Toll-Like Receptor 2/6 Agonist Macrophage-Activating Lipopeptide-2 Promotes Reendothelialization and Inhibits Neointima Formation After Vascular Injury

Karsten Grote, Kristina Sonnenschein, Piyushkumar R. Kapopara, Anja Hillmer, Christina Grothusen, Gustavo Salguero, Daniel Kotlarz, Harald Schuett, Udo Bavendiek, Bernhard Schieffer

Objective—Reendothelialization after vascular injury (ie, balloon angioplasty or stent implantation) is clinically extremely relevant to promote vascular healing. We here investigated the therapeutic potential of the toll-like receptor 2/6 agonist macrophage-activating lipopeptide (MALP)-2 on reendothelialization and neointima formation in a murine model of vascular injury.

Approach and Results—The left common carotid artery was electrically injured, and reendothelialization was quantified by Evans blue staining after 3 days. A single injection of MALP-2 (1 or 10 µg, IV) after vascular injury accelerated reendothelialization (P<0.001). Proliferation of endothelial cells at the wound margins determined by 5-ethyl-2'-deoxyuridine incorporation was significantly higher in MALP-2–treated animals (P<0.05). Furthermore, wire injury–induced neointima formation of the left common carotid artery was completely prevented by a single injection of MALP-2 (10 µg, IV). In vitro, MALP-2 induced proliferation (BrdU incorporation) and closure of an artificial wound of endothelial cells (P<0.05) but not of smooth muscle cells. Protein array and ELISA analysis of isolated primary endothelial cells and ex vivo stimulated carotid segments revealed that MALP-2 stimulated the release of multiple growth factors and cytokines predominantly from endothelial cells. MALP-2 induced a strong activation of the mitogen-activated protein kinase cascade in endothelial cells, which was attenuated in smooth muscle cells. Furthermore, MALP-2 significantly enhanced circulating monocytes and hematopoietic progenitor cells.

Conclusions—The toll-like receptor 2/6 agonist MALP-2 promotes reendothelialization and inhibits neointima formation after experimental vascular injury via enhanced proliferation and migration of endothelial cells. Thus, MALP-2 represents a novel therapeutic option to accelerate reendothelialization after vascular injury. (Arterioscler Thromb Vasc Biol. 2013;33:2097-2104.)

Key Words: cytokines ▪ endothelial growth factors ▪ neointima formation ▪ toll-like receptors ▪ vascular system injuries

Interventional angioplasty procedures for revascularization of patients with coronary artery disease or peripheral artery disease have dramatically increased during the past 3 decades and are still in progress. However, techniques such as balloon angioplasty and stent implantation provoke a massive disruption of the vascular integrity, thus requiring vascular wound healing including reendothelialization. Particularly, insufficient or decelerated reendothelialization amplifies vascular inflammation and impairs vascular wound healing, thereby increasing the risk of clinically fatal events, such as stent thrombosis and restenosis. Despite novel drug-eluting stent technologies, in the majority of cases with anti-inflammatory/antiproliferative substances, restenosis as a result of inflammation-driven proliferation of smooth muscle cells with subsequent neointima formation is still a clinical problem. Because of their antiproliferative nature, the widely used drugs not only suppress neointima formation but also retard reendothelialization. Because incompletely reendothelialized vessels are highly thrombogenic, extended dual platelet aggregation inhibition with consecutively increased bleeding risk in those patients is needed. Therefore, new strategies to promote reendothelialization after vascular injury are warranted.

Mammalian toll-like receptors (TLRs) were initially described as receptors of the innate immune recognizing...
pathogen-associated molecular patterns of bacterial and viral origin to organize the body’s immune defense. More than 10 members were identified so far activating classical inflammatory signaling pathways, such as the mitogen-activated protein kinase (MAPK) cascade or nuclear factor-κB. Especially monocytes/macrophages and granulocytes are stimulated by TLR ligands to produce reactive oxygen species, cytokines, and costimulatory molecules for pathogen killing and clearance. Their important role in dendritic cell maturation and T-cell activation established TLRs as an important link between innate and adaptive immunity. The detection of TLR members in multiple tissues and cell types, besides cells of the immune system, put a new complexion on TLRs. Inflammatory disorders, such as atherosclerosis and liver disease, are critically influenced by TLR signaling. In this regard, the ability of TLRs to recognize endogenous ligands that accumulate during tissue damage and fibrosis has already been discovered and seems to be essential for their function in regulating noninfectious inflammation. Of importance, a novel role of TLRs in wound healing and tissue regeneration has been reported, also indicating a regenerative aspect of TLR signaling.

