

Mobilization of Endothelial Progenitors by Recurrent Bacteremias with a Periodontal Pathogen

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Abstract

Background: Periodontal infections are independent risk factors for atherosclerosis. However, the exact mechanisms underlying this link are yet unclear. Here, we evaluate the *in vivo* effects of bacteremia with a periodontal pathogen on endothelial progenitors, bone marrow-derived cells capable of endothelial regeneration, and delineate the critical pathways for these effects.

Methods: 12-week old C57bl6 wildtype or toll-like receptor (TLR)-2 deficient mice were repeatedly intravenously challenged with 10⁹ live *P. gingivalis* 381 or vehicle. Numbers of Sca1+/flk1+ progenitors, circulating angiogenic cells, CFU-Hill, and late-outgrowth EPC were measured by FACS/culture. Endothelial function was assessed using isolated organ baths, reendothelization was measured in a carotid injury model. RANKL/osteoprotegerin levels were assessed by ELISA/qPCR.

Results: In wildtype mice challenged with intravenous *P. gingivalis*, numbers of Sca1+/flk1+ progenitors, CAC, CFU-Hill, and late-outgrowth EPC were strongly increased in peripheral circulation and spleen, whereas Sca1+/flk1+ progenitor numbers in bone marrow decreased. Circulating EPCs were functional, as indicated by improved endothelial function and improved reendothelization in infected mice. The osteoprotegerin/RANKL ratio was increased after *P. gingivalis* challenge in the bone marrow niche of wildtype mice and late-outgrowth EPC *in vitro*. Conversely, in mice deficient in TLR2, no increase in progenitor mobilization or osteoprotegerin/RANKL ratio was detected.

Conclusion: Recurrent transient bacteremias, a feature of periodontitis, increase peripheral EPC counts and decrease EPC pools in the bone marrow, thereby possibly reducing overall endothelial regeneration capacity, conceivably explaining pro-atherogenic properties of periodontal infections. These effects are seemingly mediated by toll-like receptor (TLR)-2.

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Introduction

Cardiovascular diseases are the leading cause of mortality in the western world. Their underlying pathological condition is atherosclerosis [1]. Risk factors for the development or acceleration of atherosclerosis include established predictors identified in the Framingham study [2], but also chronic infections, most notably periodontitis, a highly prevalent chronic inflammatory condition of the tooth-supporting tissues caused by specific periodontal pathogens in a susceptible host [3,4].

There is ample evidence from epidemiological studies suggesting that periodontal infections are an independent risk factor for atherosclerosis [5–7]. Despite the overall modest association, the consistency of data across different study populations, exposures and outcome variables suggests that these findings are not spurious or attributable to confounders.

In the past, several potential mechanisms for a periodontal-cardiovascular link have been proposed (for review, see [6,8]). These are conceptually based on the fact that the sizable ulcerated epithelium of the periodontal pockets [9] mediates persistent,

recurrent bacteremia with pathogens (for review, see [10]). The mechanisms include direct effects of periodontal pathogens or their components on vascular cells, auto-immune reactions, and oxidative stress. A causative link of infections with activation of the innate immune system, and increased atherogenesis is strongly suggested by recent studies demonstrating the necessity of pattern-recognizing receptors, e.g. the Toll-like receptors, for atherosclerotic lesion formation [11–16]. Specifically, it was shown that TLR2, the receptor recognizing a principal pathogen in human periodontitis, the gram-negative anaerobe *Porphyromonas gingivalis*, is critical for both the effects of the pathogen in the oral cavity [17,18] and in atherogenesis [19,20]. Interaction of *P. gingivalis* fimbriae with TLR2 is necessary to mediate invasion of the pathogen into endothelial cells, where it was shown to persist and replicate [21], and eventually induce endothelial dysfunction [22].

A critical first step in atherogenesis is the activation of vascular endothelial cells and the development of endothelial dysfunction with subsequent apoptosis of endothelial cells [23]. Cross-sectional studies have demonstrated increased endothelial dysfunction in otherwise healthy patients with periodontitis [24,25]. Periodontal

therapy could improve endothelial dysfunction [26]. However, the pathways underlying these effects are not yet fully understood.

Vascular health is maintained by healthy endothelium that can in part be regenerated by circulating endothelial-regenerating cells (e.g. Sca1+/flk1+ progenitors) [27]. Numbers of these regenerating cells are associated with endothelial function [28] and cardiovascular outcomes [29]. Impaired endothelial regeneration after endothelial cell damage – as known to be elicited by periodontal pathogens [30] – is closely connected to the development of atherosclerotic lesions [31].

However, no mechanistic studies evaluating the effect of periodontal infections on endothelial regeneration have been conducted so far. The data available to date are limited to a single, cross-sectional study showing increased endothelial progenitor cell (EPC) counts in otherwise healthy periodontal patients when compared to controls without periodontitis [32], and an intervention study in the same population that showed a decrease of CD34 positive cells by periodontal therapy [33]. Importantly, these studies were neither designed nor suitable to prove causality, or to investigate the underlying mechanisms of a potential association.

