain, 2011; Johansson et al., 2014). However, vessel normalization remains a highly descriptive entity that refers to parameters such as quantitative mural cell or pericyte coverage of endothelial cells, basement membrane composition, and vessel permeability (Goel et al., 2011). Most current genetic and pharmacological normalization strategies focus on endothelial cells (Jain, 2014; Magrini et al., 2014; Rolny et al., 2011). However, we and others have demonstrated a crucial role for pericytes in the normalization process that, for instance, involves platelet-derived growth factor B (PDGFB)/PDGFB receptor β (PDGFRβ) interactions (Abramsson et al., 2003; di Tomaso et al., 2009) or modulation of the regulator of G protein signaling 5 (RGS5) (Hamzah et al., 2008a). Induction of a more mature and normal tumor vasculature by manipulating either endothelial cells or pericytes is consistent with the notion of reciprocal interactions between all vascular components (Armulik et al., 2005). Pericytes are unique mural cells of the microvasculature that share with vascular smooth muscle cells (vSMC) the ability to contract and regulate endothelial proliferation (Gaengel et al., 2009; Rucker et al., 2000). However, this capacity is altered or lost in intratumoral pericytes. Indeed, weak or absent expression of contractile proteins in human tumors is associated with aggressive growth, metastatic dissemination, and poor survival (Islam et al., 2004). The role of pericyte contractile phenotype in vascular normalization has so far not been explored.

LIGHT, an acronym for homologous to lymphotoxins, exhibits inducible expression, and competes with HSV glycoprotein D for HVEM, a receptor expressed by T lymphocytes, or TNFSF14, is a transmembrane glycoprotein of the tumor-necrosis factor (TNF) super family. LIGHT interacts with herpes virus entry mediator (HVEM) and lymphotoxin beta receptor (LTβR). HVEM is predominantly, but not exclusively, expressed on immune cells, including monocytes/macrophages, and a co-stimulatory activator of T cells (Morel et al., 2000); LTβR is constitutive on most cell types but absent on T and B lymphocytes that produce LTβR ligands (Force et al., 1995). Stromal cells such as endothelial cells, fibroblasts, and vSMC signal through both HVEM and LTβR (Chang et al., 2005; Wei et al., 2006). Thus, LIGHT is a potent immune co-stimulator that is also associated with tissue remodeling and wound healing (Doherty et al., 2011; Petreaca



(legend on next page)

et al., 2012). This dual capacity makes LIGHT an attractive cytokine to explore in cancer (Tamada et al., 2000). Indeed, ectopic expression of LIGHT in tumors facilitates T cell-dependent tumor rejection, an effect mediated in part by changes in the tumor stroma (Yu et al., 2004). Specifically, LIGHT overexpression in cancer cells upregulates chemokines and adhesion molecules in the tumor environment concomitant with recruitment and priming of naive anti-tumor T cells. While LIGHT-mediated anti-tumor immune effects have been well established (Fan et al., 2006; Kanodia et al., 2010; Zhang et al., 2008; Zou et al., 2012), it is less clear which stromal cells are activated by LIGHT and whether modulation of the vascular barrier contributes to anti-tumor immunity. Furthermore, experimental delivery of LIGHT into solid tumors is so far limited to direct injection of adenoviral constructs or ectopic expression in mesenchymal stem cells that spontaneously home into tumors (Yu et al., 2007; Zou et al., 2012).

Here, we developed an approach that employs peptide-targeting to specifically deliver physiological amounts of LIGHT into the tumor environment of spontaneously arising pancreatic neuroendocrine and orthotopic breast cancers. Surprisingly, LIGHT has profound direct and indirect effects on the angiogenic vasculature that simultaneously increases tumor perfusion and anti-tumor effector cell penetration. Our data also demonstrate an exquisite interplay between tumor resident peri-vascular macrophages and the vascular bed that can be exploited to reverse angiogenesis and restore vessel function.

RESULTS

Intratumoral Low Dose LIGHT Acts as Adjuvant to Promote Anti-tumor Immunity

LIGHT is a proinflammatory factor that induces expression of cytokines and adhesion molecules on vascular endothelial cells in vitro (Chang et al., 2005). To specifically target LIGHT into solid tumors, a recombinant LIGHT-RGR peptide fusion compound was produced (Figure S1A). Intravenously (i.v.) injected RGR peptide (CRGRRST) binds to ~60% of angiogenic but not normal vessels (Figure S1B) and has been shown to deliver fusion compounds into pancreatic neuroendocrine tumors of rat insulin gene promoter-large SV40 T antigen (RIP-Tag) mice (Hamzah et al., 2008b, 2009; Johansson et al., 2012a; Joyce et al., 2003). Recombinant LIGHT-RGR, in contrast to unconjugated LIGHT, homes into RIP1-Tag5 tumors and is specifically retained on blood vessels (Figure 1A). To assess potential vascular effects of LIGHT-RGR in vivo, tumor bearing RIP1-Tag5 mice were treated for 2 weeks with bi-weekly i.v. injections. A dose-response analysis revealed that a very low dose of LIGHT-RGR (0.2 ng or ~6 ng/kg body weight), but not unconjugated LIGHT, is sufficient to induce expression of ICAM-1 on tumor endothelial cells (Figure 1B). This low dose is insufficient to stimulate spontaneous anti-tumor immunity as assessed by the number of infiltrating $CD8^+$ T cells (Figure 1C). However, combining low dose LIGHT-RGR treatment with adoptive transfers of ex vivo-activated anti-tumor $CD8^+$ effector cells, which normally do not extravasate into the tumor tissue (Ganss et al., 2002), significantly increases tumor infiltration (Figure 1C). This result demonstrates that LIGHT-RGR pre-treatment renders tumors permissive for T cell infiltration.

RIP1-Tag5 mice develop tumors over a period of 20 weeks and, without treatment, die between 26 to 32 weeks of age (Ganss et al., 2002). To assess the potency of LIGHT-RGR as an adjuvant, 23-week-old tumor-bearing RIP1-Tag5 mice were treated with LIGHT-RGR and adoptive transfers or anti-tumor vaccination (Figure 1D). For adoptive transfer experiments, mice were treated for a total of 8 weeks; survival was recorded at the pre-set endpoint of 30 weeks. Mice undergoing combination treatment are 5.5 times more likely (odds ratio, $p = 0.038$, Pearson's chi-square test, $p = 0.045$ Fisher's exact test) to survive until week 30 than groups treated with adoptive transfers only (Figures 1E and S1C). Similarly, therapeutic anti-Tag vaccination is ineffective in RIP1-Tag5 mice (Garbi et al., 2004) (Figure 1F). However, combination of LIGHT-RGR and vaccine significantly prolongs survival of tumor-bearing mice when compared to vaccine groups or LIGHT combined with vaccine ($p = 0.006$, $p = 0.04$, respectively, Figure 1F). Interestingly, the majority of tumors from LIGHT-RGR/adoptive transfer combination treatment groups at 30 weeks are typically "white" in appearance indicative

Figure 1. LIGHT-RGR Activates the Tumor Vasculature and Facilitates T Cell Access

(A) Biotinylated LIGHT or LIGHT-RGR (LR) were injected i.v. into tumor-bearing RIP1-Tag5 mice. LIGHT or LIGHT-RGR binding to tumor vessels ($CD31^+$, red) was assessed by histology. Arrows point to LIGHT-RGR attached to vessels. Scale bar, 50 μm. Representative images from three independent experiments are shown.

(B) Specific activation of tumor endothelial cells in untreated groups (Untr), or following a short-term (2 weeks) treatment regimen with 0.2 ng LIGHT or LR, as assessed by ICAM-1 immunohistochemistry. Intratumoral ICAM-1 staining was quantified in all groups; n = 3–7 mice. *$p < 0.05$ compared to all experimental groups, Student's t test. Scale bar, 100 μm. Representative images from three independent experiments are shown.

(C) Immunohistochemical analysis of tumor infiltrating $CD8^+$ T cells in untreated tumors, LIGHT, LR-treated tumors, or tumors from mice treated with adoptive transfer (AdT) of ex vivo-activated TagTCR8 cells only, or a combination of LIGHT or LR and adoptive transfers. Intratumoral $CD8^+$ T cells were quantified in all groups; n = 4–6 mice. *$p < 0.05$ compared to all experimental groups, Student's t test. Scale bar, 50 μm. Representative images from three independent experiments are shown.

(D) Schematic representation of AdT (end point study, 30 weeks) or vaccination (survival study) treatment regimens in RIP1-Tag5 mice. Both groups were treated ± bi-weekly LR or LIGHT i.v. injections.

(E) Survival of untreated mice (Untr; n = 8 mice), mice treated with LIGHT (n = 6), LR (n = 17), AdT only (n = 12), or mice treated with LR in combination with AdT (n = 15) was determined at a pre-defined end point (30 weeks). *$p = 0.038$ (Pearson's chi-square test) or $p = 0.045$ (Fisher's exact test), AdT versus AdT + LR.

(F) Kaplan-Meier survival analysis of untreated mice (n = 10), anti-Tag vaccinated mice (n = 7), vaccination combined with LIGHT (n = 8), or LIGHT-RGR (LR, n = 8). *$p = 0.006$ vaccine + LR compared to vaccine alone, *$p = 0.0003$ vaccine + LR compared to untreated, *$p = 0.04$ vaccine + LIGHT.

(G) The majority of tumors isolated from LR + AdT mice appear macroscopically pale in contrast to more hemorrhagic tumors in untreated and AdT groups.

Data are presented as mean ± SD.

See also Figure S1 and Supplemental Experimental Procedures for details.

