Endogenous Transmembrane TNF-Alpha Protects Against Premature Senescence in Endothelial Colony Forming Cells

Linden A. Green, Victor Njoku, Julie Mund, Jaime Case, Mervin Yoder, Michael P. Murphy, Matthias Clauss

Rationale: Transmembrane tumor necrosis factor-α (tmTNF-α) is the prime ligand for TNF receptor 2, which has been shown to mediate angiogenic and blood vessel repair activities in mice. We have previously reported that the angiogenic potential of highly proliferative endothelial colony-forming cells (ECFCs) can be explained by the absence of senescent cells, which in mature endothelial cells occupy >30% of the population, and that exposure to a chronic inflammatory environment induced premature, telomere-independent senescence in ECFCs.

Objective: The goal of this study was to determine the role of tmTNF-α in the proliferation of ECFCs.

Methods and Results: Here, we show that tmTNF-α expression on ECFCs selects for higher proliferative potential and when removed from the cell surface promotes ECFC senescence. Moreover, the induction of premature senescence by chronic inflammatory conditions is blocked by inhibition of tmTNF-α cleavage. Indeed, the mechanism of chronic inflammation-induced premature senescence involves an abrogation of tmTNF/TNF receptor 2 signaling. This process is mediated by activation of the tmTNF cleavage metalloprotease TNF-α–converting enzyme via p38 MAP kinase activation and its concurrent export to the cell surface by means of increased iRhom2 expression.

Conclusions: Thus, we conclude that tmTNF-α on the surface of highly proliferative ECFCs plays an important role in the regulation of their proliferative capacity. (Circ Res. 2016;118:1512-1524. DOI: 10.1161/CIRCRESAHA.116.308332.)

Key Words: apoptosis ■ endothelium ■ inflammation ■ metalloprotease ■ vasodilation

Despite improvements in treatment and prevention, cardiovasculare disease remains one of the leading causes of death, disability, and healthcare expenditure in the United States. Vascular changes such as the decline in passive compliance of arterial blood vessels and impaired endothelium-dependent vasodilation response are reliable markers for aging.1 Mathematical modeling based on known rates of endothelial turnover and proliferation rates have calculated that a reservoir of highly proliferative endothelial progenitor cells is required to maintain vascular function.2 Therefore, endothelial progenitor cells, including nonmyeloid highly proliferative endothelial progenitor cells, also described as endothelial colony–forming cells (ECFCs) or outgrowth endothelial cells, and bone marrow–derived circulating progenitor cells are thought to play an important role in maintenance of a viable endothelial layer in the vascular system.1-6 ECFCs define a novel hierarchy of endothelial cells and are nearly identical to mature ECs, with the exception of greatly enhanced proliferative potential.3,7 Deregulation of ECFCs and circulating progenitor cells have been shown to correlate with vascular diseases and diabetes mellitus.8-11 Therefore, further investigation into their biology is important to both general vascular biology and investigations into potential cell therapies.

Tumor necrosis factor-α (TNF-α) is predominately associated with an inflammatory response, vascular dysfunction, and eventually endothelial apoptosis. However, most of the studies on TNF-α have been performed with soluble TNF-α, which is cleaved from precursor transmembrane TNF-α (TNF-α) by the matrix metalloproteinase TNF-α–converting enzyme (TACE).12 Soluble TNF-α binds to both TNF receptor 1 (TNFR1) and TNFR2, although in endothelial cells it predominantly signals through TNFR1.13 In contrast to soluble TNF-α, tmTNF-α binds preferentially to TNFR2 in endothelial cells.14 TNFR1 and TNFR2 are different in their biology. TNFR1 contains a death signaling domain while TNFR2 does not; this leads to profound differences in terms of cell proliferation and survival in endothelial cells.15 In fact, studies comparing TNFR1 and TNFR2 knockout mice demonstrated that
TNFR1 is antiangiogenic, whereas the tmTNF-α-selective TNFR2 confers a survival signal, mediating angiogenic and blood vessel repair activities. In addition, transgenic mice that only express tmTNF develop fewer inflammatory atherosclerotic plaques, and transgenic uncleavable tmTNF-α expression in endothelial cells elicits angiogenesis in vivo.