Therefore, in this study we evaluated the effects of infection with the periodontal model pathogen *P. gingivalis* on numbers of different endothelial progenitor cell populations in an *in vivo* model. We aimed to evaluate whether progenitor cell numbers were in fact higher in infected groups than controls, and to determine the biological significance of this finding.

Materials and Methods

Ethics Statement

All animal experiments were performed in accordance with institutional guidelines and the German animal protection law. The study protocol was approved by the appropriate authority (North-Rhine Westphalia State Environment Agency (*Landesamt für Natur, Umwelt und Verbraucherschutz/LANUV*), Recklinghausen, Germany, permit no #8.87-50.10.35.08.013).

Mice

Female, 12-week-old C57bl6 mice (Charles River, Sulzfeld, Germany) or age- and gender-matched TLR2^{-/-} mice (a kind gift of Dr. Sabine Specht, Bonn) were used for this study. The animals were maintained in a 22°C room with a 12-hour light/dark cycle and received chow and water *ad libitum*. The mice were killed at day 12, and blood and tissue samples were recovered immediately. To account for potential gender-specific effects in the wildtype mice, we also tested several age-matched male mice, with very similar results than in female mice (data not shown).

Bacteria

Porphyromonas gingivalis strain 381 (a kind gift of Dr. Evie Lalla, New York, NY, USA) was cultured under anaerobic conditions, as described previously [34].

In vivo Infection (Bacteremia Model)

2×10^9 live *P. gingivalis* resuspended in 200 μ l saline or saline alone (control) were injected into the tail veins on days 0, 2, 4, 6, 8, and 10.

Flow Cytometry

Peripheral blood was collected from the inferior vena cava at sacrifice. Bone marrow cells were flushed from both femurs using sterile saline.

After red blood cell lysis (BD Pharm Lyse, BD, Heidelberg, Germany) and blocking of the Fc γ II/III receptors (CD16/CD32, BD), the viable lymphocyte population was assessed for the expression of Sca-1-FITC (BD Pharmingen) and vascular endothelial growth factor receptor-2 coupled to PE (VEGFR2/flk-1, BD). Isotype-identical antibodies served as controls (BD). Analyses were run on a BD FACScalibur flow cytometer (BD), data were analyzed using FloJo (Treestar, Ashland, OR, USA).

Data were presented as %gated, relative to levels found in control mice.

Preparation of Spleen-derived Mononuclear Cells

Spleens were minced and gently homogenized. The resulting single cell suspension was fractionated using Ficoll (Percoll, Biochrom, Berlin, Germany) gradient centrifugation.

Preparation of Circulating Angiogenic Cells (CAC)

1×10^6 spleen-derived mononuclear cells were seeded into fibronectin-coated (Sigma-Aldrich, St. Louis, MO, USA) 24-well plates in 500 μ l of endothelial basal medium 2 (Lonza) with supplements. After 7 days in culture, cells were assayed for Dil-Ac-LDL uptake and lectin staining (UEA-1, Sigma-Aldrich). Per well, 5 high-power fields were analyzed by a blinded observer (author MH) for Dil-Ac-LDL+/lectin+ staining.

Preparation of CFU–Hill

CFU-Hill were cultured from splenic MNCs, as described [35,36]. In brief, 1×10^7 cells were seeded on 6 cm dishes in complete EBM-2 medium, after 48 hours, 1×10^6 non-adherent cells were collected and replated on fibronectin-coated 24-well plates for 7 days. CFU-Hill were counted by a blinded observer (author MH) using a phase-contrast microscope using a mosaic of 10×10 high power fields.

Preparation of Late-outgrowth EPC

Late-outgrowth EPC were grown from splenic MNCs, as described [37,38]. In brief, 1×10^7 cells were seeded in complete EBM-2 medium on a 6 cm dish, non-adherent cells were removed after 48 hours, and cells were cultured for a total of 21 days. Colonies were identified by visual inspection using phase-contrast microscopy. Results of the dichotomous decision (presence of differentiated colonies) by a blinded observer (author MH) were statistically tested using Fishers exact test.

In vitro Infection

1×10^5 phenotyped [39] late-outgrowth EPC were seeded in 6 cm dishes in EBM-2 medium with growth supplements, but without antibiotics. 5×10^6 , 1×10^7 , or 5×10^7 live *P. gingivalis*381 were added, corresponding to a multiplicity-of-infection (MOI) of 50, 100, or 500 bacteria per EPC. The infection was maintained for 24 hours.

ELISA

Bone marrow supernatants were produced by flushing both femurs with chilled saline and subsequent removal of bone marrow cells by centrifugation. Levels of RANKL and osteoprotegerin protein were assessed using commercially available ELISAs (#DY462 and #DY805, R&D Systems, Abingdon, UK) and normalized for total protein content, as assessed by Bradford assay.

