VASCULAR BIOLOGY, ATHEROSCLEROSIS, AND ENDOTHELIUM BIOLOGY

M1 Macrophage—Induced Endothelial-to-Mesenchymal Transition Promotes Infantile Hemangioma Regression

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Infantile hemangiomas are benign tumors of vascular endothelial cells (ECs), characterized by three distinct stages: proliferating phase, involuting phase, and involuted phase. The mechanisms that trigger involution of hemangioma into fibro-fatty tissue remain unknown. We report a novel mechanism by which M1-polarized macrophages induce endothelial-to-mesenchymal transition (EndMT) and promote hemangioma regression. M1- but not M2-polarized macrophages induced EndMT in ECs. Tumor necrosis factor- α and, to a lesser extent, IL-1 β and interferon- γ were the most potent cytokines produced by the M1 macrophages that induce *in vitro* EndMT. Western blot analysis and gene expression profiling showed that ECs treated with M1 macrophages, tumor necrosis factor- α , or IL-1 β decreased the expression of endothelial markers, whereas mesenchymal markers increased concomitantly. Immunohistochemical staining of patient samples revealed that a significant perivascular infiltration of M1, but not M2, macrophages coincides with endothelial expression of the critical EndMT transcription factors Snail/Slug in involuting hemangiomas. Most strikingly, M1 macrophage-treated ECs isolated from patient hemangiomas (HemECs) but not untreated HemECs readily differentiated into adipocytes on adipogenic induction. Thus, *in vitro* EndMT and adipogenesis of HemECs have, in part, recapitulated the natural history of hemangioma regression. In conclusion, our findings indicate that EndMT induced by M1 macrophages promotes infantile hemangioma regression and may lead to novel therapeutic treatments for this vascular tumor. (*Am J Pathol* 2017, 187: 2102–2111; <http://dx.doi.org/10.1016/j.ajpath.2017.05.014>)

Infantile hemangiomas (IHs) are benign tumors of vascular endothelial cells, which occur as sporadic, solitary cutaneous lesions in up to 5% of newborns. The natural history of IHs is characterized by three distinct stages: the proliferating phase (postnatal 1 to 2 weeks to 6 to 12 months), which consists of an immature vascular mass; the involuting phase (1 to 5 years old), which is structured with a matured vascular phenotype; and the involuted phase, which starts between 5 and 10 years of age and is manifested by collapsed blood vessels surrounded by fibroadipose tissue.^{1,2} The pathogenesis of IH remains to be elucidated. A unifying theory is that IHs result from aberrant proliferation and differentiation of a pluripotent progenitor cell, which embolizes in areas of relative hypoxia and grows into a

vascular tumor.^{3,4} The vascular endothelial growth factor (VEGF) signaling pathway has been linked to IH pathogenesis. The relative low expression of decoy VEGF receptor-1 in hemangioma endothelial cells (HemECs) results in increased VEGF-dependent activation of VEGF receptor-2 and downstream signaling.⁵ To date, the most accurate diagnostic confirmation of IH can be achieved by immunohistochemical staining for glucose transporter-1.⁶ It

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is unknown why IHs follow a three-stage life cycle and what causes the hemangioma self-regression. Although most IHs involute without leaving major sequelae, a significant minority of these tumors cause disfigurement or lead to severe or life-threatening complications, such as ulceration, visual impairment, airway obstruction, and congestive heart failure.¹ Propranolol is the most common β -blocker used to treat IHs, but its mechanism of action remains uncertain. Rapamycin was suggested as a possible treatment for IHs that could potentially be curative because of its ability to suppress self-renewal of stem cells and deplete hemangioma stem cells from which they originate.⁷ However, none of the current treatments are etiology based,⁸ which underlines the critical need for a better understanding of disease mechanisms in IHs. One of the most intriguing questions remains unanswered: what triggers the involution of hemangioma? Some apoptosis was observed in involuting compared to proliferating hemangiomas.⁹ However, concomitant adipogenesis appears to be the predominant event during hemangioma regression, as the vascular endothelial tumor is replaced by a mass of fat tissue.