We here used the N-terminal diacetylated lipopeptide macrophage-activating lipopeptide of 2 kDa (MALP-2) that was originally isolated from Mycoplasma species. MALP-2 is recognized by a heterodimer of TLR2 and TLR6, and is to date one of the few definitely identified natural ligands signaling via this pathway. Initial studies were performed in cells of the innate immune system, that is, macrophages. Of note, immune activation with MALP-2 has potential therapeutic implications, for example, as a mucosal adjuvant for vaccination or for the induction of lipopolysaccharide cross-tolerance. Furthermore, MALP-2 also exhibits potential for regenerative processes because MALP-2 has already been successfully used to promote dermal wound healing in mice. Wound repair involves not only recruitment of inflammatory cells but also the reestablishment of a capillary network by endothelial cells, termed angiogenesis. In this regard, we recently reported that MALP-2 exhibits a considerable potential to induce angiogenesis in a TLR2/6-dependent manner, which represents an endogenous mechanism to restore capillaries in infected tissues to recruit immune cells for pathogen defense.

The luminal endothelium plays a critical role in the regulation of the vascular permeability, cellular adhesion to the arterial wall, and the local production of growth factors. Thus, injury to the luminal endothelium, either metabolic or mechanical, definitely results in a misbalance of these important features. Because we already established endothelial cells as target cells for a TLR2/6-dependent pathway, we now investigated a potential therapeutic application of MALP-2 to promote luminal reendothelialization after vascular injury.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
MALP-2 Promotes Reendothelialization After Vascular Electric Injury
To induce an endothelial damage, we performed an electric injury of the left common carotid artery in C57BL/6N wild-type mice, a precise and reliable method to determine reendothelialization (Figure IA in the online-only Data Supplement). Subsequently, 2 hours after injury, either 1 or 10 µg MALP-2 was injected (intravenously), and reendothelialization was quantified on Evans blue–stained en face prepared carotid arteries 3 days after injury by morphometric analysis. Compared with the initial wound area, we observed 7.9% reendothelialization under control conditions, which was significantly enhanced by MALP-2 treatment to 23.7% (1 µg; P<0.001) and 28.9% (10 µg; P<0.001). Of note, MALP-2 treatment enhanced reendothelialization ≤3.5-fold. Combined injection of neutralizing antibodies against the MALP-2 receptors TLR2 and TLR6 significantly suppressed MALP-2–induced reendothelialization (P<0.001), proving the relevance of the TLR2/6-dependent pathway for MALP-2–induced endothelial regeneration (Figure IA).

In addition, we investigated reendothelialization in response to different TLR ligands. However, neither Pam_CS, that signals via TLR2/6 just as MALP-2 nor the TLR2/1 agonist Pam_CS, nor the TLR4 agonist LPS promoted reendothelialization in this model (Figure II in the online-only Data Supplement). Obviously, this effect is not a class effect of TLR agonists and not even characteristic of TLR2/6 agonists. Next, we investigated whether enhanced proliferation of endothelial cells is responsible for MALP-2–induced reendothelialization after vascular injury. Therefore, we analyzed cell proliferation at the endothelial wound margins in en face prepared carotid arteries (Figure IB in the online-only Data Supplement) 3 days after injury by quantifying 5-ethyl-2′-deoxyuridine incorporation using confocal microscopy. Proliferating 5-ethyl-2′-deoxyuridine-positive cells could be clearly identified as endothelial cells by immunofluorescent staining of the endothelial marker CD31. Indeed, compared with control conditions, MALP-2 significantly enhanced endothelial cell proliferation at the denuded wound margins (P<0.05; Figure IB).