Quantitative RT-PCR

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) and subsequent spin-column purification (RNeasy

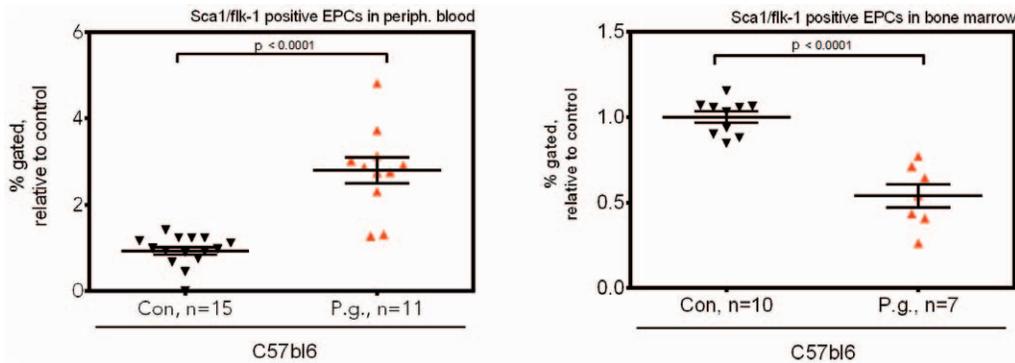


Figure 1. Bacteremia with *P. gingivalis* leads to mobilization of Sca1/flk1 progenitors from the bone marrow into peripheral blood of wildtype mice. Sca-1/flk1 progenitor cells (EPC) were quantified by flow cytometry in peripheral blood (left panel) or bone marrow (right panel) of C56bl6 wildtype mice after intravenous application of the periodontal pathogen *P. gingivalis* or saline (control). Data are presented as mean percentages of gated cells \pm SEM normalized to control, statistical testing was performed using unpaired t-tests. doi:10.1371/journal.pone.0054860.g001

Mini Kit, Qiagen, Hilden, Germany), reverse-transcribed using Superscript III (Invitrogen) and analyzed using Taqman chemistry (Applied Biosystems, Foster City, CA, USA) using the probes Mm00441906_m1 for RANKL, Mm01205928_m1 for osteoprotegerin, and Mm99999915_g1 for GAPDH on a ABI 7500 Fast cyclor (Applied Biosystems). Data were analyzed using the $\Delta\Delta C_t$ method.

Aortic Ring Preparation and ex vivo Measurement of Endothelial Function

To assess endothelial function ex vivo, vasoconstriction and endothelium-dependent and -independent vasodilation were measured in isolated organ baths, as described previously [40]. In brief, the thoracic aorta was carefully dissected, adventitial tissue was removed, and 3 mm segments were prepared for investigation (3–4 replicates/mouse) in organ baths filled with oxygenated modified Tyrode buffer at 37°C. After administration of a resting tension of 10 mN, drugs were added in increasing concentrations and cumulative concentration response curves were recorded. We used 20 and 40 mM KCl and 1 nM–10 μ M phenylephrine to induce aortic ring contraction. 10 nM–100 μ M carbachol were then added to assess endothelium-dependent vasodilation after precontraction with phenylephrine. Finally, endothelium-independent vasodilation was assessed by application of 1 nM–10 μ M nitroglycerine. At each drug concentration, a plateau phase was observed before addition of a higher dose of the drug. Before addition of the next substance, drugs were washed out.

Reendothelization after Defined Carotid Artery Injury

Reendothelization was assessed in an electric injury model of the common carotid artery, as described previously [41]. In brief, on day 7 of the experimental protocol, a defined area of 4 mm length of the distal common carotid artery was denuded of endothelium using a bipolar microregulator.

The extent of endothelial repair was measured after 5 days by staining of the denuded areas by intravenous injection of 50 μ l Evan's blue dye (5% in saline). The yet unrepaired area – stained blue by the dye – was quantified by a blinded observer (author MH) using a calibrated stereomicroscope and expressed as percentage of the total injured area. Data are presented relative to the mean unrepaired area in sham-infected control animals.

Statistical Analysis

All data were analyzed using GraphPad Prism V (GraphPad, San Diego, CA, USA). Normality of data was assessed using d'Agostino K^2 tests, where appropriate. For normally distributed data, in comparisons of two groups, two-tailed, unpaired or paired (analysis of circulating angiogenic cells) t-tests were used, for comparisons of three or more groups, one-way ANOVA and *post-hoc* Tukey tests or two-way ANOVA with *post-hoc* Bonferroni tests were utilized. Late-outgrowth EPC experiments yielding dichotomous decisions were analyzed by Fisher's exact test. All data are presented as means \pm SEM. A p-value of <0.05 was considered significant.

Results

In C57bl6 wildtype animals, recurrent bacteremia with *P. gingivalis* did not impair clinical status of the animals, but led to strongly increased peripheral count of Sca1+/flk1+ progenitors (control vs. test: 1.0 ± 0.09 vs. 2.80 ± 0.30 , $p < 0.0001$) with concomitantly decreased counts in bone marrow (control vs. test: 1.0 ± 0.03 vs. 0.54 ± 0.06 , $p < 0.0001$; figure 1). This increase in progenitor counts was dependent on the numbers of periodontal pathogens injected (data not shown).