Endothelial cell plasticity is a regulatory mechanism of both embryonic development and disease progression. It has been well established that endothelial-to-mesenchymal transition (EndMT) participates in atrioventricular valve development of the heart,¹⁰ as well as in a number of pathogenic processes, such as cardiac fibrosis,¹¹ renal fibrosis,¹² atherosclerosis,¹³ and cancer.¹⁴ A recent finding further demonstrates that vascular endothelial cells can acquire a stem cell phenotype and differentiate into other cell types to mediate the progression of human disease.¹⁵ Hence, we hypothesize that the immature vascular endothelial cells within hemangiomas undergo EndMT, possibly as remnants of their embryonic plasticity, to generate a mesenchymal stem cell–like phenotype and subsequently differentiate into adipocytes, leading to tumor regression. Previous reports showed that macrophage infiltration increased in the proliferative phase along with an up-regulated level of several cytokines, including monocyte chemoattractant protein-1 (MCP-1), a major component for recruitment of macrophage on the affected site,¹⁶ and macrophage inflammatory protein 1 β .¹⁷ Emerging evidence also revealed that a large number of infiltrating macrophages are present in both proliferating and involuting hemangiomas,¹⁸ and macrophages participated in the proliferation of hemangioma stem cells.¹⁹ We reason that IH progression must involve interactions between the defective endothelial cells and the infiltrating macrophages. As an essential component of the innate immunity, classic M1- and alternative M2-polarized macrophages can either inhibit or promote cell proliferation and tissue repair, depending on the proper microenvironmental stimuli.²⁰ In this report, we provide evidence for a novel mechanism that proinflammatory cytokines tumor necrosis factor (TNF)- α , IL-1 β , and interferon (IFN)- γ , produced by M1-polarized macrophages, induce endothelial-to-mesenchymal transition and promote hemangioma regression.

Materials and Methods

In Vitro Assays of EndMT

Endothelial cells were seeded at 5×10^4 cells per well in a 6-well plate overnight. The complete endothelial cell growth medium (EBM-2 CC-3156 with EGM-2 BulletKit CC-3162; Lonza, Walkersville, MD) was then removed, and the cells were washed twice with phosphate-buffered saline. Human Endothelial-Serum Free Medium (11111-044; Thermo Fisher Scientific, Waltham, MA) supplemented with 20 ng/mL of basic fibroblast growth factor and 10 ng/mL of EGF was added to each well. Bone morphogenetic protein 4 (10 to 100 ng/mL), transforming growth factor- β 2 (10 to 100 ng/mL), TNF- α (10 to 100 ng/mL), IL-1 β (10 to 100 ng/mL), IFN- γ (10 to 100 ng/mL), growth-regulated oncogene- α (20 to 320 ng/mL), IL-6 (10 to 160 ng/mL), IL-8 (20 to 320 ng/mL), interferon γ -induced protein-10 (20 to 320 ng/mL), MCP-1 (10 to 160 ng/mL), MCP-2 (20 to 320 ng/mL), macrophage inflammatory protein-3 α (10 to 160 ng/mL), or osteopontin (100 to 1600 ng/mL) was then added to the culture medium. Alternatively, M1 macrophage conditioned medium (M1-CM) or M2-CM containing 20 ng/mL of basic fibroblast growth factor and 10 ng/mL of EGF was added to the endothelial culture. All cytokines and growth factors as well as a neutralizing antibody against TNF- α were purchased from R&D Systems (Minneapolis, MN). Morphological changes of the cells were imaged at 24 and 48 hours using a Nikon Eclipse TS100 inverted microscope (Nikon Instruments Inc., Melville, NY) equipped with a camera and SPOT Advanced software version 4.7 (Diagnostic Instruments Inc., Sterling Heights, MI).

Macrophage Polarization

A macrophage polarization model was used with a human acute monocytic leukemia cell line THP-1 (ATCC, Manassas, VA).²¹ For M1 macrophage polarization, approximately 3×10^6 of THP-1 cells were seeded in a T-75 flask containing RPMI 1640 medium. The cells were treated with 300 nmol/L of phorbol 12-myristate 13-acetate for 48 hours, followed by exposure to 20 ng/mL of lipopolysaccharide and 20 ng/mL of IFN- γ in RPMI 1640 medium for 24 hours. The culture medium was then replaced with Human Endothelial Serum-Free Medium containing the same amount of lipopolysaccharide and IFN- γ for an additional 24 hours. For M1-CM, the cell culture supernatant was then collected by centrifugation, filtered, and used immediately or stored at -80°C until use. For M2 macrophage polarization, the THP-1 cells were treated with 300 nmol/L phorbol 12-myristate 13-acetate and then 20 ng/mL IL-4 and 20 ng/mL IL-13 in RPMI 1640 medium for 24 hours. The culture medium was then replaced with Human Endothelial-Serum Free Medium containing the same amount of IL-4 and IL-13

for an additional 24 hours. M2-CM was then collected by centrifugation, filtered, and used immediately or stored at -80°C until use.