MALP-2 Inhibits Neointima Formation After Vascular Wire Injury
The method of perivascular electric injury is mainly used to investigate reendothelialization of the carotid artery, where normally no neointima formation is observed. However, because MALP-2 seems to have highly proliferative properties, we likewise took the possibility of MALP-2–induced smooth muscle proliferation even in this model into consideration. To this end, we investigated the morphology of the carotid artery ≤28 days after electric injury. Morphometric analyses in hematoxylin and eosin–stained cross sections of the injured carotid artery revealed no abnormalities under control or MALP-2 conditions. We did not detect any neointima or media hyperplasia or other signs of maladaptive vascular remodeling (Figure IIIA in the online-only Data Supplement). Immunofluorescent staining of the endothelial marker CD31 showed that the luminal site of the vessel wall was not yet covered with endothelial cells early on at day 2 but was already completely reendothelialized 14 days after vascular injury (Figure IIIB in the online-only Data Supplement).

To address especially neointima proliferation, we took advantage of the vascular wire injury model in FVB/N mice.
Figure 1. Macrophage-activating lipopeptide (MALP)-2 promotes reendothelialization after vascular injury. A, Reendothelialization was quantified in Evans blue–stained en face prepared carotid arteries 3 days after vascular injury. PBS (control) or MALP-2 was injected intravenously 2 hours after injury, and antibodies targeted against toll-like receptor (TLR) 2 and TLR6 or control IgG were injected intraperitoneally injected 2, 24, and 48 hours after injury. Representative pictures are shown. Scale bar, 50 μm; *P<0.01 vs control, #P<0.001 vs control MALP-2 (10 μg). Numbers in columns indicate number of investigated carotid arteries. B, Proliferation of endothelial cells was quantified at the endothelial wound margins in en face prepared carotid arteries 3 days after vascular injury. Proliferating nuclei are indicated by 5-ethynyl-2′-deoxyuridine (EdU) incorporation (purple) and endothelial cells by CD31 staining (green). PBS (control) or MALP-2 (10 μg) was intravenously injected 2 hours after injury, and EdU was intraperitoneally injected 2, 24, and 48 hours after injury. Representative pictures are shown. Scale bar, 50 μm; *P<0.01 vs control (n=5). AB indicates antibody; and IgG, immunoglobulin G.

Figure 2. Macrophage-activating lipopeptide (MALP)-2 inhibits neointima formation after vascular wire injury. Neointima formation was determined on hematoxylin and eosin–stained cross sections of prepared carotid arteries 28 days after vascular wire injury. PBS (control) or MALP-2 (10 μg) was injected intravenously 2 hours after injury. Representative pictures are shown. Scale bar, 100 μm (n=5). n.d. indicates not detected.

Wire injury was performed at the same site of the left common carotid artery as before in the electric injury model (Figure IA in the online-only Data Supplement). Subsequently, 10 μg MALP-2 was administered 2 hours after injury (intravenously), and morphometric analysis was performed on hematoxylin and eosin–stained cross sections of the carotid artery 28 days later. Of note, wire injury–induced neointima formation was completely inhibited on MALP-2 treatment (Figure 2). In addition, MALP-2 did not induce platelet aggregation of human platelet-rich plasma (Figure IV in the online-only Data Supplement), suggesting that adverse effects on thrombosis after MALP-2 treatment are not to be expected.

MALP-2 Enhances In Vitro Wound Closure and Proliferation of Endothelial Cells But Not Smooth Muscle Cells

To study cellular processes that are presumably involved in enhanced reendothelialization after MALP-2 injection, we studied proliferation and migration of endothelial cells in response to MALP-2 in vitro. Although MALP-2 treatment did not provoke neointimal proliferation in vivo, we likewise expanded our in vitro studies to smooth muscle cells to further study potentially undesirable side effects of MALP-2 in this regard. Similar to the in vivo situation, MALP-2 accelerated the closure of an artificial wound scratch in endothelial cells 24, 48, and 72 hours after experimental induction of the wound compared with control conditions (P<0.05; Figure 3A). However, we did not observe any significant effect of MALP-2 to enhance the ability of smooth muscle cells to close the artificial wound (Figure 3B). Of note, during an observation period of 3 days, wound closure in this model implies both cell migration and cell proliferation. To address particularly cell proliferation, we additionally investigated nuclear BrdU incorporation. Consistent with the scratch experiments, we observed similar effects of MALP-2 on these vascular cell types. Compared with control, stimulation with MALP-2 for 24 hours increased proliferation of endothelial cells (P<0.05; Figure 3C) but not of smooth muscle cells (Figure 3D).