Spleen-derived early and late-outgrowth endothelial progenitor population counts were also increased by *P. gingivalis* bacteremia. Specifically, numbers of Dil-Ac-LDL+/lectin+ circulating angiogenic cells (CAC) were increased more than 5-fold in the infected group (control vs. test: 6.2 ± 0.55 vs. 38.66 ± 2.28 double-positive cells/HPF, $p < 0.0001$; figure 2), numbers of CFU-Hill were increased (control vs. test: 1.00 ± 0.18 vs. 2.71 ± 0.19 CFU/HPF (normalized to control), $p = 0.0002$; figure 3a), and almost all infected mice yielded differentiated late-outgrowth EPC, whilst those in the control group showed no late-outgrowth EPC development after 21 days in culture (figure 3b).

To assess the biological functional relevance of the observed strong increases in counts of the different endothelial progenitor populations, we determined the impact of a recurrent bacteremia and subsequent progenitor mobilization on endothelial function and reendothelization.

Endothelium-dependent vasodilation, a measure of endothelial function, was significantly improved in the bacteremia group (figure 4, left panel). Endothelium-independent vasodilation on the other hand was similar in the bacteremia and the control group (figure 4, right panel).

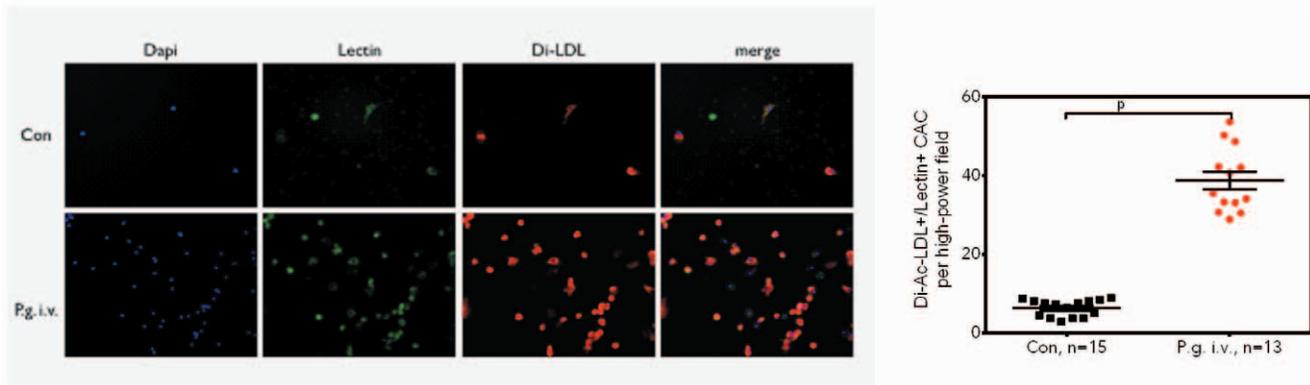


Figure 2. Bacteremia with *P. gingivalis* leads to increased numbers of circulating angiogenic cells (CACs) in wildtype mice. Strongly increased counts of DiI-Ac-LDL+/Lectin+ spleen-derived CACs in *P. gingivalis* infected mice. Data are presented as means of five high-power fields/mouse \pm SEM, statistical testing was performed using paired t-tests to account for day-to-day staining variability. doi:10.1371/journal.pone.0054860.g002

Similarly, reendothelization after electric/thermic denudation of the endothelium of the common carotid artery was improved in the bacteremia group characterized by high numbers of peripheral progenitors (figure 5).

Lastly, we assessed how critical mediators of bone marrow cell mobilization were affected by *P. gingivalis* infection and the subsequent strong mobilization of progenitors from bone marrow into peripheral blood.

In the bone marrow niche, we found an increased osteoprotegerin/RANKL protein ratio in *P. gingivalis* infected mice (control vs. test: 0.27 ± 0.03 vs. 0.51 ± 0.05 , $p = 0.0035$; figure 6a). In line with this observation, an increased osteoprotegerin/RANKL mRNA ratio ($+339 \pm 0.18\%$ at MOI 100, $p < 0.0001$) was observed in *P. gingivalis* infected late-outgrowth EPC *in vitro* (figure 6b).

Finally, we evaluated the molecular pathway underlying the demonstrated mobilization and concomitant depletion of functional endothelial progenitors by recurrent bacteremia with the periodontal pathogen *P. gingivalis*. Unlike most gram-negative species, *P. gingivalis* was described to primarily utilize toll-like receptor (TLR)-2, rather than TLR4, to invade into host cells and exert its primary biological effects. Indeed, in mice deficient in TLR2, we could not observe the pronounced mobilization found in C56bl6 wildtype mice (figures 7&8). In line with these observations, the aforementioned increase in osteoprotegerin/RANKL protein ratios in the bone marrow niche of *P. gingivalis* infected mice was not found in the absence of TLR2 (figure 9). These data indicate that the observed biological effects on

progenitor mobilization are in fact primarily mediated by interactions of *P. gingivalis* with the TLR2 receptor.