Human Cytokine Antibody Array Analysis

Human cytokine antibody array experiments were performed according to the manufacturer's instructions (Abcam, Cambridge, MA). Briefly, the array membranes were blocked with blocking buffer for 30 minutes at room temperature and were each incubated with 2 mL of conditioned medium in a tray overnight at 4°C . After incubation, the membranes were washed with Washing Buffer I and Washing Buffer II (Abcam) and were incubated with biotin-conjugated antibodies for 2 hours at room temperature. After another round of washing, the membranes were incubated with horseradish peroxidase-conjugated streptavidin for 2 hours at room temperature and the chemiluminescence signal was detected with the ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA). The signal intensity of individual spots was analyzed with the Bio-Rad Image Lab software version 5.2.1.

RT² Profiler PCR Array Analysis

Gene profiling at the transcription level was performed by using the RT² Profiler PCR array of 84 genes with emphasis on human endothelial cell biology (Qiagen, Germantown, MD). Human umbilical vein endothelial cells (HUVECs) were treated with M1-CM for 48 hours. Total RNA was isolated by using the RNeasy Mini Kit (Qiagen). Reverse transcription was performed with the RT² First Strand Kit (Qiagen), and target genes were amplified with the RT² CYBR Green qPCR Mastermix (Qiagen) on a CFX96 Real Time Thermocycler (Bio-Rad): 95°C for 10 minutes, 95°C for 15 seconds, and 60°C for 60 seconds, with 40 cycles. The C_T cutoff was set to 30, and the real-time quantitative PCR data were analyzed at the web portal of the Gene Globe Data Analysis Center (Qiagen). A twofold change of differentially up-regulated or down-regulated gene expression level was considered significant. Three separate experiments were performed.

Immunohistochemical Staining

Surgically resected human infantile hemangioma tissues were obtained from the Hasbro Children's Hospital/Rhode Island Hospital (Providence, RI) with appropriate institutional review board approval. Frozen sections of both proliferating and involuting hemangioma samples ([Supplemental Table S1](#)) were probed with a mouse monoclonal antibody against human leukocyte antigen D related (ab80658; Abcam) to identify M1 macrophages,^{19,22–24} a rabbit monoclonal antibody against CD163 (ab182422; Abcam) to identify M2 macrophages,^{19,22–24} rabbit polyclonal antibodies against transcription factors Snail/Slug (ab180714;

Abcam), which are required for EndMT,²⁵ or rabbit polyclonal antibodies against transcription factor peroxisome proliferator-activated receptor γ (ab45036; Abcam), which is a key regulator for adipogenesis.²⁶ Immunostaining was performed following manufacturer's specific immunohistochemistry (frozen sections) instructions for each antibody. Negative controls without the primary antibodies were also included. Slides were then incubated with the EnVision+ Dual Link System-HRP solution (Dako, Santa Clara, CA) containing goat anti-mouse and anti-rabbit immunoglobulins conjugated to peroxidase-labeled polymer. After chromogenic development, the slides were counterstained with hematoxylin. Images were taken by using a Nikon Eclipse E800 microscope equipped with a camera and SPOT Advanced software.

Isolation of HemECs

Hemangiomas at the proliferating stage and at the involuting stage ([Supplemental Table S1](#)) were used for HemEC isolation. The tissues were minced and digested with 1 mg/mL of collagenase A for 1 hour. The homogenates were suspended in red blood cell lysis buffer (0.8% NH_4Cl /0.1 mmol/L EDTA) and were filtered through a 40 μm cell strainer. HemECs were isolated with Ulex europaeus agglutinin I lectin-coated Dynabeads (M-450 Tosylactivated; Thermo Fisher Scientific).²⁷ The isolated HemECs were plated in a dish coated with 1 $\mu\text{g}/\text{cm}^2$ fibronectin in EBM-2 complete endothelial cell growth medium (EBM-2 CC-3156 with EGM-2 BulletKit CC-3162; Lonza), which was changed every 3 days. To characterize the isolated cells, cells from early passages of HemECs were stained with antibodies against endothelial marker CD31 conjugated to fluorescein isothiocyanate, and mesenchymal markers neuron-glia antigen 2 conjugated to fluorescein isothiocyanate and platelet-derived growth factor receptor β conjugated to allophycocyanin. Flow cytometry analyses were performed on a BD LSR II flow cytometer (BD Biosciences, San Jose, CA), and the flow cytometry data were analyzed using FlowJo software version 7.6.5 (Tree Star, Inc., Ashland, OR).