MALP-2 Stimulates the Release of Growth Factors and Cytokines and MAPK Cascade Activation in Endothelial Cells

During vascular injury and endothelial denudation, smooth muscle cells are uncovered and unnaturally exposed to the blood flow in the carotid artery. Thus, circulating MALP-2
could activate TLR2 and TLR6 on endothelial cells and smooth muscle cells to initiate a regenerative program of local endothelial wound healing. To investigate which of these vascular cell types may release factors as a potential underlying mechanism for reendothelialization in response to MALP-2, we isolated primary endothelial cells and smooth muscle cells. Protein array analyses for selected cytokines and growth factors revealed that endothelial cells released numerous factors on MALP-2 stimulation. In contrast, these factors were not enhanced or even downregulated by MALP-2 stimulation in smooth muscle cells (Figure 4A). In addition, we performed ELISA analyses to validate the protein array data. Therefore, we investigated granulocyte colony-stimulating factor (G-CSF) and interleukin-6 (IL-6), which were the most prominent MALP-2–induced factors in endothelial cells. In fact, we were able to confirm the results of the protein array. MALP-2 significantly enhanced the secretion of G-CSF (Figure 4B) and interleukin-6 (Figure 4C) from endothelial cells (P<0.05), whereas MALP-2 had no effect on G-CSF and interleukin-6 secretion from smooth muscle cells. Expression of both MALP-2 receptors was detected in endothelial cells and smooth muscle cells, although TLR6 was found to be only weakly expressed in smooth muscle cells (Figure 4D).

Endothelial cells seem to be the main vascular source of soluble factors mediating the regenerative effects of MALP-2 after vascular injury. To further address this issue, we isolated the left carotid artery immediately after injury and at the same time the noninjured right carotid artery. Both vessels were dissected and subsequently cultured and stimulated with MALP-2 ex vivo. Secretion of numerous investigated factors was strongly induced by MALP-2 in vessels with an intact endothelium and either attenuated or downregulated in vessels with endothelial damage (Figure 5A). Again, we confirmed the strongest MALP-2–induced factors by ELISA, which were G-CSF and granulocyte macrophage colony-stimulating factor. Secretion of both growth factors was induced after MALP-2 stimulation in the noninjured carotid artery that was significantly less pronounced in the injured carotid artery (P<0.05; Figure 5B and 5C).

Furthermore, we investigated MAPK signaling in isolated murine endothelial cells and smooth muscle cells in response to MALP-2 by Western blot. All 3 major pathways, the extracellular signal-regulated kinase 1/2, p38 MAPK, and c-Jun N-terminal kinase, were strongly induced in endothelial cells on MALP-2 stimulation, with a maximal peak at 15 minutes. Interestingly, MALP-2–dependent MAPK activation was attenuated and decelerated in smooth muscle cells (Figure 6A–6C).