Discussion

Here, we show that recurrent bacteremias with the periodontal model pathogen *P. gingivalis* induce Sca1+/flk1+ endothelial progenitor mobilization from the bone marrow into the peripheral circulation *in vivo* and result in higher levels of both early and late EPC, distinct progenitor cell subtypes with dissimilar properties [42]. These data corroborate reports from a cross-sectional study showing higher peripheral EPC counts in otherwise healthy periodontal patients [32]. Increased levels of endothelial progenitors have also been associated with other inflammatory conditions exhibiting similarities in pathobiological mechanisms with periodontal infections, such as rheumatoid arthritis [43], or in situations of acute tissue damage, such as myocardial infarction [44–47], percutaneous coronary intervention [48], or excessive exercise [49].

The infection-induced mobilization was dependent on the presence of toll-like receptor 2 (TLR2), the receptor primarily mediating the invasion of *P. gingivalis* into host cells [11], seemingly a prerequisite for exerting its biological effects. These data are in line with prior observations of reduced periodontal infection-mediated atherosclerosis in a mouse model deficient in TLR2 [19,20] and point to a potential role for TLR2 as a therapeutic target in host modulation [50]. Still, it needs to be noted that since

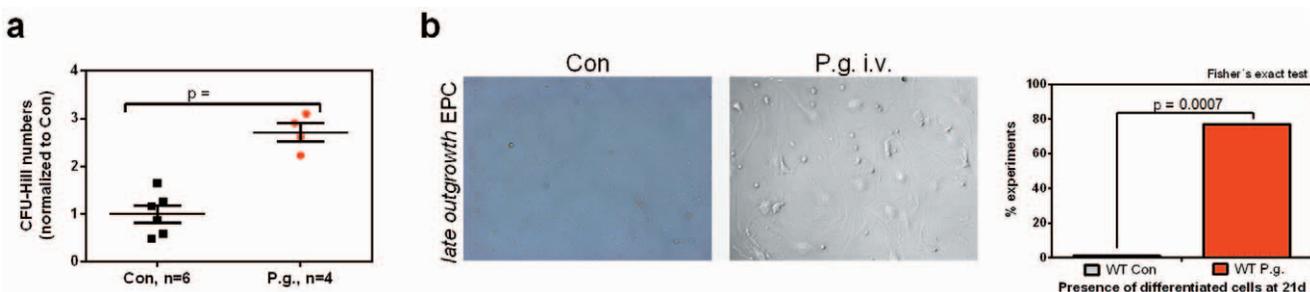


Figure 3. Bacteremia with *P. gingivalis* leads to increased numbers of CFU-Hill and late-outgrowth EPC in wildtype mice. (a) Increased numbers of CFU-Hill, (b) high proportion of presence of differentiated late-outgrowth EPC in *P. gingivalis* infected mice. Data are presented as mean colony counts \pm SEM relative to controls (CFU-Hill) or as numbers of experiments yielding differentiated colonies (late EPC). Data were analyzed using unpaired t-tests (CFU-Hill) or Fishers exact test (late EPC). doi:10.1371/journal.pone.0054860.g003

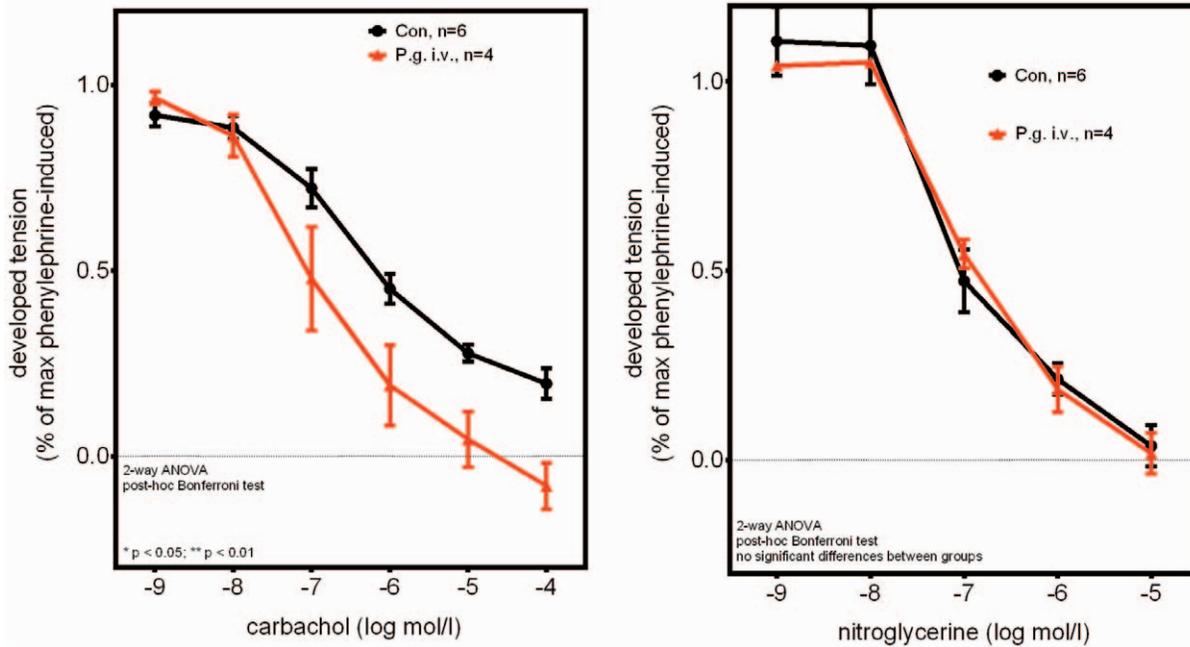


Figure 4. Improved endothelium-dependent vasodilation in *P. gingivalis* infected mice (left panel). No difference between groups in endothelium-independent vasodilation (right panel). Data are presented as means of 3–4 aortic ring preparations/mouse ± SEM. Statistical testing was performed using 2-way ANOVA and post-hoc Bonferroni tests. doi:10.1371/journal.pone.0054860.g004