The proliferative potential of ECs is limited by the processes of senescence. Endothelial injury in the absence of sufficient circulating progenitor cells may affect the progression of cardiovascular disease because increases in senescent vascular wall cells may lead to the inability of the endothelium to maintain a continuous functional monolayer. We have previously shown that ECFCs have extremely low levels of senescence but undergo stress-induced cellular senescence when exposed to chronic inflammatory conditions, a process which is independent of telomeric shortening-dependent replicative senescence.

We show here that ECFCs unexpectedly express high levels of surface tmTNF-α. Moreover, sorting freshly isolated cord blood using tmTNF-specific magnetic beads results in an increase in the number of ECFC colonies recovered, and these colonies contain a greater distribution of highly proliferative cells than unsorted ECFC colonies. We also show that when tmTNF/TNFR2 signaling is perturbed, ECFCs undergo premature senescence, resulting in loss of the highly proliferative phenotype. Furthermore, inflammation-induced premature senescence is blocked by inhibition of tmTNF-α cleavage. Our data show for the first time that tmTNF-α plays an important role in ECFC proliferative capacity.

### Methods

#### Reagents and Cells

Antibodies directed against tmTNF-α, TACE ectodomain, TNFR1, and TNFR2 were purchased from R&D Biotechnologies, and secondary AlexaFluor antibodies were from Invitrogen. p16, p65, and p-Erk antibodies were purchased from Abcam. TACE activity detection kit was from AnaSpec, and β-gal senescence detection kit was obtained from BioVision (Mountain View, CA). All other reagents were purchased from Sigma.

ECFC were isolated from fresh cord blood as previously described. Briefly, mononuclear cells (MNC) were isolated using standard Ficoll purification, then grown on collagen-coated tissue culture plates in endothelial growth media-2 MV media. Isolation of Endothelium From Arteries

Tibial arteries (diseased) and internal mammary arteries (IMA; healthy) were obtained as medical waste from amputations because of critical limb ischemia or cardiac bypass surgeries, respectively. Samples were obtained from males aged 55 to 68 years and matched for comorbidities. There was no significant difference in demographic variables or risk factors associated with critical limb ischemia or heart disease, including smoking, diabetes mellitus, hyperlipidemia, and hypertension. Vessels were cut longitudinally, rinsed with saline, and the endothelium was removed by passing gently over the surface with a cell scraper. Recovered cells were washed and then stained for CD31 and tmTNF for flow cytometry analysis.

#### Immunofluorescence Staining and Flow Cytometry

For detection of tmTNF in vessel walls, healthy IMA (healthy) or tibial arteries from patients with peripheral vascular disease (diseased) were obtained. Cells were gently removed from the vessel using a collagenase solution and then stained for tmTNF and CD31. Cells were fixed in 1% paraformaldehyde and blocked with 1% bovine serum albumin in phosphate-buffered saline, then stained with tmTNF-α, TACE, TNFR2, or CD31 and appropriate secondary antibodies. Fluorescence was detected using a FACSCalibur II (BecktonDickenson) and analyzed using CellQuest software. Percent positive signal was gated based on isotype control.

For detection of tmTNF+ cells in the CD34+/CD45− fraction, MNCs were stained using a 7 color assay with positive gating based on fluorescence minus one controls as described. Data were collected on a BD LSRII and analyzed using Flowjo software.

#### Staining for Senescence-Associated β-Galactosidase

To assess senescence in cord blood-derived ECFC, senescence-associated β-galactosidase activity was measured using a standard senescence detection kit (Biovision) according to the manufacturer’s instructions. Briefly, culture media was removed, and cells were washed once with phosphate-buffered saline then fixed with the fixation solution for 15 minutes at room temperature. After 2 additional washes with phosphate-buffered saline, the staining solution containing 1 mg/mL 5-bromo-4-chloro-3-indolyl-d galactoside was added to each well. Cells were incubated at 37°C overnight and then observed under a microscope for development of blue color. The percentage of blue cells versus total cells was measured by choosing 25 random microscopic fields.

#### Western Blot Analysis

For iRhom2 detection, ECFC were treated for ≤ 6 days with 10 ng/mL soluble TNF-α. Cells were lysed in cell lysis buffer (Cell Signaling) containing protease inhibitor cocktail (ThermoScientific, no. 88663) with protein concentrations determined by Pierce bichinchoninic acid assay. Proteins (20 μg) were separated by SDS-PAGE, followed by immunoblotting using appropriate antibodies. The chemiluminescent signals were quantified by densitometry using Adobe Photoshop.