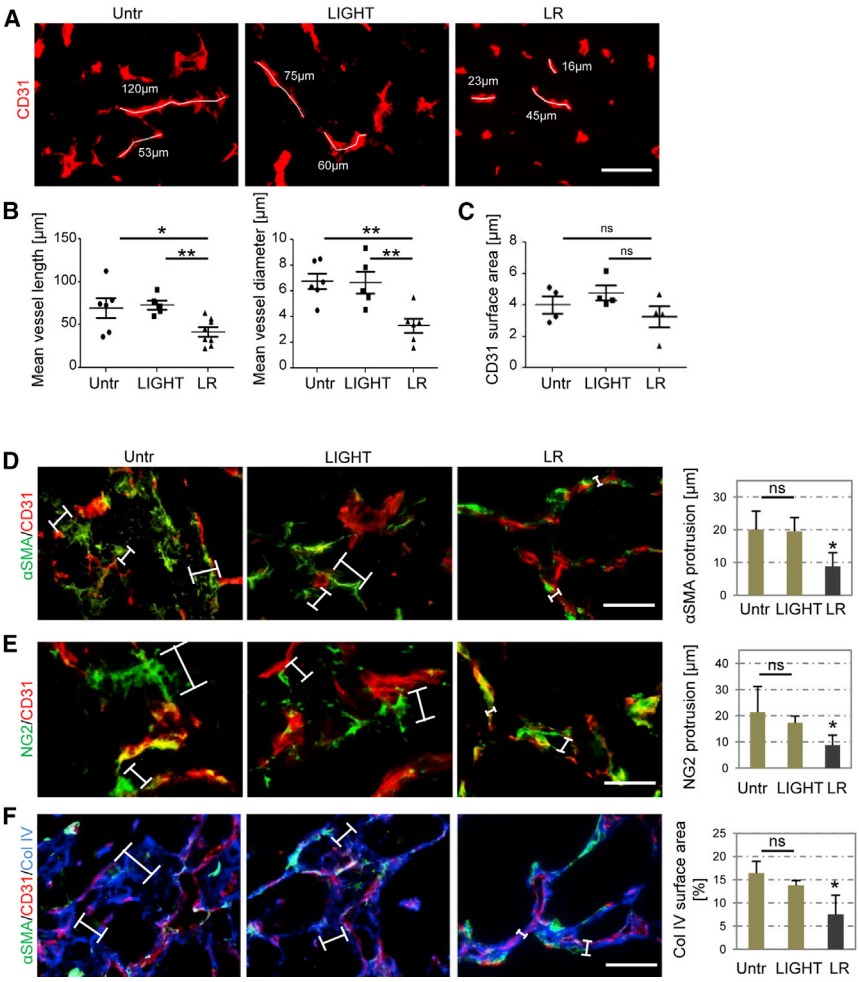


Figure 2. LIGHT-RGR Treatment Normalizes Tumor Vessels

Vascular changes in RIP1-Tag5 tumors, analyzed after 2 weeks of treatment with 0.2 ng LIGHT or LIGHT-RGR (LR) in comparison to untreated tumors (Untr). Representative images from at least three independent experiments are shown. *$p < 0.05$, **$p < 0.01$. Student's t test. Data are presented as mean ± SD.

(A) Representative vascular staining ($CD31^+$) in untreated, LIGHT and LR treatment groups and indication of vessel size (two to three representative vessels per group are demarcated). Scale bar, 50 μm.

(B) Quantification of mean vessel lengths (left) and vessel diameters (right) in all treatment groups, n = 6 mice, 100–150 vessels/group were assessed.

(C) Quantification of overall vascularity (total $CD31^+$ vessels) in all treatment groups.

(D) Endothelial ($CD31^+$) coverage by αSMA^+ pericytes and quantification of pericyte protrusion into parenchyma. Bars indicate broad/fuzzy appearance in contrast to closely aligned vascular components; n = 4–10 mice. Scale bar, 50 μm.

(E) Endothelial ($CD31^+$) coverage by $NG2^+$ pericytes and quantification of pericyte protrusion into parenchyma. Bars indicate broad/fuzzy appearance in contrast to closely aligned vascular components; n = 4–10 mice. Scale bar, 50 μm.

(F) Association of collagen IV (Col IV) with the vascular bed ($CD31^+/\alpha SMA^+$) and quantification of total tumor Col IV content. Bars indicate broad/fuzzy appearance in contrast to closely aligned vascular components; n = 4–10 mice. Scale bar, 50 μm.

See also Figure S2.

of reduced hemorrhaging and tumor vessel remodeling in the context of a Th1 immune response (Figure 1G).

Intratumoral LIGHT Normalizes the Vasculature and Increases Tumor Perfusion

The "white" tumor appearance prompted us to explore a potential role of LIGHT-RGR in vessel remodeling. Tumor bearing 27-week-old RIP1-Tag5 mice were treated for 2 weeks with biweekly i.v. LIGHT-RGR injections. In treated tumors, $CD31^+$ vessels are smaller with decreased lengths and diameters (Figures 2A and 2B). Importantly, the shift in vessel caliber is not associated with an overall change in vascularity demonstrating that LIGHT-RGR treatment does not deplete tumor vessels (Figure 2C). To assess pericyte coverage, tumors were co-stained with the vessel marker CD31 and the pericyte markers αSMA and NG2. In RIP-Tag tumors, αSMA^+ cells are almost exclusively located around blood vessels and also positive for NG2, thus representing αSMA^+ pericytes (Figures S2A–S2C). Furthermore, $CD31^+$ endothelial cells and $NG2^+$ pericytes express both HEVM and LTβR, immune cells predominantly express HVEM, whereas expression of the receptors in epithelial cells is negligible (Figures S2D and S2E). Upon LIGHT-RGR treatment, αSMA or NG2 vessel coverage remains unchanged (Figures S2F and S2G). However, protrusion of αSMA^+ or $NG2^+$ pericyte sheaths away from endothelial cells into the tumor parenchyma, a hallmark of angiogenic vessels (Morikawa et al., 2002; Xian et al., 2006), is significantly diminished (Figures 2D and 2E). Similarly, the typically aberrant thickness of the vascular basement membrane (Baluk et al., 2003), which connects pericytes and endothelial cells, is reduced upon LIGHT-RGR treatment as assessed by collagen IV staining (Figure 2F). These morphological changes are consistent with features of a normalized vasculature (Goel et al., 2011). Importantly, LIGHT-induced vascular changes significantly reduce dextran extravasation into the interstitium, a marker for vascular leakiness (Figure 3A). This correlates with enhanced tumor perfusion as inferred by increased FITC-lectin binding to tumor vessels (Figure 3B). Consistently, LIGHT-RGR treatment increases the efficacy of the cytotoxic drug cyclophosphamide when administered continuously through the drinking water following an 8-week metronomic treatment regimen (Pietras and Hanahan, 2005). Specifically, combination treatment induces tumor cell apoptosis at higher frequency (2-fold increase, Figure 3C) and decreases tumor progression compared to cyclophosphamide and LIGHT-RGR

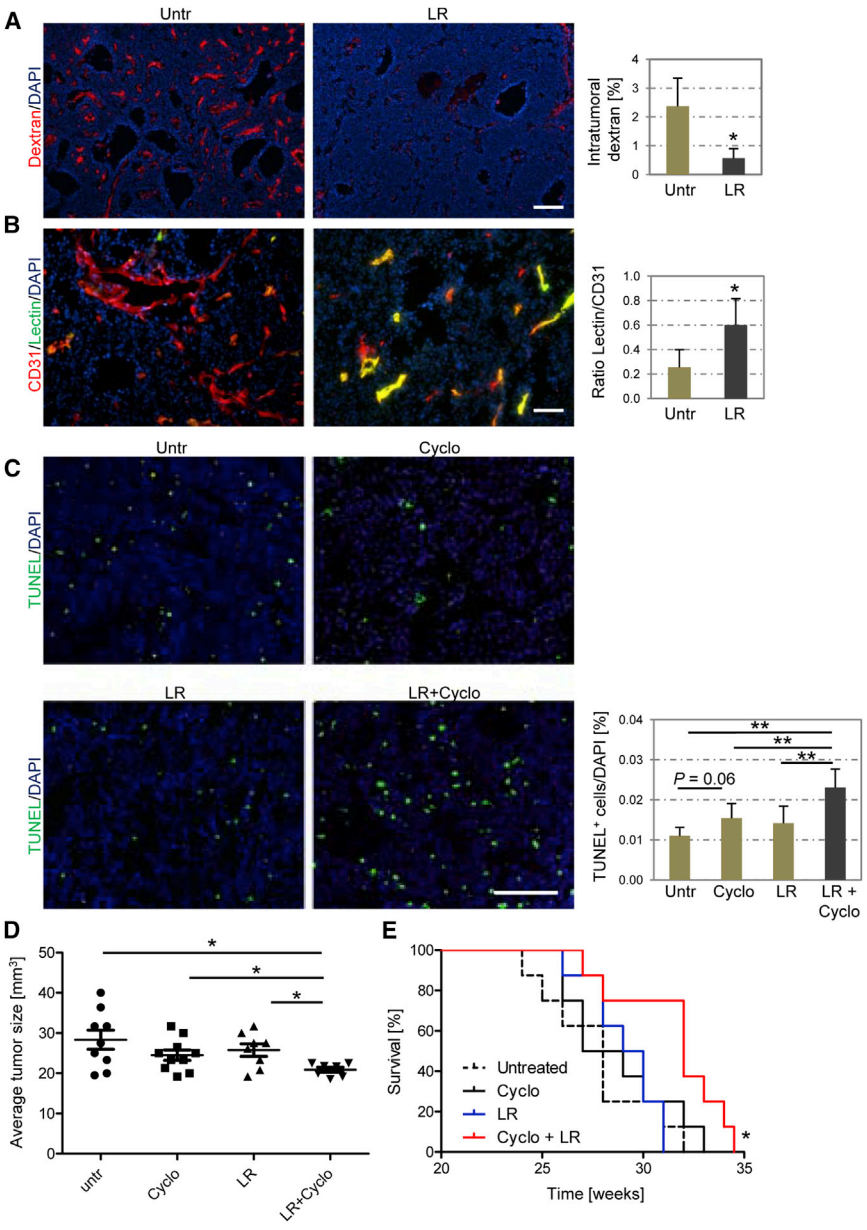


Figure 3. LIGHT-RGR Treatment Improves Tumor Perfusion and Drug Delivery

(A) Histology image of intratumoral dextran (red) following injection with 70 kDa Texas Red dextran and quantification of extravasated dextran as surrogate marker for vascular leakiness in untreated (Untr) and LIGHT-RGR (LR)-treated tumors. Representative images from at least three independent experiments are shown. Data are presented as mean ± SD. n = 4–7 mice, *p < 0.05, Student's t test. Scale bar, 100 μm.