Detection of Endothelial and Mesenchymal Markers by Western Blot

HUVECs, human dermal microvascular endothelial cells (HDMECs), or HemECs were plated in 100-mm dishes at a density of 6×10^5 cells per dish overnight and were treated with M1-CM, $\text{TNF-}\alpha$, or $\text{IL-1}\beta$ for 8, 16, 24, or 48 hours, respectively. The cells were harvested at indicated time points in radioimmunoprecipitation assay lysis buffer plus Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). The protein concentration was determined by BCA Protein Assay (Thermo Fisher Scientific). Each sample (10 μg) was resolved in 10%

Figure 1 Tumor necrosis factor (TNF)- α is the key cytokine produced by M1-polarized macrophages inducing *in vitro* endothelial-to-mesenchymal transition (EndMT) in human umbilical vein endothelial cells (HUVECs). **A:** M1- but not M2-polarized macrophages were able to induce *in vitro* EndMT. Untreated endothelial cells as well as polarization agents lipopolysaccharide (LPS) and interferon (IFN)- γ treated cells were used as controls. Representative images of at least three independent experiments are shown. **B:** Human cytokine array analysis was performed to identify cytokines produced by the M1-polarized macrophages. Although one cytokine [tissue inhibitor of metalloproteinases (TIMP)-2] was found down-regulated (green), 12 cytokines were up-regulated (red) in comparison to 300 nmol/L phorbol 12-myristate 13-acetate (PMA)-treated THP-1 control. Representative arrays of three independent experiments are shown. **C:** TNF- α is a potent inducer, to a lesser extent also IL-1 β and IFN- γ , of *in vitro* EndMT in HUVECs. Synergistic effects of the three cytokines appear to be comparable to that of M1-CM. Representative images of at least three independent experiments are shown. **D:** A TNF- α antibody blocks TNF- α -induced *in vitro* EndMT in HUVECs in a dose-dependent manner. Representative images of at least three independent experiments are shown. Original magnification, $\times 100$ (**A**, **C**, and **D**). Ab, antibody; GRO, growth-regulated oncogene; IP, interferon γ -induced protein; NEG, negative; POS, positive.

SDS-PAGE and was blotted onto a polyvinylidene difluoride membrane (Bio-Rad). Primary antibodies were diluted in Tris-buffered saline containing 5% bovine serum albumin and incubated at 4°C overnight. After washing with Tris-buffered saline with 0.1% Tween-20, the membranes were incubated with IRDye 800CW Goat (polyclonal) Anti-Mouse IgG (H+L) or IRDye 800CW Goat (polyclonal) Anti-Rabbit IgG (H+L) secondary antibody (LI-COR, Lincoln, NE) for 1 hour. The signals were detected by Odyssey Clx Infrared Imager (LI-COR). The protein bands were quantified using ImageJ software version 1.45s (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>) to determine the integrated density of each band, and the ratio to α -tubulin was then calculated.

Adipogenesis and Oil Red O Staining

HemECs were treated with M1-CM, TNF- α , IL-1 β , IFN- γ , or a combination of the three cytokines for 48 hours. The treated cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1 μ mol/L insulin, 1 μ mol/L dexamethasone, and 500 μ mol/L 3-isobutyl-1-methylxanthine for 14 to 21 days to induce adipogenesis. The cells were then fixed with 10% formalin for 30 minutes, incubated with 60% isopropanol for 5 minutes, stained with Oil Red O solution (Sigma-Aldrich, St. Louis, MO) for 5 minutes, and counterstained with hematoxylin (Sigma-Aldrich) for 2 minutes. The stained cells were imaged with a Nikon Eclipse inverted microscope.

Results

M1- But Not M2-Polarized Macrophages Induce *in Vitro* EndMT

In vitro assays of EndMT have been previously reported,¹⁵ but their method was not well described, which has become a challenge for many investigators to pursue similar studies. Herein, a reproducible *in vitro* EndMT assay in both HUVECs and HDMECs has been established by treating them with either growth factor bone morphogenetic protein 4 or transforming growth factor- β 2, which is known to induce EndMT.¹⁵ The results demonstrate a quantifiable morphological change in HUVECs, where the endothelial cells lose their cobblestone morphology and took on a spindle shape or fibroblast-like appearance, a hallmark of EndMT (Supplemental Figure S1). We then tested whether M1- or M2-polarized macrophages could induce EndMT in HUVECs and HDMECs. We treated the endothelial cells for 48 hours with M1-CM or M2-CM. The results clearly demonstrate that M1- but not M2-polarized macrophages could induce EndMT in HUVECs (Figure 1A) and HDMECs (Supplemental Figure S2).