#### Nuclear Factor κB and TNFR2 Silencing With Small-Interfering RNA

For nuclear factor κB (NFκB) gene knockdown by RNAi, we used Ambion Silencer Select Custom Designed siRNA against NFκB using a protocol as previously described. TNFR2 siRNA was purchased from Life Technologies. Briefly, cells were transfected with Genehammer transfection reagent (Agilent Technologies) and after incubation for 2 days at 37°C, total cell lysate was used to determine the knockdown of NFκB or TNFR2 by Western blotting. Cells were then used in proliferation assays.

#### Proliferation Assays

To determine proliferation potential of ECFC, single cells were plated on collagen-coated 96-well plates, 1 cell/well. After 14 days, incubation colony size was determined.

Population doubling time was determined by seeding cells in 12-well plates. The number of population doublings (PDs) occurring between passages was calculated according to the equation PD=log(CH/CS), where CH is the number of viable cells at harvest and CS, the number of cells seeded. The population doubling time was derived using the time interval between cell seeding and harvest divided by the number of PDs for that passage.

### Nonstandard Abbreviations and Acronyms

<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>ECFCs</td>
<td>endothelial colony–forming cells</td>
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<tr>
<td>NFκB</td>
<td>nuclear factor κB</td>
</tr>
<tr>
<td>IMA</td>
<td>internal mammary arteries</td>
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<tr>
<td>MNC</td>
<td>mononuclear cells</td>
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<tr>
<td>TACE</td>
<td>TNF-α-converting enzyme</td>
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<tr>
<td>tmTNF</td>
<td>transmembrane tumor necrosis factor</td>
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For carboxyfluorescein diacetate succinimidyl ester determination of proliferation, cells were labeled with 5 μmol/L carboxyfluorescein diacetate succinimidyl ester for 15 minutes at 37°C, then washed twice with phosphate-buffered saline and incubated for 3 days with the appropriate treatments, and the percentage of proliferating cells was determined by dilution of carboxyfluorescein diacetate succinimidyl ester signal as measured by flow cytometry.

Sprouting Assay
ECFC sprouting ability was assessed as previously described.17 Briefly, cells were seeded onto collagen-coated Cytodex beads and embedded into fibrinogen gel overlaid with growth media. After 5 days the number of sprouts longer than the diameter of the bead were determined.

TACE Activation
TACE activity in whole-cell lysates was determined with Sensolyte 520 TACE Activity Assay Kit (Anaspec, Fremont, CA) at 490 nm/520 nm according to the manufacturer’s instructions.

Statistical Analysis
Each experiment was performed in triplicate, with a minimum of 3 independent experiments. The differences between groups were compared using paired Student t test or ANOVA with Bonferroni corrections. Where applicable, mean±SEM of multiple measurements is reported as indicated.

Results

tmTNF Correlates With ECFC Proliferation
To assess the association of tmTNF with endothelial cell proliferative capacity, we first compared the surface expression levels of tmTNF-α in highly proliferative ECFCs with mature ECs. As shown in Figure 1, ECFCs isolated from cord blood express significant levels of tmTNF-α, whereas human microvascular endothelial cells or human coronary arterial endothelial cells express tmTNF-α.

Figure 1. Transmembrane tumor necrosis factor (tmTNF) is expressed on a subset of EC. A and B, endothelial colony–forming cells (ECFC), human coronary arterial endothelial cells (HCAEC), or human microvascular endothelial cells (HMVEC; passage 3) were stained for tmTNF and analyzed by fluorescence-activated cell sorting (FACS), with percentage tmTNF+ determined based on IgG controls. C, tmTNF was detected in HMVEC and ECFC cell lysates by Western blot. D, Freshly isolated cord blood MNCs were stained for CD45, CD34, and tmTNF, and the percentage of CD45+/CD34+ cells that were positive for tmTNF was determined (blue dots in blue box). E and F, Age-matched internal mammary arteries (healthy) were obtained from patients undergoing cardiac bypass surgery and tibial arteries (diseased) from patients with critical limb ischemia. Endothelial lining was gently removed from the vessel wall and costained for CD31 and tmTNF-α then analyzed by FACS. Data are representative of 3 to 4 independent experiments.
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cells (HCAECs) exhibit low amounts (Figure 1A and 1B). We further confirmed this expression by Western blot (Figure 1C). To confirm that tmTNF-α expression was not an artifact of tissue culture, we isolated MNCs from cord blood and stained for CD34, CD45, and tmTNF-α (Figure 1D). The majority of tmTNF staining (blue dots) was located in the CD34+/CD45− population, which is enriched for ECFCs and circulating angiogenic ECs. In fact, >25% of this population was positive for tmTNF-α. Next, we obtained sections of arteries from patients with peripheral vascular disease or IMA as age-matched healthy controls. IMA is a particularly good control in our case as we were able to control for age and other demographic variables, yet IMA is seemingly exempt from development of atherosclerosis. We detached the endothelial cells, stained for tmTNF-α with CD31 as an endothelial marker and determined CD31+tmTNF-α cells by fluorescence-activated cell sorting (FACS; Figure 1E). As shown in Figure 1F, CD31/tmTNF-α costaining in healthy arteries showed a small percentage (~6% to 8%) of endothelial cells expressing tmTNF-α. Interestingly, in diseased arteries (tibial artery from patients with critical limb ischemia) the number of CD31/tmTNF-α double-positive cells is lower than in healthy arteries, which correlates with current literature suggesting that ECFCs are decreased in diseased vessels. Together, these data suggest that a small subpopulation of endothelial cells expresses tmTNF-α, and that this subpopulation correlates with ECFCs.