(B) Overlay of CD31$^+$ vessels with FITC-lectin delineates perfused (yellow) and non-perfused (red) tumor vessels in untreated and LR-treated groups. Representative images from at least three independent experiments are shown. Data are presented as mean ± SD. n = 4–7 mice, *p < 0.05, Student's t test. Scale bar, 50 μm.

(C) Intratumoral apoptosis (TUNEL, green) in untreated RIP1-Tag5 mice and after long term treatment (8 weeks) with cyclophosphamide (Cyclo), LR, or a combination of Cyclo + LR. Quantification of TUNEL$^+$ cells in relation to total cells (DAPI$^+$ nuclei). Representative images from at least three independent experiments are shown. Data are presented as mean ± SD. n = 5–7 mice. **p ≤ 0.01, Student's t test. Scale bar, 100 μm.

(D) Quantification of mean tumor size in untreated and treated RIP1-Tag5 mice (age at endpoint: 30 weeks), n = 10–12 mice. *p ≤ 0.04, Student's t test.

(E) Kaplan-Meier survival analysis of untreated RIP1-Tag5 mice and after treatment with Cyclo, LR, or a combination of Cyclo + LR (n = 8 for all groups). *p = 0.05, Cyclo + LR compared to Cyclo. *p = 0.01, Cyclo + LR compared to all other groups.

See also Figure S3.

alone, or untreated controls (Figure 3D). This translates into a significant survival advantage in the combination treatment group as compared to untreated or cyclophosphamide groups (Figure 3E, p = 0.01). Since insulinomas are resistant to chemotherapy, this finding is remarkable and indicates that LIGHT-RGR treatment substantially increases tumor perfusion and drug access. Importantly, after 8 weeks of continuous treatment, tumor vessels and margins remain intact, thus demonstrating that long-term treatment does not promote vessel death or tumor invasiveness (Figures S3A and S3B).

LIGHT Reprograms Pericyte Phenotype

Molecular mechanisms of vascular normalization are poorly defined. Surprisingly, gene expression profiling of untreated and LIGHT-RGR-treated tumors, which most significantly differ in tumor perfusion, revealed a prominent gene signature of vSMC differentiation markers as well as transforming growth factor β (TGF-β) that is a potent inducer of pericyte maturation (Hirschi et al., 1998) (Figure 4A). Specifically, vSMC contractile markers such as *myocardin*, *calponin*, *SM22α*, and *smooth muscle myosin heavy chain* (*Myh11*) are upregulated, whereas in proliferating/synthetic vSMC, these markers are generally decreased (Owens et al., 2004). Given the prominent role of pericytes in angiogenic vessel remodeling (Hamzah et al., 2008a; Song et al., 2005; Winkler et al., 2004; Xian et al., 2006), we hypothesized that LIGHT may induce vessel normalization by regulating pericyte contractile phenotype. Strikingly, CD31$^+$ blood vessels in LIGHT-RGR-treated tumors are covered with increased numbers of myocardin-, caldesmon-, and calponin-positive cells (Figures S4A–S4D); the vast majority of these cells co-localize with NG2$^+$/αSMA$^+$ pericytes (Figures 4B–4D). Conversely, collagen I, a marker for synthetic vSMC is downregulated (Figures 4E and S4E). Both, contractile marker expression and tumor

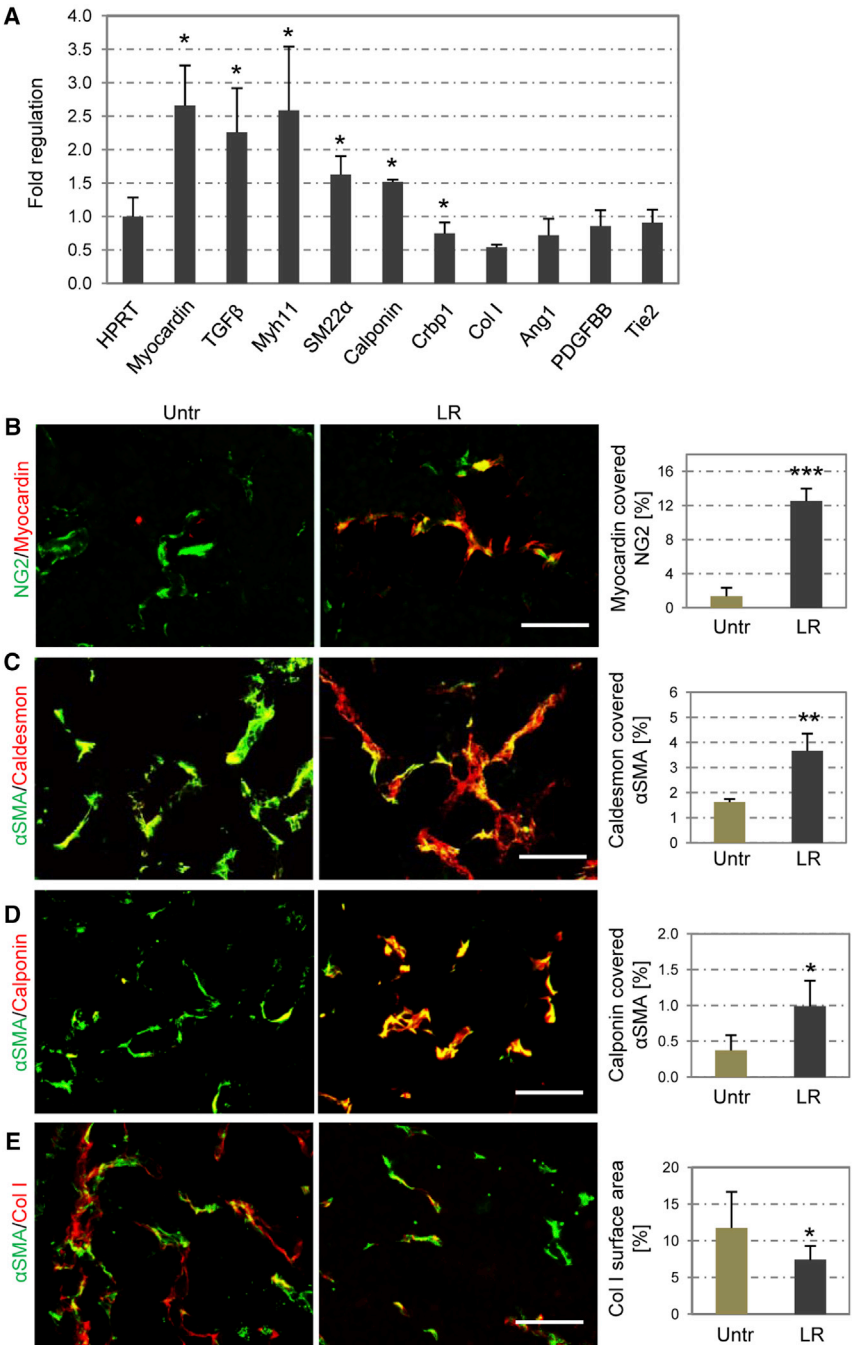


Figure 4. LIGHT-RGR Treatment Induces Contractile Properties in Intratumoral Pericytes

(A) Comparative gene expression analysis of tumors isolated from untreated 27-week-old RIP1-Tag5 mice and mice treated with 0.2 ng LIGHT-RGR for 2 weeks. Pericyte contractile and differentiation markers were selected for qPCR analysis. Data are shown as fold regulation in LIGHT-RGR tumors compared to untreated controls, n = 3–6 mice, *p < 0.05, Student's t test.

(B–E) RIP1-Tag5 mice (27 weeks old) were left untreated (Untr) or treated for 2 weeks with 0.2 ng LIGHT-RGR (LR). (B) Myocardin co-localization with $NG2^+$ pericytes in controls and LR-treated groups and quantification of myocardin induction. (C) Caldesmon, (D) calponin, (E) collagen I (Col I) co-localization with αSMA^+ pericytes and quantification. Representative images from three independent experiments are shown. n = 5–10 mice, *p < 0.05, **p < 0.01, ***p < 0.0001, Student's t test. Data are presented as mean ± SD. Scale bars, 50 μm. See also Figures S4 and S5.