**MALP-2 Increases Numbers of Circulating Monocytes, Granulocytes, and Progenitor Cells**

Because leukocyte subsets and progenitors cells have been shown to contribute to vascular wound healing, we additionally investigated the effect of MALP-2 on peripheral blood levels of these cell populations. A single injection of 10 µg MALP-2 (intravenously) significantly increased monocytes (F4/80<sup>+</sup>CD11b<sup>+</sup>CD14<sup>+</sup>) after 24 and 48 hours and trend to increase granulocytes (Gr-1<sup>+</sup>CD11b<sup>+</sup>CD123<sup>+</sup>) after 24 hours but had no effect on T cells (CD3<sup>+</sup>CD220<sup>+</sup>) in peripheral blood samples (Figure V in the online-only Data Supplement). In addition,
colony-forming unit assays revealed that repeated intravenous injection of MALP-2 increased hematopoietic progenitor cells in peripheral blood after 24 hours (Figure VIA in the online-only Data Supplement). Colony-forming unit was similarly increased by a single injection of MALP-2 with a peak 48 hours after injection (Figure VIB in the online-only Data Supplement). Protein array analyses 24 hours after injection of MALP-2 showed no relevant changes in plasma cytokines and growth factors compared with PBS injection (Figure VICA in the online-only Data Supplement). An exemplary and time-dependent analysis of G-CSF plasma levels by ELISA revealed only a moderate and transient increase 1 hour after MALP-2 injection (Figure VIIB in the online-only Data Supplement). Thus, systemic application of MALP-2 did not result in severe systemic inflammation.

**Discussion**

Endothelial cells play a pivotal role in vascular homeostasis. The endothelium produces nitric oxide for vasorelaxation, controls leukocyte adherence, regulates vascular permeability, and much more. In this regard, endothelial dysfunction is an initial event in arterial hypertension, atherosclerosis, and diabetes mellitus. All kinds of interventional angioplasty techniques induce massive vascular injuries, including extensive endothelial damage. Therefore, cardiologists have to deal with the consequences of revascularization in patients with coronary artery disease or peripheral artery disease because vascular injury results in severe vascular inflammation. Insufficient or delayed reendothelialization further aggravates and extends vascular inflammation and wound healing in these patients (as known from drug-eluting stents). The inflammatory process includes activation of inflammatory signaling pathways, enhanced expression of cellular and soluble adhesion molecules, local release of cytokines and chemokines, and enhanced generation of reactive oxygen species in the vessel wall. A major problem of this exaggerated inflammation is the neointima formation because of excessive proliferation of smooth muscle cells from the media bearing the risk of stent thrombosis and restenosis. Therefore, drug-eluting stent technologies using antiproliferative substances, such as sirolimus or paclitaxel, are frequently being used to inhibit excessive smooth muscle cell proliferation and thereby neointima formation and restenosis. The drawback of these drugs is a further retarded reendothelialization requiring a dual antiplatelet aggregation therapy, usually with aspirin and P2Y12 inhibitors, for ≤1 year to prevent coagulation at sites of vascular injury. Until now, studies testing substances to improve reendothelialization are very rare. We here describe for the first time that a TLR agonist significantly improves reendothelialization.

TLRs represent a major protein receptor class of pattern recognition receptors of the innate immunity with fundamental functions for pathogen recognition. However, TLRs are likewise activated by danger-associated molecular patterns that do not only include bacterial and viral
pathogen-associated molecular patterns but also a variety of endogenous substances, such as proteins, peptides, polysaccharides, proteoglycans, nucleic acids, and phospholipids. The latter are particularly extracellular matrix degradation products that accumulate after tissue damage. Thus, TLRs are activated not only in response to tissue-invading pathogens but are also pathogen independent, for example, after tissue damage or ischemic conditions. In both cases, TLRs have important functions in the recruitment of immune cells to initiate a regenerative program: in the first case mainly to eliminate invading pathogens and in the second case to clear the affected tissue from apoptotic cells and cellular debris.