Previous studies suggest that tmTNF-α is associated with increased angiogenesis. Because of the known role of ECFCs in angiogenesis, we examined the role of tmTNF-α in ECFC proliferation. We separated MNCs from cord blood into tmTNF-positive and tmTNF-negative fractions by magnetic bead separation. We determined colony formation, and there were significantly more highly proliferative colonies formed from the tmTNF+ fraction (Figure 2A) along with a...
Figure 3. Loss of transmembrane tumor necrosis factor (tmTNF)/TNF receptor 2 (TNFR2) axis results in less endothelial colony-forming cells (ECFC) proliferation. A, ECFCs were stained with antibodies for tmTNF and TNFR2 or IgG controls and expression analyzed by FACS (fluorescence-activated cell sorting). B, ECFCs were labeled with 5 μmol/L carboxyfluorescein diacetate succinimidyld ester (CFSE) for 10 minutes at room temperature and incubated for 4 days with nuclear factor κB (NFκB) inhibitor (100 nmol/L), TNF-α-converting enzyme (TACE), or αTNFR2 (500 ng/mL). The percentage of proliferating cells was determined by dilution of the CFSE signal. C, ECFCs were seeded into 12-well plates and cultured for 4 days with NFκB inhibitor (100 nmol/L), TACE, or αTNFR2 (500 ng/mL), after which cell number was determined and the population doubling time (pdt) was calculated. ECFCs were transfected with siRNA targeted to TNFR2 (D) or the p65 subunit of NFκB (E) and knockdown confirmed by Western blot. F, ECFCs were treated with TNFR2 siRNA, αTNFR2, or TACE and phosphorylation of TNFR2-specific Etk determined by Western blot. Percent proliferation (G) and pdt (H) were determined 2 days after transfection with p65 NFκB siRNA or TNFR2 siRNA. Data are representative of 3 to 4 independent experiments.
green number of cells per colony than in the tmTNF fraction. Conversely, when we added TACE, a metalloprotease that cleaves tmTNF, to the culture media there were significantly fewer colonies recovered (Figure 2B). Re-seeding single cells from similar-sized colonies showed a marked shift toward greater proliferative potential in cells from tmTNF+ colonies compared with those from tmTNF colonies (Figure 2C). Moreover, when we sorted ECFC colonies according to tmTNF intensity (Figure 2D) and seeded each fraction in single-cell assays to determine proliferative potential, the stronger tmTNF-expressing cells exhibited greater proliferative capacity (Figure 2E). Thus, our data show a strong association between tmTNF expression and ECFC proliferative potential.

To assess the role of tmTNF signaling in ECFC proliferation, we first confirmed that both tmTNF and its preferred receptor, TNFR2, are expressed on ECFCs (Figure 3A). Next, we determined ECFC proliferative capacity using carboxyfluorescein diacetate succinimidyl ester, a cell permeable dye which covalently couples to intracellular molecules and is diluted with each cell division, thus allowing quantitative assessment of cell proliferation (Figure 3B) and by determining population doubling times (Figure 3C) in the presence of TACE or an anti-TNFR2 neutralizing antibody. Because NFkB is known to be a downstream mediator of TNFR2 but not TNFR1, we also included an NFkB inhibitor. In all cases, interference with the tmTNF-TNFR2 signaling axis or perturbation of TNFR2 downstream signaling resulted in decreased proliferation, as well as significantly reduced phosphorylation of Etk, a TNFR2-specific kinase (Figure 3F). To confirm the specificity of this inhibition, we transfected ECFCs with siRNA directed against TNFR2 (Figure 3D) or the p65 subunit of NFkB (Figure 3E), both of which resulted in strongly reduced proliferation (Figure 3G and 3H).