Pericyte Contractile Properties Are Regulated by Macrophage-Derived TGF-β

Having shown active pericyte remodeling in vivo, we next sought to determine the factor(s) involved in this process by using a well-established model of undifferentiated mesenchymal 10T1/2 cells (Hirschi et al., 1998). Although 10T1/2 cells express LTβR, LIGHT in itself does not induce upregulation of contractile proteins (Figures S6A and S6B), thus suggesting possible indirect effects of LIGHT-RGR on pericyte contractile phenotype in vivo. Interestingly, macrophages that predominantly express the LIGHT receptor HVEM (Figure S6C) increase in LIGHT-RGR-treated tumors and also align more closely with the tumor vasculature (Figures 5A and 5B). Moreover, as indicated by our gene signature (Figure 4A), TGF-β is induced around the remodeling vasculature and almost exclusively co-localizes with perivascular $CD68^+$ macrophages following LIGHT-RGR treatment (Figures 5C and S6D–S6F). TGF-β has been shown to mature mesenchymal precursor cells such as 10T1/2 into vSMC (Hirschi et al., 1998) (Figure S6G) consistent with TGF-β being a direct transcriptional activator of SMC differentiation genes (Alexander and Owens, 2012). This prompted us to investigate the role of macrophages in vessel differentiation. Indeed, tissue culture supernatant of macrophages isolated ex vivo from LIGHT-RGR-treated tumors induces contractile markers in 10T1/2 cells as exemplified by myocardin and caldesmon staining. Simultaneous incubation with macrophage-conditioned medium and TGF-β blocking

perfusion, increase over time in direct correlation with LIGHT-RGR therapy (Figures S4F–S4H). These findings suggest that loss of contractile properties in pericytes is part of the angiogenic switch in RIP1-Tag5 tumors, and restoration of a contractile phenotype normalizes the vascular bed. Importantly, LIGHT-RGR treatment of orthotopically implanted breast tumors recapitulates key features of LIGHT-RGR-treated insulinomas. This includes vascular stabilization and enhanced perfusion that in turn improves oxygenation of highly hypoxic breast cancers and also induces a downward trend for lung metastases (p = 0.1, Figures S5A–S5E).

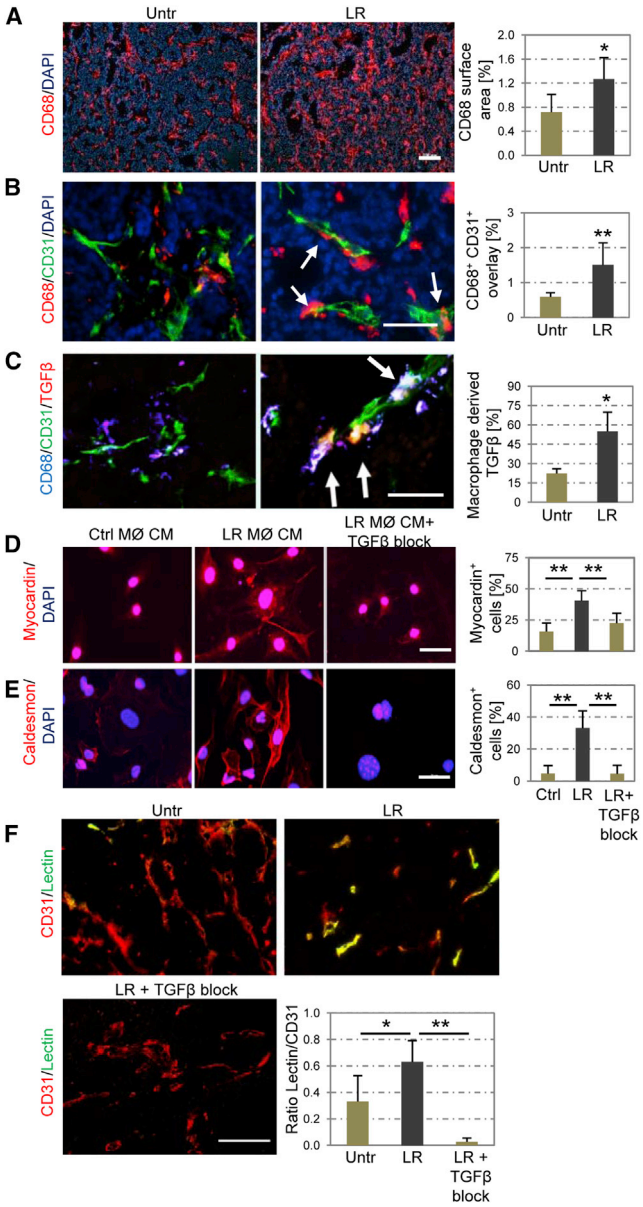


Figure 5. LIGHT-Activated Macrophages in the Peri-Vascular Niche Secret TGF-β and Induce Pericyte Differentiation

(A) Histology image of CD68$^+$ macrophages in untreated RIP1-Tag5 mice (Untr), or mice treated for 2 weeks with 0.2 ng LIGHT-RGR (LR) and quantification of intratumoral CD68$^+$ signals. n = 4–5 mice, *p < 0.05, **p = 0.006, Student's t test. Scale bar, 100 μm.

(B) Images depict CD68$^+$ macrophage location in relation to CD31$^+$ vessels and quantification of CD68/CD31 association as % total surface area. Arrows point at direct overlay (yellow). n = 4–5 mice, *p < 0.05, **p = 0.006, Student's t test. Scale bar, 20 μm.

(C) Histology image of TGF-β$^+$/CD68$^+$ double-positive macrophages in relation to CD31$^+$ vessels (arrows point at triple overlay, white). Quantification of TGF-β$^+$/CD68$^+$ double positive macrophages (found exclusively around vessels) as % overlay in relation to total TGF-β signals in both treatment groups. n = 4–5 mice, *p < 0.05, **p = 0.006, Student's t test. Scale bar, 20 μm.

(D and E) In vitro stimulation of 10T1/2 cells with conditioned medium (CM) from macrophages isolated from untreated RIP1-Tag5 tumors (Ctrl MØ) or tumors after 2 weeks of LIGHT-RGR treatment (LR MØ) induces (D) myocardin and (E) caldesmon expression that is inhibited by TGF-β-blocking antibodies (TGF-β block); n = 10–11. Representative images from three independent experiments are shown. **p < 0.001, Student's t test. Scale bars, 50 μm.

(F) Mice were treated with four injections of LR and TGF-β-blocking antibodies (TGF-β block) for 2 weeks followed by assessment of tumor perfusion with FITC-lectin. Quantification of perfused vessels (yellow) (n = 3–7). *p = 0.01, LR versus untreated; **p < 0.0001, LR versus LR + TGF-β block; Student's t test. Scale bar, 100 μm. Data are presented as mean ± SD.

See also Figure S6.

antibodies abolishes myocardin/caldesmon induction in vitro (Figures 5D and 5E). In addition, in vivo treatment of tumor-bearing RIP1-Tag5 mice with LIGHT-RGR and TGF-β-blocking antibodies neutralizes LIGHT-RGR effects and impairs tumor perfusion (Figure 5F). These results suggest that tumor-targeted LIGHT induces macrophages to secrete TGF-β that, in turn, activates contractile properties in pericytes. LIGHT-induced effects are remarkably specific and do not induce a general shift in macrophage polarization (Figures S6H and S6I). Moreover, potential TGF-β-mediated endothelial-mesenchymal transition as assessed by phenotypic conversion of endothelial cells into FSP-1$^+$ fibroblast-like cells (Zeisberg et al., 2007) or loss of endothelial cell contact integrity (Krizbai et al., 2015) were not observed consistent with absence of local invasion or increased distant metastases (Figures S6J and S6K).

To further assess the critical role of macrophages in vivo, peritoneal macrophages from syngeneic donor mice were isolated and stimulated with LIGHT overnight. Consistent with our in vivo data, LIGHT induces peritoneal macrophages to secrete TGFβ (Figure 6A). Ex vivo-stimulated macrophages (CD11b$^+$) were adoptively transferred into tumor-bearing RIP1-Tag5 mice (Figure 6B). Both, control and LIGHT-treated macrophages, home into tumors and form clusters within the tumor stroma (Figures S7A and S7B). Remarkably, only ex vivo LIGHT-stimulated, TGF-β-secreting peritoneal macrophages recapitulate LIGHT-RGR treatment by reducing hemorrhaging and restoring vessel functionality; in contrast, control macrophages do not change tumor appearance or perfusion. Furthermore, transfer of LIGHT-stimulated macrophages with simultaneous injection of TGF-β-blocking antibodies abolishes improved vessel functionality (Figures 6C–6E). Overall, these experiments demonstrate the crucial role of TGF-β-secreting macrophages in LIGHT-induced vessel normalization.