Identification of TNF- α , IL-1 β , and IFN- γ as Key Cytokines Inducing EndMT

To identify which cytokines produced by M1-polarized macrophages were able to induce EndMT, 80 cytokines on a human cytokine array were screened for potential candidates in the M1-CM (Figure 1B and Supplemental Figure S3). Among the up-regulated cytokines listed in Table 1 (note that some IFN- γ may come from the induction medium), we tested them individually at different concentrations using an *in vitro* EndMT assay. Although significantly elevated in

Table 1 Up-Regulated Cytokines in M1 Macrophage Conditioned Medium

Cytokine (array position)	Fold*
IL-6 (H2)	23.99
GRO (J1)	14.39
GRO- α (K1)	7.02
MCP-2 (F3)	6.29
MCP-1 (E3)	5.44
IL-8 (J2)	4.34
MIP-3 α (I7)	3.91
IFN- γ (D3)	2.86
Osteopontin (B8)	2.64
TNF- α (G4)	2.48
IP-10 (D7)	1.96
IL-1 β (C2)	1.73

*Fold changes are average values of three independent experiments.
GRO, growth-regulated oncogene; IFN, interferon; IP, interferon γ -induced protein; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; TNF, tumor necrosis factor.

M1-CM, growth-regulated oncogene- α , IL-6, IL-8, interferon γ -induced protein-10, MCP-1, MCP-2, macrophage inflammatory protein-3 α , and osteopontin had no effect on HUVECs in the *in vitro* EndMT assay (data not shown). However, TNF- α dramatically transformed HUVECs from a cobblestone to spindle shape at 48 hours (Figure 1C). To a lesser extent, IL-1 β and IFN- γ also promoted morphological changes in HUVECs. When TNF- α , IL-1 β , and IFN- γ were combined, the synergistic effect of these three cytokines in inducing *in vitro* EndMT in HUVECs was observed comparable to M1-CM (Figure 1C). A neutralizing antibody against TNF- α blocked TNF- α -induced EndMT in HUVECs in a dose-dependent manner, demonstrating the specificity of the effects by TNF- α (Figure 1D).

Decrease of Endothelial Markers and Increase of Mesenchymal Markers during EndMT

Western blot analysis showed that in M1-CM-treated HUVECs (Figure 2A) and HDMECs (Figure 2B), the amount of endothelial markers vascular endothelial (VE)-cadherin and CD31 decreased, whereas the amount of mesenchymal markers N-cadherin, α -smooth muscle actin, and vimentin increased. Similar results were obtained in TNF- α - or IL-1 β -treated HUVECs, where the amount of endothelial markers CD31, endoglin, and VE-cadherin decreased, whereas the amount of mesenchymal marker vimentin increased (Figure 2, C and D). Neutralizing TNF- α antibody blocked the decrease of endothelial marker endoglin and the concomitant increase of mesenchymal marker N-cadherin (Figure 2E).

Decrease of Endothelial Gene Expression on EndMT Induction

Gene expression profiling was performed on 84 genes, with emphasis on human endothelial cell biology. On M1-CM treatment of HUVECs, endothelial-specific markers, such as kinase insert domain receptor (down 2.7-fold), platelet endothelial cell adhesion molecule 1 (CD31; down 2.7-fold), nitric oxide synthase 3 (down 6.08-fold), and E-selectin (down 7.08-fold), decreased significantly, whereas leukocyte adhesion molecules intercellular adhesion molecule 1 (up 10.98-fold) and vascular cell adhesion molecule 1 (up 2.88-fold), proinflammatory cytokine IL-6 (up 25.35-fold), and MCP-1 [chemokine (C-C motif) ligand 2; up 3.09-fold] markedly up-regulated (Supplemental Figure S4). Notably, VEGF-A expression also increased dramatically (up 6.51-fold). These changes on gene expression level in HUVECs on EndMT induction indicate a substantial departure of the endothelial phenotype. Similar results were obtained in additional two sets of experiments (data not shown).