Next, we determined the effect of perturbing tmTNF signaling on the angiogenic capacity of ECFCs. We coated Cytodex beads with ECFCs, embedded them in fibrin gel, and incubated in ECFC media ± TACE or α-converting enzyme (TACE, 0.2 μg/mL, B) or αTNFR2 (500 ng/mL) for 5 days. Sprouts longer than the diameter of the beads (dashed line, A) were counted. Representative pictures of untreated (left) and TACE (right) sprouts are shown in A. C. Sprouting was determined using ECFCs transfected with TNFR2 siRNA. Data are representative of 3 to 4 independent experiments.
using ECFCs transfected with either TNFR2 siRNA or scr control siRNA and found that knockdown of TNFR2 resulted in a similar decrease in angiogenic capacity (Figure 4D).

**Loss of tmTNF Results in Premature Senescence**

Our previous work has shown that ECFCs undergo premature senescence, resulting in a loss of proliferative potential, when exposed to chronic inflammatory conditions. Because of the observed relationship between tmTNF and ECFC proliferative capacity, we next determined the relationship between tmTNF and premature senescence, which is a major cause of decreased proliferation in endothelial cells. We incubated ECFCs with either recombinant TACE (Figure 5A) or an anti-TNFR2 neutralizing antibody (Figure 5B) for 6 days and determined development of premature senescence by staining for senescence-associated β-galactosidase (SA-β-gal), and senescent cells were quantified. C, Representative SA-β-gal staining. D, To confirm senescence with another marker of senescence, p16ink (p16) ECFCs were treated with TACE for 6 days, and p16 expression was determined by Western blot. Data are representative of 3 to 4 independent experiments.

To determine the role of tmTNF–TNFR2 signaling in our previously established inflammation-induced senescence model, we treated ECFCs with soluble TNF or lipopolysaccharide for 6 days to simulate a chronic inflammatory state and then determined senescence (Figure 6A) and tmTNF surface expression (Figure 6B). Interestingly, we found that senescence correlated with a downregulation of tmTNF. Next, we exposed ECFCs to chronic inflammation (soluble TNF or lipopolysaccharide) for 6 days with or without the TACE inhibitor TAPI (TNF-α processing inhibitor) and determined both tmTNF expression (Figure 6C) and senescence (Figure 6D). Importantly, we found that when tmTNF expression is maintained the development of inflammation-induced senescence is blocked even in chronic inflammatory conditions. An ELISA of the ECFC supernatant showed a dramatic increase in soluble TNF-α after 6 days of culture in chronic

![Figure 5](http://circres.ahajournals.org/)

**Figure 5.** Loss of transmembrane tumor necrosis factor (tmTNF)/TNF receptor 2 (TNFR2) axis induces premature senescence in endothelial colony-forming cells (ECFCs). ECFCs were treated with TNF-α–converting enzyme (TACE, 10 ng/mL, A) or α-TNFR2 (500 ng/mL, B) for 6 days, then stained for senescence-associated β-galactosidase (SA-β-gal), and senescent cells were quantified. C, Representative SA-β-gal staining. D, To confirm senescence with another marker of senescence, p16ink (p16) ECFCs were treated with TACE for 6 days, and p16 expression was determined by Western blot. Data are representative of 3 to 4 independent experiments.
Figure 6. Inflammation-induced premature senescence correlates with loss of transmembrane tumor necrosis factor (tmTNF).
Endothelial colony-forming cells (ECFCs) were treated with soluble TNF (10 ng/mL) or lipopolysaccharide (LPS; 100 ng/mL) for 6 days, then stained for senescence-associated β-galactosidase (SA-β-gal; A) or tmTNF expression was determined by fluorescence-activated (Continued)
inflammatory conditions, further demonstrating that these conditions resulted in a loss of tmTNF (Figure 6E). Moreover, ECFCs experience dramatic changes in TNFR expression during the course of chronic inflammation treatment, shifting from a predominantly TNFR2 profile to strongly expressing TNFR1 (Figure 6F). Together, these data describe a situation in which chronic inflammation dramatically alters TNF signaling, resulting in a shift from tmTNF–TNFR2 signaling to soluble TNF–TNFR1 signaling and concomitant development of premature senescence.