With respect to the cardiovascular system, TLR2 and TLR4 expression has been documented in cardiomyocytes, endothelial cells, and smooth muscle cells. Accumulating evidence, in most instances from genetically modified mouse models, suggests that TLRs play important roles in different cardiovascular pathologies, such as septic cardiomyopathy, viral myocarditis, atherosclerosis, ischemia/reperfusion injury after myocardial infarction, dilated cardiomyopathy, and cardiac allograft rejection. These disorders are of noninfectious cause, and inflammatory pathways are triggered by endogenous TLR ligands that usually accumulate under these conditions. Experimental loss-of-function studies, mainly on TLR2 and TLR4, demonstrated that inhibition of TLRs has beneficial effects on the pathology of atherosclerosis and on ischemia/reperfusion injury after myocardial infarction. This is in accordance with findings that prolonged administration of TLR2 agonists dramatically increases experimental atherosclerosis. Prolonged TLR3 activation by polyI:C has even been shown to impair reendothelialization and endothelial function, finally aggravating atherosclerosis. In contrast, unique and selective pretreatment with TLR2 or TLR4 ligands reduced infarct size and improved cardiac function after ischemia/reperfusion. To date, different TLR ligands (eg, TLR9) have been described to accelerate wound healing involving angiogenesis by endothelial cells. These data suggest an ambivalent role for TLRs in cardiovascular pathology. On the one hand, sustained TLR stimulation exacerbates inflammation. Therefore, blockade of TLRs may represent a promising strategy for therapeutic intervention. On the other hand, administration of defined TLR agonists in a timely controlled and limited manner could have beneficial effects. This may occur via a singular inflammatory trigger that initiates regenerative pathways, and this strategy could even be used for preconditioning.

We here used the lipopeptide MALP-2 that represents a well-characterized TLR agonist with already reported regenerative properties. The synthesis of the compound is completely synthetic, excluding contaminations with other TLR agonists, a well-known problem in the field. Initially, MALP-2 was shown to stimulate mainly cells of the innate immunity by binding to a heterodimeric complex of TLR2 and TLR6. On receptor binding, MALP-2 activates the inflammatory pathways, such as MAPK and nuclear factor-κB, with subsequent release of growth factors and cytokines regulated by MALP-2.

**Figure 5.** Macrophage-activating lipopeptide (MALP)-2 stimulates the release of growth factors and cytokines from the noninjured endothelium. A. Protein array analyses of secreted cytokines and growth factors in the supernatants of isolated noninjured and injured carotid arteries that were left unstimulated (control) or stimulated with MALP-2 (1 µg/mL) for 24 hours. B. Granulocyte colony-stimulating factor (G-CSF) levels and (C) granulocyte macrophage colony-stimulating factor (GM-CSF) levels in the supernatants of isolated noninjured and injured carotid arteries that were left unstimulated (control) or stimulated with MALP-2 (1 µg/mL) for 24 hours were measured by ELISA. *P<0.05 vs control, #P<0.05 vs noninjured (n=3). bFGF indicates basic fibroblast growth factor; b.t., below threshold; C, control; IFN, interferon; M-CSF, macrophage colony stimulating factor; MCP, monocyte chemoattractant protein; MIG, monokine induced by IFN-gamma; IGF, insulin-like growth factor; PF, platelet factor; TIMP, tissue inhibitor of metalloproteinases; TNF, tumor necrosis factor; and VEGF, vascular endothelial growth factor.
cytokines, such as interleukin-6 and tumor necrosis factor-α.\textsuperscript{16,17} In the meantime, MALP-2 has even been successfully used for therapeutic applications in different experimental animals models (eg, for cancer immunotherapy)\textsuperscript{16,17} as a mucosal adjuvant for therapeutic applications in different experimental animals.\textsuperscript{16,17} MALP-2,\textsuperscript{16,17} when applied to the skin of volunteers in a phase 1 clinical trial\textsuperscript{38} Topically applied MALP-2 has been proved to be well tolerable when applied to the skin of volunteers in a phase 1 clinical trial\textsuperscript{38} for the induction of lipopolysaccharide cross-tolerance,\textsuperscript{19} and for dermal wound healing.\textsuperscript{11} Remarkably, clinical data for a therapeutic potential of MALP-2 are already available.\textsuperscript{11} Topically applied MALP-2 has been proved to be well tolerable when applied to the skin of volunteers in a phase 1 clinical trial\textsuperscript{38} and exhibits beneficial effects in a phase 1/2 study with patients having developed aggressive pancreas adenocarcinoma.\textsuperscript{39}