TLRs play a vital role in host defense, there exist several challenges that future TLR-targeting drugs need to overcome [51]. Importantly, our data suggesting a specific reaction towards the periodontal pathogen also corroborate reports showing that unspecific acute systemic inflammation *per se* does not lead to increased mobilization of endothelial progenitors [52].

The beneficial effects of acute bacteremia with the periodontal pathogen on endothelial function and reendothelization point to a functional relevance of mobilized endothelial progenitors. Our group has previously demonstrated that increased levels of endothelial progenitors by systemic transfusion in fact significantly improve endothelial function [40].

In addition to the hitherto demonstrated beneficial effect of regenerating progenitors, the observed significantly improved endothelial-dependent vasodilation in infected mice could also be attributed in part to the excessive vasodilatation in sepsis [53]. However, it must be noted (i) that our treatment regimen did not at all induce a septic shock, a prerequisite for abundant NO production and vasodilatory state, and (ii) that we also demonstrated an improved re-endothelization in the infected group, an outcome independent of endothelial vasodilatory state.

Thus, the observed enhanced mobilization of endothelial progenitors leads to a short-term functional improvement, likely acting as a counter-measure against pathogen-mediated endothelial damage. However, since during the course of periodontal

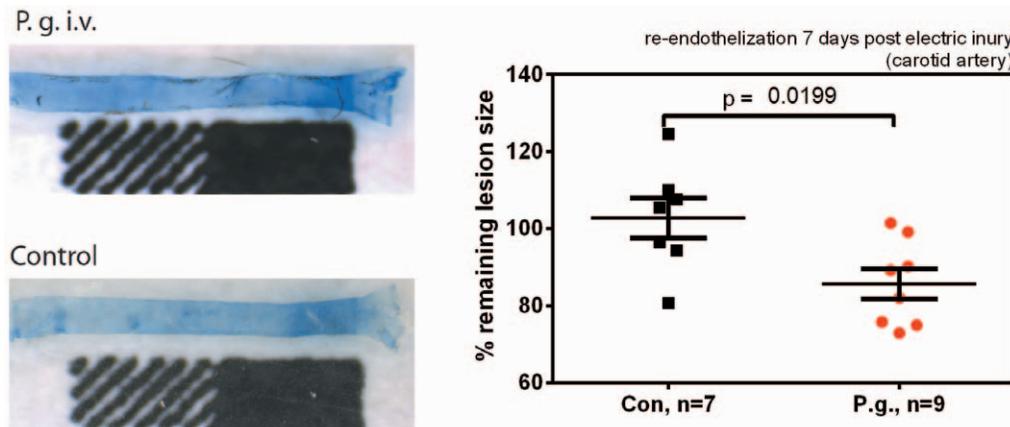


Figure 5. Improved reendothelization in *P. gingivalis* infected mice 5 days after electric injury of the common carotid artery. Data are expressed as proportions of non-regenerated to regenerated areas relative to saline-treated controls. Micrographs show representative results, dark blue areas mark denuded areas. Data are presented as means ± SEM, statistical testing was performed using an unpaired t-test. doi:10.1371/journal.pone.0054860.g005

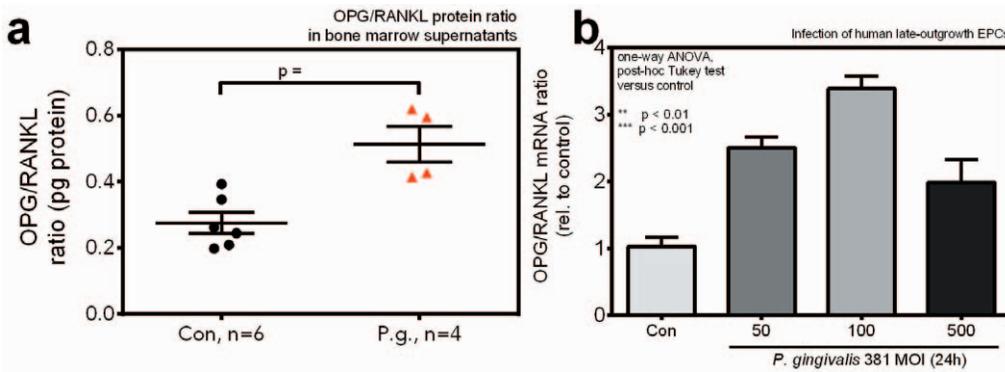


Figure 6. Increased osteoprotegerin/RANKL protein ratio within the bone marrow niche in *P. gingivalis* infected mice (a), increased osteoprotegerin/RANKL mRNA ratio in *P. gingivalis* infected late-outgrowth EPC *in vitro* (b). Osteoprotegerin induces bone marrow cell retention and expansion, RANKL triggers mobilization. Increased osteoprotegerin/RANKL ratios may counteract a strong mobilization and depletion of bone marrow EPC. Data are presented as means \pm SEM, statistical testing was performed using an unpaired t-test (protein data) and ANOVA and post-hoc Tukey test (mRNA data). MOI, multiplicity-of-infection (ratio of prokaryotic/eukaryotic cells). doi:10.1371/journal.pone.0054860.g006