Mechanisms of Premature Senescence

Next, we addressed the mechanisms responsible for loss of tmTNF, and therefore premature ECFC senescence. Because of our previous observation that chronic inflammation–induced senescence required p38 MAP kinase activation,22 we examined the effect of p38 inhibition on the expression of tmTNF and found that blocking p38 completely prevented the loss of tmTNF and subsequent development of premature senescence (Figure 7A and 7B). We then determined surface expression of TACE by FACS during the course of 6-day treatment with soluble TNF (Figure 7C) and found that TACE expression increased until almost 100% of ECFCs expressed TACE. However, upregulated surface expression of TACE was not p38 dependent because inclusion of a p38 inhibitor did not prevent this. We determined TACE activity and found that soluble TNF treatment led to increased activation of TACE, corresponding to the same time-frame as TACE translocation to the plasma membrane. TACE activity, in contrast to surface expression, was p38 dependent (Figure 7D). Endothelial cells do not constitutively express TACE on their surface; instead, on stimulus the chaperone protein iRhom2 enables its release from the ER and allows transport to the surface.28 Therefore, we examined the expression of iRhom2 on addition of soluble TNF and found that expression increased dramatically during the course of the 6-day treatment (Figure 7E and 7F). Increased iRhom2 expression was not p38 dependent, further confirming that translocation of TACE to the cell surface does not require p38. Taken together, our data indicate that under chronic inflammatory conditions ECFCs lose surface tmTNF because of a combination of iRhom2-dependent TACE surface upregulation and p38-dependent increased TACE activation, resulting in the loss of a tmTNF/TNFR2-dependent proliferative signal and premature development of senescence.

Discussion

Our data address for the first time that tmTNF–α may have an important role in ECFC proliferative capacity. We show here that ECFCs express higher levels of tmTNF than mature endothelial cells. Moreover, the majority of tmTNF signal is found in populations of cord blood that have previously been shown to be enriched for ECFCs and circulating ECs, both of which exhibit high proliferative potential.23 Selection of tmTNF+ MNCs from cord blood enriches for ECFCs as shown by an almost 300-fold increase in ECFC colonies per million MNCs over tmTNF– MNCs. Not only were more colonies obtained but also individual cells from these colonies exhibited a greater proportion of highly proliferative ECFCs than cells from the tmTNF– fraction. Finally, we found a positive correlation between the intensity of tmTNF expression and proliferative potential of ECFCs. This is in agreement with previous studies showing that human umbilical vein endothelial cells not only contain a higher percentage of ECFCs than other vessels but also that the tmTNF expression in the population with human umbilical vein endothelial cells not only contain a higher percentage of ECFCs than other vessels but also that the tmTNF expression in the population with human umbilical vein endothelial cells is heterogenous and contains numerous highly positive cells, in contrast to mature endothelium which exhibits extremely low expression of tmTNF. Importantly, detection of tmTNF expression cells may provide a novel technique for selecting highly proliferative ECFCs for both experimental procedures and potential cell therapies.

Our finding that tmTNF comprises both a marker for and a functional maintenance mechanism of ECFCs is surprising and on the first glance seems to be counterintuitive. This is because the bulk of literature on TNF–α is on its soluble form, which is converted by cleavage from cell surface tmTNF and plays a major role in immunity and inflammation.29 Comparably, few publications analyzes its precursor, tmTNF. Among those are publications demonstrating that in immune and vascular endothelial cells tmTNF binds preferentially to TNFR2, whereas soluble TNF–α binds to both TNFR1 and TNFR2. However, soluble TNF–α binds weakly to TNFR2 and disassociates quickly resulting in minimal receptor activation. Conversely, tmTNF binds strongly to TNFR2 and disassociates at a much slower rate, resulting in a strong and sustained signal transduction.30 One of the functions of the tmTNF–TNFR2 axis in endothelial cells is to protect against atherosclerosis formation and to promote angiogenesis and repair as demonstrated by studies using uncleavable tmTNF transgenic and TNFR2 knockout mice.17,18,30 Another proposed function of tmTNF is regulating responsiveness to vascular endothelial growth factor for the induction of vascular permeability as previously shown by our group’s and others’ TNF knockout and in vitro studies.31,32 Interestingly, in a previous study we observed a pronounced upregulation of tmTNF in angiogenic tumor blood vessels,31 which is in line with studies demonstrating involvement of endothelial progenitors in tumor angiogenesis, a process also referred to as vasculogenesis.33–35 In contrast to the proposed maintenance function of tmTNF in ECFC in vascular repair and in angiogenesis, soluble TNF–α is predominately associated with inflammation, vascular dysfunction, and impaired repair,25 and according to our group and others acts overwhelmingly through TNFR1 in endothelial cells.33 Our data reported here show that removal of either tmTNF or TNFR2 causes ECFCs to lose their proliferative potential and develop premature senescence, which provides a mechanism for the observed role of TNFR2 in angiogenesis and vascular repair.