Figure 2 Western blot analysis of endothelial and mesenchymal markers on *in vitro* endothelial-to-mesenchymal transition (EndMT) induction. **A** and **B**: Western blot analysis (left panels) shows that in M1-conditioned medium (CM)-treated human umbilical vein endothelial cells (HUVECs; **A**) and human dermal microvascular endothelial cells (HDMECs; **B**), the amount of endothelial markers vascular endothelial (VE)-cadherin and CD31 decreases, whereas the amount of mesenchymal markers N-cadherin, α -smooth muscle actin (α -SMA), and vimentin increases concomitantly. α -Tubulin was used as a loading control. Protein bands were quantified using ImageJ software version 1.45s (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>) to determine integrated density of each band, and the ratio to α -tubulin is shown in the right panels. **C** and **D**: Western blot analysis (left panels) shows that in tumor necrosis factor (TNF)- α - (C) and IL-1 β - (D) treated HUVECs, the amount of endothelial markers CD31, endoglin, and VE-cadherin decreases, whereas the amount of mesenchymal marker vimentin increases concomitantly. Similarly, α -tubulin was used as a loading control, and the ratio to α -tubulin is shown in the right panels. **E**: A TNF- α antibody can block the decrease of endothelial marker endoglin and the concomitant increase of mesenchymal marker N-cadherin. Lane 1, untreated HUVEC control. Lane 2, 10 ng/mL TNF- α -treated HUVECs. Lane 3, 10 ng/mL TNF- α and 2 μ g/mL anti-TNF- α antibody treated HUVECs. Lane 4, 10 ng/mL TNF- α - and 3 μ g/mL anti-TNF- α antibody-treated HUVECs. Representative Western blot results of at least three independent experiments are shown. Ctrl, control; h, hour.

Perivascular Infiltration of M1 Macrophages and Endothelial Expression of Snail/Slug in Hemangioma Tissues

Immunohistochemical staining of surgically resected infantile hemangioma tissues revealed that a significant amount of M1, but not M2, macrophages were present in the perivascular

regions of blood vessels of all sizes. In the proliferating hemangioma tissues, numerous small blood vessels could be seen and were associated with strong human leukocyte antigen D related⁺ M1 macrophage staining (Figure 3A and Supplemental Figure S5A). There was modest CD163⁺ M2 macrophage staining throughout the proliferating hemangioma tissues (Figure 3B and Supplemental Figure S5B). In the

Figure 3 Immunohistochemical staining of infantile hemangioma tissues. **A:** Immunostaining of human leukocyte antigen D related (HLA-DR) to demonstrate the presence of M1 macrophages in a proliferating hemangioma. Numerous small blood vessels were visible and associated with strong perivascular staining of HLA-DR (brown), indicating a significant infiltration of M1 macrophages. **B:** Immunostaining of human CD163 to demonstrate the presence of M2 macrophages in a proliferating hemangioma. Interstitial staining of CD163 (brown) suggests a modest presence of M2 macrophages throughout the tissue. **C:** A negative control staining of the same proliferating hemangioma without primary antibodies was also performed. **D:** Immunostaining of human HLA-DR to demonstrate the presence of M1 macrophages in an involuting hemangioma. Large (**asterisk**) and medium (**filled and unfilled arrows**) blood vessels were visible and associated with strong perivascular staining of HLA-DR (brown), indicating a significant infiltration of M1 macrophages. **E:** Immunostaining of human CD163 for M2 macrophages in the same involuting hemangioma sample as in **D**. Large (**asterisk**) and medium (**filled and unfilled arrows**) blood vessels can be identified, and minimal staining of CD163 (brown) suggests negligible presence of M2 macrophages in the involuting hemangioma. **F:** A negative control staining of the involuting hemangioma without primary antibodies was also performed. **G:** Immunostaining of human Snail/Slug in sequential sections of the same involuting hemangioma sample as in **D**. Large (**asterisk**) and medium (**filled arrow**) blood vessels were visible and associated with luminal staining of Snail/Slug (brown), indicating their endothelial expression. **H:** Immunostaining of human peroxisome proliferator-activated receptor γ (PPAR γ) in sequential sections of the same involuting hemangioma sample as in **G**. Large (**asterisk**) and medium (**filled and unfilled arrows**) blood vessels can be identified, and sporadic PPAR γ staining (brown) is evident in a number of cells in the proximity. **I:** A negative control staining of this involuting hemangioma without primary antibodies was also performed. The tissues were counterstained with hematoxylin (blue). Representative images are shown from several experiments. Scale bars = 50 μ m. Original magnification, $\times 400$.