Rho Kinase Signaling Is Crucial for TGF-β-Induced Vessel Normalization

To further elucidate the mechanism of TGF-β-induced vSMC differentiation, Rho kinase-dependent signaling was assessed. Interestingly, the phosphorylated form of the Rho kinase downstream effector myosin light-chain (MLC) is significantly upregulated around vessels under treatment. Indeed, pMLC strongly co-localizes with peri-endothelial pericytes from LIGHT-RGR-treated but not control tumors (Figure 7A). Thus, TGF-β-induced pericyte contractile phenotype may be Rho kinase-dependent. In vitro blockade of Rho kinase during incubation of 10T1/2 cells with macrophage conditioned medium abolishes myocardin expression similar to TGF-β-blocking antibodies (Figure 7B). Importantly, simultaneous treatment of RIP1-Tag5 mice with LIGHT-RGR and Rho kinase inhibitor in vivo decreases tumor perfusion in

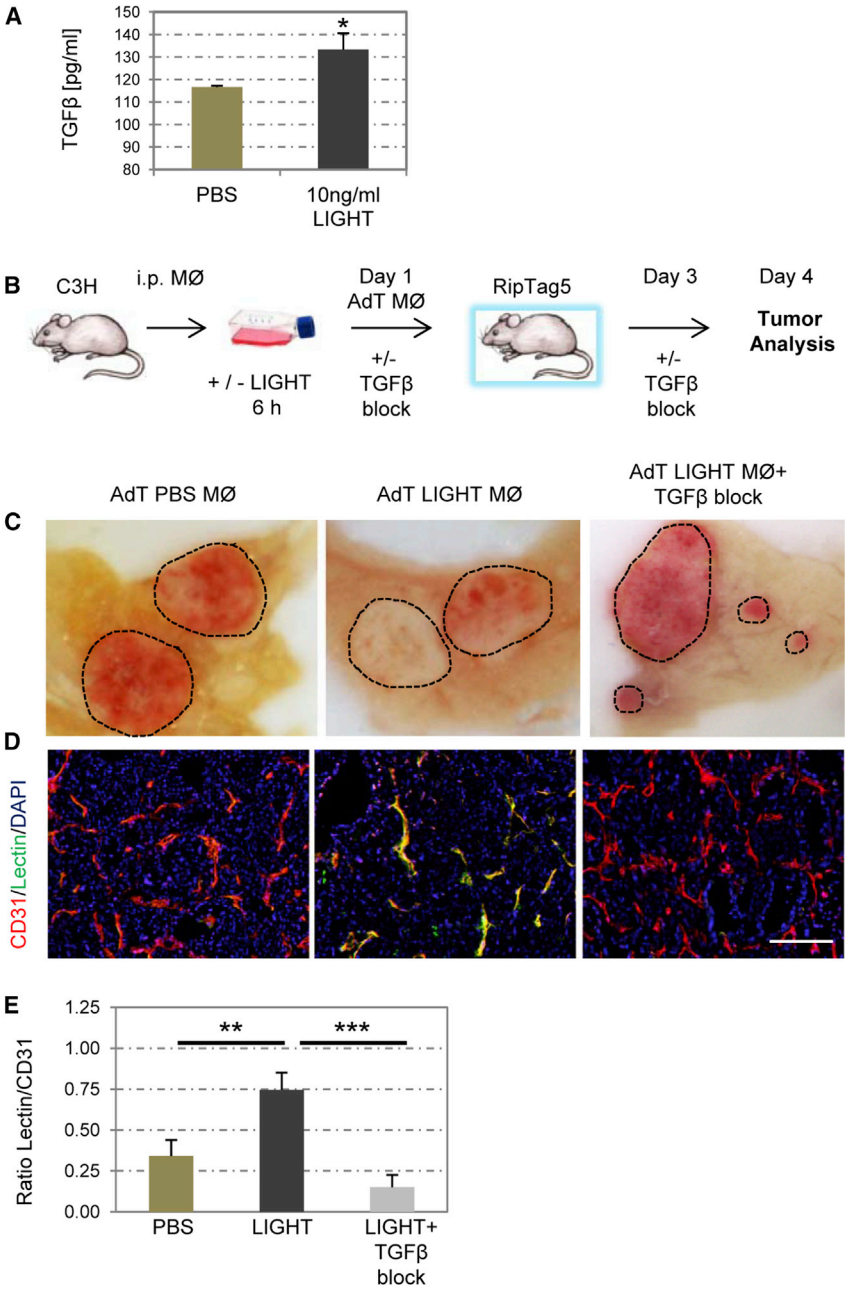


Figure 6. Adoptive Transfer of LIGHT-Activated Macrophages Mimics LIGHT-RGR Treatment

(A) Quantification of TGF-β secreted by ex vivo LIGHT-stimulated peritoneal macrophages, n = 3, *p < 0.05, Student's t test.

(B) Schematic outline of adoptive transfer of peritoneal macrophages into tumor-bearing RIP1-Tag5 mice.

(C) Macroscopic appearance of control tumors (AdT PBS MØ), tumors after adoptive transfer of LIGHT-stimulated macrophages (AdT LIGHT MØ), or tumors after adoptive transfer of LIGHT-stimulated macrophages with TGF-β-blocking antibodies (AdT LIGHT MØ + TGF-β block). Dotted circles indicate tumors that are embedded in exocrine pancreatic tissue.

(D) Overlay of $CD31^+$ vessels with FITC-lectin delineates perfused (yellow) and non-perfused (red) tumor vessels after adoptive transfers (AdT) of peritoneal macrophages stimulated with PBS (AdT PBS MØ), LIGHT without (AdT LIGHT MØ) or with TGF-β-blocking antibodies (AdT LIGHT MØ + TGF-β block) into RIP1-Tag5 mice. Scale bar, 100 μm.

(E) Quantification of ratio lectin/CD31 in all groups; n = 3 mice. **p < 0.001, ***p < 0.0001, Student's t test. Data are presented as mean ± SD.

See also Figure S7.

LIGHT-RGR-treated animals to untreated control levels (Figure 7C). Collectively, these data demonstrate that targeting low doses of LIGHT into tumors triggers TGF-β secretion by resident macrophages; this in turn induces pericyte differentiation and normalizes tumor vessels in a Rho kinase-dependent manner.

DISCUSSION

To exploit the inherent plasticity of stromal cells as adjuvant cancer therapy is an attractive concept (Coussens et al., 2013; Johansson et al., 2014). Here, we developed a strategy to specifically deliver LIGHT into solid tumors and uncovered its potential to normalize vessels by inducing pericyte differentiation. Smooth muscle cell phenotypic switching is an integral part of embryonic development and wound healing. For instance, during vascular injury, vSMC dedifferentiate and increase proliferation, migration, and matrix synthesis; upon resolution of the insult a differentiated, contractile state is reinstalled (Owens et al., 2004). Pericytes share many features of vSMC but so far little is known about their contractile phenotype in tumors.

Blood vessels in RIP1-Tag5 tumors are highly angiogenic, lack contractile markers, and tumors are notoriously difficult to treat. As documented here, low dose LIGHT-RGR treatment normalizes tumor vessels in a pericyte-dependent process that in turn reduces hemorrhaging, increases tumor perfusion, and efficacy of chemo- and immunotherapy. Similar effects are observed in orthotopic breast cancer where LIGHT-RGR significantly reduces tumor hypoxia. Pericyte-mediated vascular normalization has previously been shown to enhance the effectiveness of adoptive immunotherapy (Hamzah et al., 2008a; Huang et al., 2013; Shrimali et al., 2010), possibly by reducing intratumoral pressure or alleviating hypoxia. Alternatively, active pericyte-leukocyte interactions may facilitate the transmigration process. Recently, it has been shown in models of venous inflammation that pericytes are capable of orchestrating leukocyte migration (Proebstl et al., 2012; Stark et al., 2013; Wang et al., 2012).

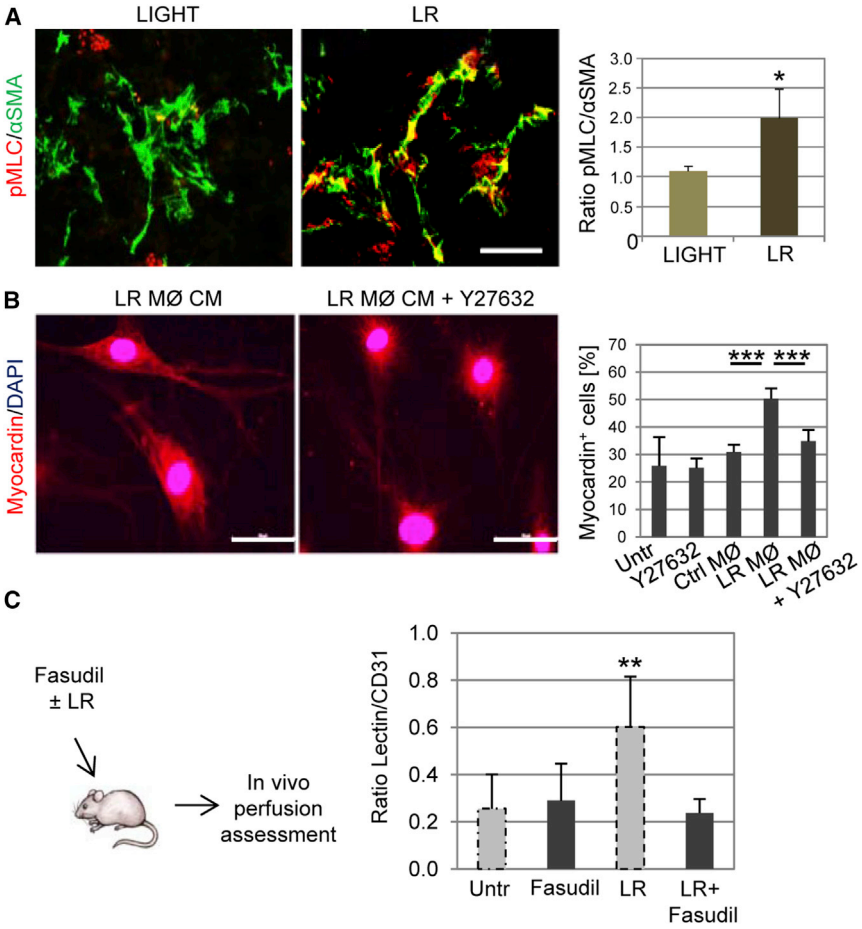


Figure 7. Rho Kinase Signaling Is Crucially Involved in LIGHT-RGR-Induced Vascular Normalization

(A) Co-localization of phosphoMLC$^+$ cells (pMLC) with αSMA$^+$ pericytes in RIP1-Tag5 mice treated with LIGHT or LIGHT-RGR (LR) for 2 weeks; quantification of pMLC-positive pericytes, n = 3–5. *$p < 0.05$, Student's t test. Scale bar, 50 μm.