infections, bacteremia with periodontal pathogens is a frequent event [10], recurrent mobilization of bone marrow-derived cells may conceivably deplete bone marrow pools over time, thereby reducing the overall regenerative potential and facilitating atherosclerosis. Still, to adequately test for the long-term consequences of our observations in the present, acute bacteremia model, appropriate atherosclerosis models need to be employed [54].

To further assess the mobilization of progenitors, we examined in mice infected with *P. gingivalis* the levels of RANKL, a cytokine triggering mobilization [55] and osteoprotegerin, a decoy receptor for RANKL that is known to mediate bone marrow cell expansion and retention that was recently established as a biomarker for cardiovascular prognosis and mortality [56,57]. Importantly, we found increased osteoprotegerin/RANKL ratios in the bone marrow niche of infected wildtype mice. In this work, we have defined this niche as a ‘cellular and molecular microenvironment that regulates (...) the engagement of specific programs in response to stress’, as proposed by Ehninger and Trumpp [58]. Contrastingly, in TLR2-deficient mice showing no mobilization, the ratio was unchanged. In this context, it needs to be mentioned that the number of control animals in this comparison was rather low (n = 3) due to the sparse availability of the knockout animals. This finding is unexpected, since an increased ratio is indicative for increased retention and decreased mobilization of bone marrow

cells [55]. Here, it could possibly act as a counter measure against the conceivable bone marrow cell depletion by recurrent bacteremias. In fact, our group has previously shown that increased systemic progenitor levels in response to physical training are accompanied by similarly increased, and not diminished, levels in the bone marrow [59].

In line with these observations *in vivo*, we could demonstrate *in vitro* that infection of human late-outgrowth EPC with *P. gingivalis* also increases the osteoprotegerin/RANKL ratio. Interestingly, the increase was less pronounced at multiplicities of infection in the range associated with apoptotic changes in endothelial cells [30], an observation in line with reports of decreased osteoprotegerin production in endothelial cells with activated p53 [60]. Increased systemic osteoprotegerin/RANKL ratios after intravascular application of *P. gingivalis* LPS have been reported before [61].

Interestingly, this is in contrast to the well-established osteoclastic phenotype dominated by increased RANKL over osteoprotegerin levels [62] that is found in periodontal lesions that are primarily characterized by bone resorption [63]. We attribute this discrepancy of effects triggered by *P. gingivalis* to the apparent strong differences in cellular composition of the different tissue compartments. The pathogen in the systemic circulation is most likely to encounter endothelial cells and progenitors or vascular smooth muscle cells, potent producers of osteoprotegerin when

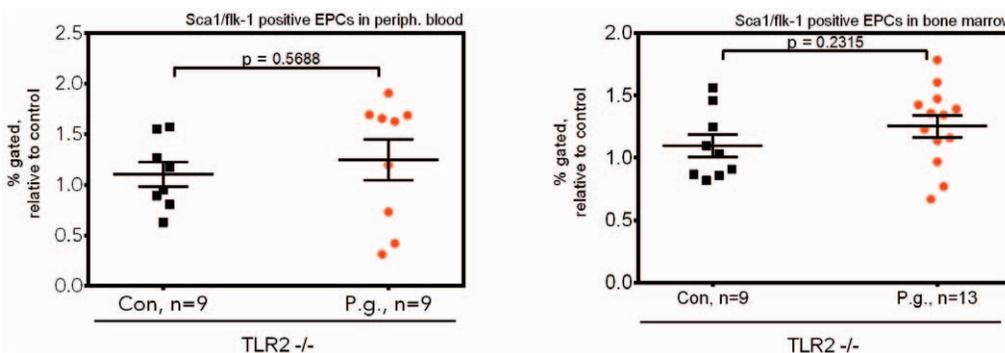


Figure 7. Sca1+/flk1+ progenitor mobilization is TLR2-dependent. In mice deficient in toll-like receptor (TLR)-2, the receptor primarily mediating the invasion of *P. gingivalis* into host cells, no increased mobilization of Sca1+/flk1+ progenitors was observed. Data are presented as mean percentages of gated cells \pm SEM normalized to control, statistical testing was performed using unpaired t-tests. doi:10.1371/journal.pone.0054860.g007