Our data demonstrate that NFκB is a key component of ECFC proliferation. This may be of relevance for...
anti-inflammatory therapies targeting NFκB as aggressive NFκB may reduce repair capacities of progenitor cells. Our findings are also in agreement with previous studies showing that NFκB is a regulator of cell proliferation and cell survival genes and indeed is upregulated or constitutively active in many cancers. Importantly, NFκB has been identified previously to be downstream of TNFR2 and is even directly activated by TNFR2. Although NFκB is also a

Figure 7. Tumor necrosis factor (TNF)-α–converting enzyme (TACE) expression is upregulated during chronic inflammatory conditions and required p38 activity and iRhom expression. Endothelial colony–forming cells (ECFC) were treated with soluble TNF (10 ng/mL) or lipopolysaccharide (LPS; 100 ng/mL) for 6 days±p38 inhibitor (10 nmol/L), then stained for tmTNF (A) or senescence–associated β-galactosidase (SA-β-gal, B). C, ECFC were treated with soluble TNF (10 ng/mL) for 6 days±p38 inhibitor. Every 2 days, cells were harvested and stained for surface TACE and analyzed by FACS (fluorescence-activated cell sorting). D, ECFC were treated with soluble TNF (10 ng/mL) for 6 days±p38 inhibitor, and TACE activity was determined using a fluorescence-based kit (Anaspec). E, ECFC were treated with soluble TNF (10 ng/mL) for 6 days±p38 inhibitor. Cell lysates were harvested every 2 days and probed for iRhom2 and GAPDH expression. F, Densitometry analysis of iRhom2, normalized to GAPDH. Data are representative of 3 to 4 independent experiments.
downstream of TNFR1, it seems to be antiapoptotic in this context because it is activated by the TNFR-associated protein with death domain/TNFR-associated factor 2 signaling, whereas the prototypical apoptotic caspase cascade associated with TNFR1 is downstream of TNFR-associated protein with death domain/Fas-associated protein with death domain activation. Interestingly, a recent report demonstrates that NFκB signaling is involved in regulating the epigenetic machinery required for the nuclear reprogramming that induces pluripotency in iPSCs, which may suggest a role for NFκB in the establishment of stemness.

Although we show here that TNFR2 signaling is necessary to prevent ECFCs from becoming senescent, further studies into the mechanism behind TNFR2-dependent prevention of senescence are needed and are ongoing in our laboratory. There are several candidate regulators of senescence in endothelial cells and various progenitor cells that could be regulated by tmTNF/TNFR2 signaling, including survivin which modulates cell cycle and proliferation in CD34+ cord blood cells and SIRT1, which has been shown to prevent the development of senescence in endothelial cells. In this context, our previous work specifically analyzing tmTNF/TNFR2 regulated genes will be useful. Importantly, we observed upregulation of several genes which promote angiogenesis such as connective tissue growth factor (Ctgf or CCN2) and endothelial plasminogen activator inhibitor (serpin E1), along with several cell signaling molecules, which promote proliferation, such as Akt1 and p65 NFκB.

Endothelial injury in the absence of sufficient circulating progenitor cells may affect the progression of vascular diseases because increases in senescent vascular wall cells may lead to the inability of the endothelium to maintain a continuous functional monolayer. ECFCs normally have low levels of senescence but undergo stress-induced cellular senescence when exposed to chronic inflammatory conditions, a process which is independent of telomeric shortening—dependent replicative senescence. We show here that this process is blocked by inhibition of tmTNF-α cleavage, and indeed the mechanism of chronic inflammation-induced premature senescence involves an abrogation of tmTNF/TNFR2 signaling. This is accomplished by the activation of the tmTNF cleavage metalloprotease TACE via p38 MAP kinase as has been shown in other situations, along with its concurrent export to the cell surface by means of increased iRhom2 expression (Figure 8). Further investigation of this pathway remains to be done, but may involve upregulation of iNOS, as this pathway has been linked to iRhom2 expression in hepatocytes.