(B) Representative images of myocardin staining in 10T1/2 cells after in vitro stimulation with conditioned media from LIGHT-RGR-treated tumor macrophages in the absence (LR MØ CM) or in the presence of Rho kinase inhibitor (LR MØ CM + Y27632). Quantification of myocardin induction in all experimental groups, n = 5–15, ***$p \leq 0.0001$, Student's t test. Scale bars, 50 μm.

(C) Simultaneous treatment of RIP1-Tag5 mice with LIGHT-RGR and Rho kinase inhibitor (LR + Fasudil) or Fasudil alone for 2 weeks and assessment of ratio FITC-lectin/CD31 as surrogate marker for tumor perfusion, n = 3 mice, **$p \leq 0.001$ compared to all experimental groups, Student's t test. Values from control and LR-treated tumors (dashed lines) are shown as reference only (original data shown in Figure 3B). Data are presented as mean ± SD.

Common underlying themes in these inflammatory models are changes in pericyte shape/cytoskeleton and basement membrane re-organization (Proebstl et al., 2012; Wang et al., 2012). Intriguingly, LIGHT-induced vessel normalization also correlates with strong induction of pericyte contractile markers and changes in basement membrane, consistent with active phenotypic remodeling. Pericytes control proliferation of capillary endothelial cells in a cell contact-dependent manner in vitro (Durham et al., 2014; Kutcher et al., 2007). Therefore, it is conceivable that loss of contractile properties in intratumoral pericytes is an integral part of excessive vessel growth and tumor angiogenesis; reversing this process may in turn induce normalization of the entire vascular bed.

Interestingly, pericyte contractile phenotype is most likely not induced by LIGHT itself, but by TGF-β secreted by LIGHT-stimulated peri-vascular cells, predominantly macrophages. Indeed, minute amounts of LIGHT trigger significant TGF-β production exclusively in the peri-vascular niche, demonstrating substantial amplification to locally effect normalization.

TGF-β is a well-known differentiation factor for mural precursors such as 10T1/2 cells (Hirschi et al., 1998). In cancer, TGF-β can suppress or promote angiogenesis, depending on the context (Liu et al., 2012; Pardali and ten Dijke, 2009; Sounni et al., 2010). In our study, TGF-β is exclusively secreted by peri-vascular cells and thus acts at short range to restore vascular stability but not endothelial to mesenchymal transition; this is in clear contrast to widespread TGF-β-induced fibrosis of activated tumor-associated fibroblasts that can promote tumorigenesis and metastatic dissemination (Massagué, 2008). The exquisite role of peri-vascular macrophages in amplifying LIGHT signals highlights their own plasticity and potential to remodel the tumor environment including angiogenic vessels for improved anti-tumor effects (De Palma and Lewis, 2013; Johansson et al., 2012b). There is precedence for a LIGHT-TGF-β signaling axis in inflammatory disease such as asthma. There, LIGHT enhances pathological tissue remodeling that involves upregulation of TGF-β, potentially as a result of LIGHT-LTRβ interactions with macrophages (Doherty et al., 2011).

Expression of vSMC differentiation markers is regulated by several pathways including RhoA/Rho kinase signaling. Rho kinase plays a crucial role in TGF-β-induced vSMC contractility since it controls both cytoskeletal activity and transcription of contractile genes (Althoff et al., 2012; Mack, 2011). Indeed, pericyte contractile properties and remodeling of the vascular bed in response to LIGHT is RhoA/Rho kinase-dependent. This is consistent with in vitro data demonstrating that TGF-β and Rho kinase signaling can control the contractile phenotype of pericytes (Kutcher et al., 2007; Sieczkiewicz and Herman, 2003). In contrast to abnormal Rho-mediated sensing of mechanical cues in the tumor microenvironment (Sanz-Moreno et al., 2011), we demonstrate here that restoring local Rho kinase activity in pericytes reduces vascular leakiness and angiogenic activity; a beneficial effect of Rho kinase signaling has also been observed in tumor endothelial cells (Mavria et al., 2006).

Pericytes are important regulators of tumor vessel function and loss of pericytes or impaired TGF-β signaling in angiogenic vessels is associated with metastasis (Anderberg et al., 2013; Cooke et al., 2012; Xian et al., 2006). However, pericyte contractile markers in cancer have been largely unexplored even though there is clinical evidence that reduction of vessel contractile proteins is a prognostic marker for metastasis. For instance, expression of caldesmon and calponin in intratumoral vSMC of malignant melanoma with metastatic potential is reduced compared to benign melanocytic tumors or normal tissue (Koganehira et al., 2003). Moreover, calponin-deficiency in mice enhances metastatic spread implying that loss of vSMC-specific contractile proteins changes vascular integrity (Taniguchi et al., 2001). Furthermore, four vSMC-specific genes, including *calponin*, are downregulated in a 17-gene signature associated with metastases of various human tumors. Conversely, components of the extracellular matrix such as *collagen type I* are upregulated (Ramaswamy et al., 2003) highlighting a causal role of vSMC dysfunction in tumor progression. Thus, as shown here, the ability to regulate peri-endothelial pericyte contractile phenotype by direct and locally-restricted targeting may have profound effects on delivery of therapeutics, response to therapy and metastatic dissemination and therefore warrants clinical evaluation.

EXPERIMENTAL PROCEDURES

For more details regarding the materials and methods used in this work, see the Supplemental Experimental Procedures.

Mice and Cell Lines

RIP1-Tag5 transgenic mice were bred on a C3HeBFe background (Johansson et al., 2012a). For adoptive transfers, mice transgenic for a Tag-specific T cell receptor (TCR), restricted to H-2K^k (referred to as TagTCR8) (Geiger et al., 1992) were used. All mice were kept under specific pathogen-free conditions at the University of Western Australia. All experimental protocols were approved by the Animal Ethics Committee of the University of Western Australia. The murine mesenchymal cell line C3H10T1/2 (10T1/2) and murine breast cancer cells (4T1) were purchased from the American Type Culture Collection (ATCC).

Recombinant LIGHT/LIGHT-RGR

Mature murine LIGHT (with or without C-terminal RGR peptide (CRGRRSTG, connected via a GGG linker) (Joyce et al., 2003) were cloned into Xho/BamH1 sides of the vector pET-44a (Novagen) to express soluble fusion proteins with N-terminal Nus × Tag/His × Tag and purified, see Supplemental Experimental Procedures.

Animal Studies

RIP1-Tag5 mice were subjected to short- or long-term treatment regimens. Short-term treatment was initiated at 26–27 weeks of age for 2 weeks. Mice were bi-weekly i.v. injected with 0.2 ng LIGHT or LIGHT-RGR in 100 μl PBS (~6 ng/kg). In some experiments TGF-β-blocking antibodies (1D11.16.8, BioXCell) were co-injected with LIGHT-RGR. At 28–29 weeks of age, mice were sacrificed and tumors were isolated for histology, RNA, or fluorescence-activated cell sorting (FACS) analysis. Long-term treatment in RIP1-Tag5 mice was started at 22–23 weeks of age with bi-weekly i.v. injections of LIGHT or LIGHT-RGR with or without cyclophosphamide, adoptive transfers of activated TagTCR8 T cells, or anti-Tag vaccine. Inhibition of Rho kinase in vivo was performed in short-term experiments with fasudil hydrochloride (LKT Laboratories) administered at 30 mg/kg, 10 min prior to LIGHT-RGR injections. For adoptive transfers of macrophages, 4% thioglycolate-induced peritoneal macrophages were harvested, washed, seeded, and stimulated with LIGHT or PBS. Six hours after stimulation, macrophages were detached, counted, and resuspended in PBS. Cells (2×10^6) were intraperitoneally (i.p.) injected into tumor-bearing RIP1-Tag5 mice with or without TGF-β-blocking antibodies as published (Klug et al., 2013). Mice were analyzed 4 days later, see Supplemental Experimental Procedures.

Statistical Analyses

Student's t test was used to distinguish differences between groups. To evaluate therapeutic efficacy based on categorical data, a 2 × 2 contingency table with survival recorded at end point (30 weeks) was used. The odds ratio was calculated by dividing the numbers of mice alive/dead in the LR + AdT group (11/4) and numbers of mice alive/dead in the AdT group (4/8). To assess significance of survival at 30 weeks, Fisher's exact test and Pearson's chi-square test were employed. A p value ≤ 0.05 was considered statistically significant. Error bars indicate SD.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.12.004.

AUTHOR CONTRIBUTIONS

A.J.-P., Z.L., and J.H. performed experiments and analyzed data. D.D.L., B.H., and X.W. performed experiments. R.G. designed research. R.G. and A.J.-P. wrote the paper.

ACKNOWLEDGMENTS

We thank C. Payne and K. Li for technical assistance and acknowledge funding from the National Health and Medical Research Foundation (NHMRC) of Australia (APP1042446, APP1006636, 634304, project grants and fellowships to R.G. and APP1058073 to J.H.), the Cancer Council of Western Australia (fellowship to A.J.-P.), and the Royal Perth Hospital Medical Research Foundation.

Received: June 29, 2015
Revised: October 12, 2015
Accepted: November 19, 2015
Published: December 17, 2015

REFERENCES

Abramsson, A., Lindblom, P., and Betsholtz, C. (2003). Endothelial and nonendothelial sources of PDGF-B regulate pericyte recruitment and influence vascular pattern formation in tumors. J. Clin. Invest. *112*, 1142–1151.