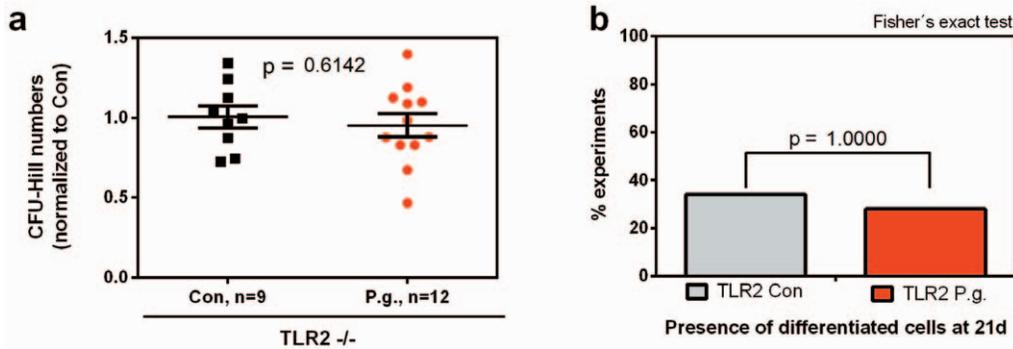


Figure 8. CFU-Hill and late-outgrowth mobilization is TLR2-dependent. No significant differences in CFU-Hill numbers (a) and the proportion of presence of differentiated late-outgrowth EPC (b) in *P. gingivalis* infected TLR2-deficient mice. Data are presented as mean colony counts \pm SEM relative to controls (CFU-Hill) or as numbers of experiments yielding differentiated colonies (late EPC). Data were analyzed using unpaired t-tests (CFU-Hill) or Fishers exact test (late EPC). doi:10.1371/journal.pone.0054860.g008

activated [64], whilst in the periodontal tissues, the pathogens are in intimate contact with bone stromal cells and infiltrating mononuclear cells, such as T-cells, all of those are known to predominantly produce RANKL [65,66].

On the other hand, osteoprotegerin, but not RANKL, was recently shown to induce endothelial cell and ECFC activation and to improve microvessel formation in vitro [67,68] – therefore, it must also be considered that the observed increase of the systemic osteoprotegerin/RANKL ratio might as well constitute another counter-measure against vascular damage inflicted by the pathogen.

When judging the inferences drawn from our data, it must be, however, noted that the utilized acute model of bacteremia with a periodontal model pathogen is neither a model of atherosclerosis nor of periodontitis. It merely mimics the bacteremia known to be a feature of active periodontal disease [6,10,69,70], and allows for exact determination of the effects exerted by these bacteria on bone marrow-derived progenitor mobilization.

An oral model of periodontal infection in atherosclerosis-prone mice [54] is more likely to adequately mimic human periodontal disease and its effects on atherosclerosis progression. However, these models all rely on genetically modified mice prone to develop atherosclerosis in due course, potentially not ideal models to shed light onto the underlying pathobiology of associations found in otherwise healthy subjects suffering from periodontitis.

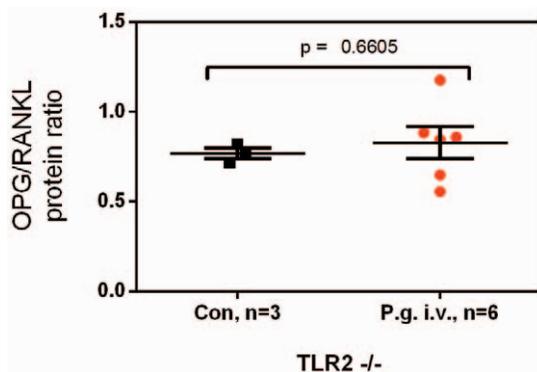


Figure 9. No differences in osteoprotegerin/RANKL ratio in the bone marrow niche of *P. gingivalis* infected vs. control mice deficient in TLR2. Data are given as means \pm SEM, data were analyzed using an unpaired t-test. doi:10.1371/journal.pone.0054860.g009

Subsequent studies in adequate models of chronic challenge with periodontal pathogens are necessary to evaluate whether the observed strong mobilization of endothelial progenitors from the bone marrow into the peripheral circulation induced by bacteremia with *P. gingivalis* will in fact lead to depletion of progenitor pools and subsequently reduced overall regeneration capacity. Alternatively, it is possible that the acute shortage of progenitors in the bone marrow is effectively countered by increasing osteoprotegerin/RANKL levels leading to stem cell expansion and retention, eventually resulting in increased EPC levels in peripheral blood and bone marrow niche, as found after repeated physical exercise [59].

These studies will unequivocally show whether, to what extent, and by what mechanisms endothelium-regenerating cells are involved in increased atherogenesis mediated by periodontal infections.

Taken together, we show that in an acute model of periodontal infection recurrent bacteremias lead to strong, TLR2-dependent mobilization of endothelial progenitors from the bone marrow to the circulation. In the short term, these cells improve endothelial function and reendothelization. Long-term studies in atherosclerosis models are needed to determine whether this recurrent mobilization is relevant for the reported increased atherosclerosis in periodontitis.

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Author Contributions

Conceived and designed the experiments: MK JD SJ GN NW. Performed the experiments: MK MH. Analyzed the data: MK MH NW. Wrote the paper: MK NW.

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