involuting hemangioma tissues, fewer but larger vessels were visible and associated with strong HLA-DR+M1 macrophage staining, suggesting a significant perivascular infiltration of M1 macrophages (Figure 3D and Supplemental Figure S5D). There was negligible CD163⁺ M2 macrophage staining in the same tissue (Figure 3E and Supplemental Figure S5E). In the sequential sections of the same involuting hemangioma sample, strong staining of the EndMT transcription factors Snail/Slug was evident at vascular endothelium of the same blood vessels (Figure 3G and Supplemental Figure S5G). A few cells in the same involuting hemangioma tissue were stained positive for the adipogenic transcription factor peroxisome proliferator-activated receptor γ , but were not associated with the blood vessels mentioned above (Figure 3H and

Supplemental Figure S5H). Negative control staining for each hemangioma was also performed (Figure 3, C, F, and I, and Supplemental Figure S5, C, F, and I). It is not clear at the present time the exact origin of these peroxisome proliferator-activated receptor γ ⁺ cells. They could be EndMT-transformed cells that have migrated away from the vasculature and are undergoing adipogenic differentiation, or they could be endogenous mesenchymal cells that have a committed adipogenic cell fate. Interestingly, there was no staining of the EndMT transcription factors Snail/Slug, or the adipogenic transcription factor peroxisome proliferator-activated receptor γ , in the proliferating hemangioma tissues (data not shown). Thus, patient samples provided *in vivo* evidence that during the involuting phase of hemangiomas, there is a significant M1

Figure 4 M1 macrophage and tumor necrosis factor (TNF)- α -induced endothelial-to-mesenchymal transition (EndMT) enables adipogenesis in patient hemangioma endothelial cells (HemECs). **A:** M1-conditioned medium (CM) and TNF- α , to a lesser extent also IL-1 β and interferon (IFN)- γ , can induce *in vitro* EndMT in HemECs after 48 hours of incubation. Representative images of at least three independent experiments are shown. **B:** Western blot analysis shows that M1-CM-treated HemECs have a reduced level of the endothelial marker vascular endothelial (VE)-cadherin and an increased level of the mesenchymal marker vimentin. Quantification of the protein bands was performed, and the ratio to β -actin control was calculated. Representative Western blot results of at least three independent experiments are shown. **C:** M1-CM and TNF- α , to a lesser extent also IL-1 β and IFN- γ , can promote adipogenesis in HemECs. Lipid droplets within adipocytes were stained with Oil Red O (red). Untreated HemECs under adipogenic conditions produce no adipocytes. Representative adipogenesis images of three independent experiments are shown. Original magnification, $\times 100$ (**A** and **C**). Ctrl, control; h, hour.

macrophage infiltration within the vasculature, which coincides with a strong endothelial expression of the critical EndMT transcription factors Snail/Slug.

EndMT Enables Adipogenesis of Infantile Hemangioma Endothelial Cells

We next tested if HemECs isolated from infant patients would respond to M1-CM or TNF- α treatment in a similar way as HUVECs and HDMECs. HemECs were isolated from five infantile hemangiomas at the proliferating or involuting stage (Supplemental Table S1). The isolated HemECs were plated in a dish coated with 1 $\mu\text{g}/\text{cm}^2$ fibronectin in EBM-2 complete endothelial cell growth medium (Supplemental Figure S6, A and B). These cells uniformly expressed endothelial marker CD31 but not mesenchymal marker neuron-glial antigen 2 or platelet-derived growth

factor receptor β (Supplemental Figure S6C). Both M1-CM and TNF- α induced significant morphological changes in HemECs at 24 hours (Supplemental Figure S7) and 48 hours (Figure 4A). In addition to M1-CM and TNF- α , IL-1 β and IFN- γ also induced noticeable morphological changes in HemECs toward a spindle shape after 48 hours (Figure 4A). The synergistic effects of TNF- α , IL-1 β , and IFN- γ in inducing *in vitro* EndMT were similar to that of the M1-CM in HemECs (Supplemental Figure S7 and Figure 4A). Western blot analysis showed that the isolated HemECs expressed endothelial-specific marker VE-cadherin, and M1-CM-treated HemECs had a reduced level of VE-cadherin and an increased level of the mesenchymal marker vimentin (Figure 4B). Most strikingly, M1-CM-treated HemECs, but not the untreated HemECs, readily differentiated into adipocytes after adipogenic induction (Figure 4C and Supplemental Figure S8). TNF- α , IL-1 β , and IFN- γ

individually or in combination also enabled adipogenesis of HemECs under adipogenic conditions (Figure 4C). The fact that only transformed HemECs were responsive to adipogenic induction, whereas untreated HemECs were unable to differentiate into adipocytes, suggests the absence of a meaningful number of endogenous mesenchymal stem cells in the HemEC isolation. Thus, our *in vitro* EndMT and adipogenesis of HemECs have, in part, recapitulated the natural history of hemangioma regression.