Alexander, M.R., and Owens, G.K. (2012). Epigenetic control of smooth muscle cell differentiation and phenotypic switching in vascular development and disease. Annu. Rev. Physiol. *74*, 13–40.

Althoff, T.F., Albarrán Juárez, J., Troidl, K., Tang, C., Wang, S., Wirth, A., Takefuji, M., Wettschureck, N., and Offermanns, S. (2012). Procontractile G protein-mediated signaling pathways antagonistically regulate smooth muscle differentiation in vascular remodeling. J. Exp. Med. *209*, 2277–2290.

Anderberg, C., Cunha, S.I., Zhai, Z., Cortez, E., Pardali, E., Johnson, J.R., Franco, M., Páez-Ribes, M., Cordiner, R., Fuxe, J., et al. (2013). Deficiency for endoglin in tumor vasculature weakens the endothelial barrier to metastatic dissemination. J. Exp. Med. *210*, 563–579.

Armulik, A., Abramsson, A., and Betsholtz, C. (2005). Endothelial/pericyte interactions. Circ. Res. *97*, 512–523.

Baluk, P., Morikawa, S., Haskell, A., Mancuso, M., and McDonald, D.M. (2003). Abnormalities of basement membrane on blood vessels and endothelial sprouts in tumors. Am. J. Pathol. *163*, 1801–1815.

Carmeliet, P., and Jain, R.K. (2011). Principles and mechanisms of vessel normalization for cancer and other angiogenic diseases. Nat. Rev. Drug Discov. *10*, 417–427.

Chang, Y.H., Hsieh, S.L., Chao, Y., Chou, Y.C., and Lin, W.W. (2005). Proinflammatory effects of LIGHT through HVEM and LTbetaR interactions in cultured human umbilical vein endothelial cells. J. Biomed. Sci. *12*, 363–375.

Cooke, V.G., LeBleu, V.S., Keskin, D., Khan, Z., O'Connell, J.T., Teng, Y., Duncan, M.B., Xie, L., Maeda, G., Vong, S., et al. (2012). Pericyte depletion results in hypoxia-associated epithelial-to-mesenchymal transition and metastasis mediated by met signaling pathway. Cancer Cell *21*, 66–81.

Coussens, L.M., Zitvogel, L., and Palucka, A.K. (2013). Neutralizing tumor-promoting chronic inflammation: a magic bullet? Science *339*, 286–291.

De Palma, M., and Lewis, C.E. (2013). Macrophage regulation of tumor responses to anticancer therapies. Cancer Cell *23*, 277–286.

De Palma, M., Mazzieri, R., Politi, L.S., Pucci, F., Zonari, E., Sitia, G., Mazzoleni, S., Moi, D., Venneri, M.A., Indraccolo, S., et al. (2008). Tumor-targeted interferon-alpha delivery by Tie2-expressing monocytes inhibits tumor growth and metastasis. Cancer Cell *14*, 299–311.

di Tomaso, E., London, N., Fuja, D., Logie, J., Tyrrell, J.A., Kamoun, W., Munn, L.L., and Jain, R.K. (2009). PDGF-C induces maturation of blood vessels in a model of glioblastoma and attenuates the response to anti-VEGF treatment. PLoS ONE *4*, e5123.

Doherty, T.A., Soroosh, P., Khorram, N., Fukuyama, S., Rosenthal, P., Cho, J.Y., Norris, P.S., Choi, H., Scheu, S., Pfeffer, K., et al. (2011). The tumor necrosis factor family member LIGHT is a target for asthmatic airway remodeling. Nat. Med. *17*, 596–603.

Durham, J.T., Surks, H.K., Dulmovits, B.M., and Herman, I.M. (2014). Pericyte contractility controls endothelial cell cycle progression and sprouting: insights into angiogenic switch mechanics. Am. J. Physiol. Cell Physiol. *307*, C878–C892.

Fan, Z., Yu, P., Wang, Y., Wang, Y., Fu, M.L., Liu, W., Sun, Y., and Fu, Y.X. (2006). NK-cell activation by LIGHT triggers tumor-specific CD8+ T-cell immunity to reject established tumors. Blood *107*, 1342–1351.

Force, W.R., Walter, B.N., Hession, C., Tizard, R., Kozak, C.A., Browning, J.L., and Ware, C.F. (1995). Mouse lymphotoxin-beta receptor. Molecular genetics, ligand binding, and expression. J. Immunol. *155*, 5280–5288.

Gaengel, K., Genové, G., Armulik, A., and Betsholtz, C. (2009). Endothelial-mural cell signaling in vascular development and angiogenesis. Arterioscler. Thromb. Vasc. Biol. *29*, 630–638.

Ganss, R., Ryschich, E., Klar, E., Arnold, B., and Hämmerling, G.J. (2002). Combination of T-cell therapy and trigger of inflammation induces remodeling of the vasculature and tumor eradication. Cancer Res. *62*, 1462–1470.

Garbi, N., Arnold, B., Gordon, S., Hämmerling, G.J., and Ganss, R. (2004). CpG motifs as proinflammatory factors render autochthonous tumors permissive for infiltration and destruction. J. Immunol. *172*, 5861–5869.

Geiger, T., Gooding, L.R., and Flavell, R.A. (1992). T-cell responsiveness to an oncogenic peripheral protein and spontaneous autoimmunity in transgenic mice. Proc. Natl. Acad. Sci. USA *89*, 2985–2989.

Goel, S., Duda, D.G., Xu, L., Munn, L.L., Boucher, Y., Fukumura, D., and Jain, R.K. (2011). Normalization of the vasculature for treatment of cancer and other diseases. Physiol. Rev. *91*, 1071–1121.

Hamzah, J., Jugold, M., Kiessling, F., Rigby, P., Manzur, M., Marti, H.H., Rabie, T., Kaden, S., Gröne, H.J., Hämmerling, G.J., et al. (2008a). Vascular normalization in Rgs5-deficient tumours promotes immune destruction. Nature *453*, 410–414.

Hamzah, J., Nelson, D., Moldenhauer, G., Arnold, B., Hämmerling, G.J., and Ganss, R. (2008b). Vascular targeting of anti-CD40 antibodies and IL-2 into autochthonous tumors enhances immunotherapy in mice. J. Clin. Invest. *118*, 1691–1699.

Hamzah, J., Altin, J.G., Herringson, T., Parish, C.R., Hämmerling, G.J., O'Donoghue, H., and Ganss, R. (2009). Targeted liposomal delivery of TLR9 ligands activates spontaneous antitumor immunity in an autochthonous cancer model. J. Immunol. *183*, 1091–1098.

Hirschi, K.K., Rohovsky, S.A., and D'Amore, P.A. (1998). PDGF, TGF-beta, and heterotypic cell-cell interactions mediate endothelial cell-induced recruitment of 10T1/2 cells and their differentiation to a smooth muscle fate. J. Cell Biol. *141*, 805–814.

Huang, Y., Goel, S., Duda, D.G., Fukumura, D., and Jain, R.K. (2013). Vascular normalization as an emerging strategy to enhance cancer immunotherapy. Cancer Res. *73*, 2943–2948.

Islam, A.H., Ehara, T., Kato, H., Hayama, M., Kobayashi, S., Igawa, Y., and Nishizawa, O. (2004). Calponin h1 expression in renal tumor vessels: correlations with multiple pathological factors of renal cell carcinoma. J. Urol. *171*, 1319–1323.

Jain, R.K. (2014). Antiangiogenesis strategies revisited: from starving tumors to alleviating hypoxia. Cancer Cell *26*, 605–622.

Johansson, A., Hamzah, J., and Ganss, R. (2012a). Intratumoral TNFα improves immunotherapy. OncoImmunology *1*, 1395–1397.

Johansson, A., Hamzah, J., Payne, C.J., and Ganss, R. (2012b). Tumor-targeted TNFα stabilizes tumor vessels and enhances active immunotherapy. Proc. Natl. Acad. Sci. USA *109*, 7841–7846.

Johansson, A., Hamzah, J., and Ganss, R. (2014). License for destruction: tumor-specific cytokine targeting. Trends Mol. Med. *20*, 16–24.

Joyce, J.A., Laakkonen, P., Bernasconi, M., Bergers, G., Ruoslahti, E., and Hanahan, D. (2003). Stage-specific vascular markers revealed by phage display in a mouse model of pancreatic islet tumorigenesis. Cancer Cell *4*, 393–403.

Kanodia, S., Da Silva, D.M., Karamanukyan, T., Bogaert, L., Fu, Y.X., and Kast, W.M. (2010). Expression of LIGHT/TNFSF14 combined with vaccination against human papillomavirus Type 16 E7 induces significant tumor regression. Cancer Res. *70*, 3955–3964.

Klug, F., Prakash, H., Huber, P.E., Seibel, T., Bender, N., Halama, N., Pfirschke, C., Voss, R.H., Timke, C., Umansky, L., et al. (2013). Low-dose irradiation programs macrophage differentiation to an iNOS$^+$/M1 phenotype that orchestrates effective T cell immunotherapy. Cancer Cell *24*, 589–602.

Koganehira, Y., Takeoka, M., Ehara, T., Sasaki, K., Murata, H., Saida, T., and Taniguchi, S. (2003). Reduced expression of actin-binding proteins, h-caldesmon and calponin h1, in the vascular smooth muscle inside melanoma lesions: an adverse prognostic factor for malignant melanoma. Br. J. Dermatol. *148*, 971–980.

Krizbai, I.A., Gasparics, Á., Nagyőszi, P., Fazakas, C., Molnár, J., Wilhelm, I., Bencs, R., Rosivall, L., and Sebe, A. (2015). Endothelial-mesenchymal transition of brain endothelial cells: possible role during metastatic extravasation. PLoS ONE *10*, e0123845.