Inflammation is involved at all stages of atherosclerosis, from the initial formation of the plaque to the time of plaque rupture resulting in acute coronary syndrome, which may explain the decreased numbers of ECFCs in diseased arteries. Furthermore, because of the prevalence of conditions resulting in chronic inflammation such as diabetes mellitus, autoimmune diseases, and the clearly documented association between aging and chronic low level systemic inflammation, investigation into the mechanism of premature ECFC senescence is extremely relevant and may enable the development of novel therapies for treating vascular disease. Protecting expression of tmTNF-α in vivo could retain a highly proliferative ECFC population even during chronic inflammatory conditions, enabling continued vascular repair, and more favorable prognosis in conditions, such as diabetes mellitus or cardiovascular disease.

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**Disclosures**

L. Green designed experiments, performed most research, analyzed data, and wrote the article. V. Njoku and J. Mund performed research. J. Case and M. Yoder provided valuable insights and reagents. M. Clauss and M.P. Murphy designed experiments.

**References**


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**Figure 8.** Schematic representation of transmembrane tumor necrosis factor (tmTNF)/TNF receptor 2 (TNFR2) regulation in endothelial colony-forming cells. Under normal conditions, tmTNF signaling through TNFR2 results in nuclear factor κB-dependent proliferation in the presence of growth factor receptors (GFRs)—mediated signaling (green arrows). On cultivation in chronic inflammatory conditions, signaling through TNFR1 results in an upregulation of iRhom2 and activation of p38 mitogen-activated protein kinase, which translocates TNF-α-converting enzyme (TACE) to the cell surface and activate it, respectively. TACE then cleaves tmTNF, resulting in a loss of tmTNF/TNFR2 signaling and subsequent development of senescence (red arrows).
The Role of tmtNF in ECFC Proliferation

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What Is Known?

• Expression of an uncleavable transmembrane tumor necrosis factor-α (tmTNF) in mice enhances angiogenesis.
• TNF receptor 1 (TNFR1) is antiangiogenic, whereas the tmTNF-α-selective TNFR2 confers a survival signal, mediating angiogenic and blood vessel repair activities.
• Endothelial colony–forming cells (ECFCs) become prematurely senescent on exposure to chronic inflammatory conditions.

What New Information Does This Article Contribute?

• Unlike mature endothelial cells, ECFCs stably express tmTNF, which corresponds to increased proliferative capacity.
• Loss of tmTNF expression or tmTNF–TNFR2 signaling results in premature senescence of ECFCs.
• The mechanism of chronic inflammation–induced senescence in ECFCs is decreased tmTNF expression because of upregulation of surface expression and activity of the TNF-cleavage enzyme TNF-α-converting enzyme.

ECFCs are thought to play an important role in the maintenance of healthy vasculature, and loss of ECFCs has been linked to development of atherosclerosis. Endothelial injury in the absence of sufficient ECFCs may affect the progression of vascular diseases because increases in senescent vascular wall cells may lead to the inability of the endothelium to maintain a functional monolayer. Because of the prevalence of conditions resulting in chronic inflammation, such as diabetes mellitus, autoimmune diseases, and the clearly documented association between aging and chronic local systemic inflammation, investigation into the mechanism of premature ECFC senescence is extremely relevant and may enable the development of novel therapies for treating vascular disease. We show here that ECFCs stably express tmTNF; that tmTNF–TNFR2 signaling is essential for ECFC proliferation; and that chronic inflammation results in a loss of tmTNF and TNFR2, triggering premature senescence. This work shows a novel aspect of tmTNF biology, pinpoints a key signal for ECFC proliferation, and identifies the molecular mechanisms of inflammation-induced senescence. Protecting expression of tmTNF in patients could retain a highly proliferative ECFC population even during chronic inflammatory conditions, enabling continued vascular repair and more favorable prognosis in conditions, such as diabetes mellitus or cardiovascular disease.
Endogenous Transmembrane TNF-Alpha Protects Against Premature Senescence in Endothelial Colony Forming Cells
Linden A. Green, Victor Njoku, Julie Mund, Jaime Case, Mervin Yoder, Michael P. Murphy and Matthias Clauss

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