Discussion

Macrophages are believed to be extremely plastic so that transcriptional programs can be dynamically reshaped in response to microenvironmental signals. Because of this plasticity, macrophages are crucial determinants of disease development and resolution.²⁸ Previous studies showed that infiltrations of myeloid progenitor–derived monocytes, mast cells, and dendritic cells were found in the tissues of IHs, particularly macrophages that may be involved in the process of endothelial proliferation and involution.^{18,19,29} An earlier investigation by Wang et al¹⁸ showed that M2-polarized phenotype macrophages are present in proliferating hemangiomas, but no staining of M1 macrophages was performed in their study. Recently, Zhang et al¹⁹ reported that more M1 than M2 macrophages were found in both proliferating and involuting hemangiomas, and that M2 macrophages inhibited adipogenesis and increased proliferation of hemangioma stem cells. In the present study, we found that a significant amount of M1 macrophages were associated with blood vessels of all sizes in both proliferating and involuting hemangiomas. Modest and minimal presence of M2 macrophages was evident in proliferating and involuting hemangiomas, respectively. Furthermore, the critical EndMT transcription factors Snail/Slug were found highly expressed in the luminal endothelium concurrently with M1 macrophage infiltration in involuting hemangioma tissues. As such, patient tissue samples provided important evidence that our proposed mechanism of EndMT may be valid *in vivo*. Although previous studies suggest that during the initial stage of IH, M2 macrophages may promote cell proliferation and tumor development,^{18,19} our current findings indicate that M1 macrophages are present at both the proliferating and involuting stages and can be responsible for the later hemangioma regression via EndMT during the involuting stage.

In the current study, we have identified several cytokines, primarily TNF- α and to a lesser extent also IL-1 β and IFN- γ , produced by M1 macrophages, as potent inducers of EndMT in HemECs. The isolated HemECs expressed endothelial-specific CD31 and VE-cadherin, which endogenous mesenchymal stem cells do not possess. Only transformed HemECs were responsive to adipogenic induction, whereas untreated HemECs were unable to differentiate into adipocytes, suggesting the absence of a meaningful number of endogenous mesenchymal stem cells in the HemEC isolation. Interestingly, attempts at adipogenesis in transformed HUVECs and

HDMECs have not been successful, despite their partial mesenchymal characteristics and despite our efforts to optimize culture conditions. The fact that transformed HUVECs and HDMECs were unable to survive the adipogenic milieu, like HemECs, suggests that HemECs are in a different and more advanced state in the EndMT process. It is possible that HemECs retain remnants of their embryonic plasticity. Recently, Nieto et al³⁰ proposed that there is a spectrum of intermediate stages between epithelial and mesenchymal when the cells undergo epithelial-to-mesenchymal transition (EMT). During the EMT process, epithelial cells partially lose their epithelial markers and gain mesenchymal markers, but remain undetermined. They may either move forward to complete their transformation into mesenchymal cells when EMT pressure persists or reverse back to the epithelial phenotype when the pressure dismisses. Whether this EMT model applies to EndMT warrants further investigation. Currently, we are in the process of overexpressing EndMT transcription factors Snail and Slug in HUVECs and HDMECs to facilitate their transformation into mesenchymal cells and their further differentiation.

Earlier investigations in cancer biology suggest that TNF- α is an important inflammatory factor that acts as a master switch in establishing an intricate link between inflammation and cancer. A recent study showed that TNF- α is the major signal that induces NF- κ B–mediated Snail stabilization and EMT.³¹ The TNF- α /NF- κ B–stabilized Snail is mediated by the transcription induction of COP9 signalosome complex subunit 2, which inhibits the phosphorylation and ubiquitination of Snail by disrupting the binding of Snail to glycogen synthase kinase-3 β and β -transducin repeat containing protein, and results in the stabilization of Snail in a nonphosphorylated and nonubiquitinated functional state. This signaling mechanism may function in a similar manner during EndMT-dependent hemangioma regression, which warrants further study.

In summary, our findings suggested that M1 macrophage cytokine TNF- α , possibly also IL-1 β and IFN- γ , induces EndMT and promotes hemangioma regression. It is plausible to administer TNF- α and IL-1 β locally as a therapeutic option to accelerate IH regression. Finally, unlocking the secret of how hemangioma vessels regress may also lead to novel ways to destroy other pathological blood vessels, such as those found in cancer, by turning on the molecular switches to induce vessel regression.

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Supplemental Data

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