Kutcher, M.E., Kolyada, A.Y., Surks, H.K., and Herman, I.M. (2007). Pericyte Rho GTPase mediates both pericyte contractile phenotype and capillary endothelial growth state. Am. J. Pathol. *171*, 693–701.

Liu, J., Liao, S., Diop-Frimpong, B., Chen, W., Goel, S., Naxerova, K., Ancukiewicz, M., Boucher, Y., Jain, R.K., and Xu, L. (2012). TGF-β blockade improves the distribution and efficacy of therapeutics in breast carcinoma by normalizing the tumor stroma. Proc. Natl. Acad. Sci. USA *109*, 16618–16623.

Mack, C.P. (2011). Signaling mechanisms that regulate smooth muscle cell differentiation. Arterioscler. Thromb. Vasc. Biol. *31*, 1495–1505.

Magrini, E., Villa, A., Angiolini, F., Doni, A., Mazzarol, G., Rudini, N., Maddaluno, L., Komuta, M., Topal, B., Prenen, H., et al. (2014). Endothelial deficiency of L1 reduces tumor angiogenesis and promotes vessel normalization. J. Clin. Invest. *124*, 4335–4350.

Massagué, J. (2008). TGFbeta in Cancer. Cell *134*, 215–230.

Mavria, G., Vercoulen, Y., Yeo, M., Paterson, H., Karasarides, M., Marais, R., Bird, D., and Marshall, C.J. (2006). ERK-MAPK signaling opposes Rho-kinase to promote endothelial cell survival and sprouting during angiogenesis. Cancer Cell *9*, 33–44.

Morel, Y., Schiano de Colella, J.M., Harrop, J., Deen, K.C., Holmes, S.D., Wattam, T.A., Khandekar, S.S., Truneh, A., Sweet, R.W., Gastaut, J.A., et al. (2000). Reciprocal expression of the TNF family receptor herpes virus entry mediator and its ligand LIGHT on activated T cells: LIGHT down-regulates its own receptor. J. Immunol. *165*, 4397–4404.

Morikawa, S., Baluk, P., Kaidoh, T., Haskell, A., Jain, R.K., and McDonald, D.M. (2002). Abnormalities in pericytes on blood vessels and endothelial sprouts in tumors. Am. J. Pathol. *160*, 985–1000.

Owens, G.K., Kumar, M.S., and Wamhoff, B.R. (2004). Molecular regulation of vascular smooth muscle cell differentiation in development and disease. Physiol. Rev. *84*, 767–801.

Pardali, E., and ten Dijke, P. (2009). Transforming growth factor-beta signaling and tumor angiogenesis. Front. Biosci. (Landmark Ed.) *14*, 4848–4861.

Petreaca, M.L., Do, D., Dhall, S., McLelland, D., Serafino, A., Lyubovitsky, J., Schiller, N., and Martins-Green, M.M. (2012). Deletion of a tumor necrosis superfamily gene in mice leads to impaired healing that mimics chronic wounds in humans. Wound Repair Regen. *20*, 353–366.

Pietras, K., and Hanahan, D. (2005). A multitargeted, metronomic, and maximum-tolerated dose "chemo-switch" regimen is antiangiogenic, producing objective responses and survival benefit in a mouse model of cancer. J. Clin. Oncol. *23*, 939–952.

Proebstl, D., Voisin, M.B., Woodfin, A., Whiteford, J., D'Acquisto, F., Jones, G.E., Rowe, D., and Nourshargh, S. (2012). Pericytes support neutrophil subendothelial cell crawling and breaching of venular walls in vivo. J. Exp. Med. *209*, 1219–1234.

Ramaswamy, S., Ross, K.N., Lander, E.S., and Golub, T.R. (2003). A molecular signature of metastasis in primary solid tumors. Nat. Genet. *33*, 49–54.

Rolny, C., Mazzone, M., Tugues, S., Laoui, D., Johansson, I., Coulon, C., Squadrito, M.L., Segura, I., Li, X., Knevels, E., et al. (2011). HRG inhibits tumor growth and metastasis by inducing macrophage polarization and vessel normalization through downregulation of PlGF. Cancer Cell *19*, 31–44.

Rucker, H.K., Wynder, H.J., and Thomas, W.E. (2000). Cellular mechanisms of CNS pericytes. Brain Res. Bull. *51*, 363–369.

Sanz-Moreno, V., Gaggioli, C., Yeo, M., Albrengues, J., Wallberg, F., Viros, A., Hooper, S., Mitter, R., Féral, C.C., Cook, M., et al. (2011). ROCK and JAK1 signaling cooperate to control actomyosin contractility in tumor cells and stroma. Cancer Cell *20*, 229–245.

Shrimali, R.K., Yu, Z., Theoret, M.R., Chinnasamy, D., Restifo, N.P., and Rosenberg, S.A. (2010). Antiangiogenic agents can increase lymphocyte infiltration into tumor and enhance the effectiveness of adoptive immunotherapy of cancer. Cancer Res. *70*, 6171–6180.

Sieczkiewicz, G.J., and Herman, I.M. (2003). TGF-beta 1 signaling controls retinal pericyte contractile protein expression. Microvasc. Res. *66*, 190–196.

Song, S., Ewald, A.J., Stallcup, W., Werb, Z., and Bergers, G. (2005). PDGFRbeta+ perivascular progenitor cells in tumours regulate pericyte differentiation and vascular survival. Nat. Cell Biol. *7*, 870–879.

Sounni, N.E., Dehne, K., van Kempen, L., Egeblad, M., Affara, N.I., Cuevas, I., Wiesen, J., Junankar, S., Korets, L., Lee, J., et al. (2010). Stromal regulation of vessel stability by MMP14 and TGFbeta. Dis. Model. Mech. *3*, 317–332.

Stark, K., Eckart, A., Haidari, S., Tirniceriu, A., Lorenz, M., von Brühl, M.L., Gärtner, F., Khandoga, A.G., Legate, K.R., Pless, R., et al. (2013). Capillary and arteriolar pericytes attract innate leukocytes exiting through venules and 'instruct' them with pattern-recognition and motility programs. Nat. Immunol. *14*, 41–51.

Tamada, K., Shimozaki, K., Chapoval, A.I., Zhu, G., Sica, G., Flies, D., Boone, T., Hsu, H., Fu, Y.X., Nagata, S., et al. (2000). Modulation of T-cell-mediated immunity in tumor and graft-versus-host disease models through the LIGHT co-stimulatory pathway. Nat. Med. *6*, 283–289.

Taniguchi, S., Takeoka, M., Ehara, T., Hashimoto, S., Shibuki, H., Yoshimura, N., Shigematsu, H., Takahashi, K., and Katsuki, M. (2001). Structural fragility of blood vessels and peritoneum in calponin h1-deficient mice, resulting in an increase in hematogenous metastasis and peritoneal dissemination of malignant tumor cells. Cancer Res. *61*, 7627–7634.

Wang, S., Cao, C., Chen, Z., Bankaitis, V., Tzima, E., Sheibani, N., and Burridge, K. (2012). Pericytes regulate vascular basement membrane remodeling and govern neutrophil extravasation during inflammation. PLoS ONE *7*, e45499.

Wei, C.Y., Chou, Y.H., Ho, F.M., Hsieh, S.L., and Lin, W.W. (2006). Signaling pathways of LIGHT induced macrophage migration and vascular smooth muscle cell proliferation. J. Cell. Physiol. *209*, 735–743.

Winkler, F., Kozin, S.V., Tong, R.T., Chae, S.S., Booth, M.F., Garkavtsev, I., Xu, L., Hicklin, D.J., Fukumura, D., di Tomaso, E., et al. (2004). Kinetics of vascular normalization by VEGFR2 blockade governs brain tumor response to radiation: role of oxygenation, angiopoietin-1, and matrix metalloproteinases. Cancer Cell *6*, 553–563.

Xian, X., Håkansson, J., Ståhlberg, A., Lindblom, P., Betsholtz, C., Gerhardt, H., and Semb, H. (2006). Pericytes limit tumor cell metastasis. J. Clin. Invest. *116*, 642–651.

Yu, P., Lee, Y., Liu, W., Chin, R.K., Wang, J., Wang, Y., Schietinger, A., Philip, M., Schreiber, H., and Fu, Y.X. (2004). Priming of naive T cells inside tumors leads to eradication of established tumors. Nat. Immunol. *5*, 141–149.

Yu, P., Lee, Y., Wang, Y., Liu, X., Auh, S., Gajewski, T.F., Schreiber, H., You, Z., Kaynor, C., Wang, X., and Fu, Y.X. (2007). Targeting the primary tumor to generate CTL for the effective eradication of spontaneous metastases. J. Immunol. *179*, 1960–1968.

Zeisberg, E.M., Potenta, S., Xie, L., Zeisberg, M., and Kalluri, R. (2007). Discovery of endothelial to mesenchymal transition as a source for carcinoma-associated fibroblasts. Cancer Res. *67*, 10123–10128.

Zhang, Y., Jiang, W., Fan, Y., Wen, J., Hao, W., and Qian, M. (2008). Engineering enhancement of the immune response to HBV DNA vaccine in mice by the use of LIGHT gene adjuvant. J. Virol. Methods *153*, 142–148.

Zou, W., Zheng, H., He, T.C., Chang, J., Fu, Y.X., and Fan, W. (2012). LIGHT delivery to tumors by mesenchymal stem cells mobilizes an effective antitumor immune response. Cancer Res. *72*, 2980–2989.