In a vascular context, we have recently identified endothelial cells as target cells for MALP-2–dependent regenerative effects. We could delineate that MALP-2 promoted angiogenesis via a TLR2/6–dependent release of granulocyte macrophage colony-stimulating factor by endothelial cells themselves, which we regard as a mechanism to restore capillaries in infected tissues to recruit immune cells for pathogen defense.\textsuperscript{39} Our findings are in accordance with a study by West et al\textsuperscript{14} showing that TLR2-knockout mice have impaired angiogenesis and endothelial cell migration. In the present study, we provide additional evidence that MALP-2 could be used for vascular regeneration. We observe that MALP-2 considerably promoted luminal reendothelialization and inhibited neointimal proliferation in unique systemic application of MALP-2 improves reendothelialization ≤3.5-fold. This seems not to be a general effect of TLR ligands, because other investigated TLR agonists did not improve reendothelialization. Interestingly, this also holds true for Pam\textsubscript{2}CSK\textsubscript{4}, that signals via TLR2/6 just as MALP-2. Both ligands share a common lipid moiety but have different peptides. Sawahata et al\textsuperscript{40} recently reported that Pam\textsubscript{2}CSK\textsubscript{4}, but not MALP-2 is capable of activating natural killer cells. Potentially, their and our observations are based on the structural difference of these otherwise similar molecules. The observed discrepancy in our study between the effects of MALP-2 and the TLR2/1 ligand Pam\textsubscript{2}CSK\textsubscript{4}, on reendothelialization is attributed to the fact that MALP-2 induces, beyond an overlapping pattern, distinct different inflammatory genes.\textsuperscript{41} MALP-2 significantly enhanced proliferation and migration of endothelial cells but not of smooth muscle cells. Further mechanistic studies suggest that improved endothelial healing is mediated by an MALP-2–dependent release of multiple factors predominantly from endothelial cells, whereas smooth muscle cells did not release factors in response to MALP-2. These differences are the result of diminished MAPK activation in smooth muscle cells. Beyond effects on vascular cells, MALP-2 as well affects circulating blood cells. We observed an early increase especially in circulating monocytes and hematopoietic progenitor cells. Potentially, these cell populations contribute to the vascular healing process in a paracrine manner. The observed regenerative effect of MALP-2 after vascular injury seems to be in line with the before observed induction of angiogenesis by MALP-2. After bacterial infection, TLRs sense pathogen-associated molecular patterns such as MALP-2, and repair mechanisms, including angiogenesis and reendothelialization, are initiated to restore damaged tissue that could potentially be used for vascular therapeutic interventions.

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**Disclosures**

None.

**References**

popeptide-2 in vascular wound healing for future therapeutic applications in patients with vascular damage after interventional revascularization.

Experimental models of vascular injury preferably in larger animals is needed to confirm potentially beneficial effects of macrophage-activating lipopeptide-2. A timely controlled manner adjacent to the site of endothelial denudation, should be tested. Furthermore, potentially additive effects of macrophage-activating lipopeptide-2 on reendothelialization may provide the opportunity to use macrophage-activating lipopeptide-2 for therapeutic endothelial wound healing after vascular injury.

**References**


**Significance**

We here describe for the first time a novel therapeutic strategy for a toll-like receptor ligand in vascular wound healing. Especially the effect on reendothelialization may provide the opportunity to use macrophage-activating lipopeptide-2 for therapeutic endothelial wound healing after vascular injury. Improved ways of application, such as degradable bioabsorbable materials to release macrophage-activating lipopeptide-2 in a timely controlled manner adjacent to the site of endothelial denudation, should be tested. Furthermore, potentially additive effects of macrophage-activating lipopeptide-2 to promote reendothelialization and angiogenesis should be explored. Thus, further research using different experimental models of vascular injury preferably in larger animals is needed to confirm potentially beneficial effects of macrophage-activating lipopeptide-2 in vascular wound healing for future therapeutic applications in patients with vascular damage after interventional recanalization.
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In the article by Grote et al, which appeared in the September 2013 issue of the journal (Arterioscler Thromb Vasc Biol. 2013;33:2097–2104. DOI: 10.1161/ATVBAHA.113.301799), figure 1 was incorrect. The correct figure is below:

The online version of the article has been corrected and is available at http://atvb.ahajournals.org/content/33/9/2097.full.
Supplemental Figure I

Supplemental Figure I: Overview of the carotid artery injury model. A, Scheme of the aortic arch with all arterial outlets. The experimental site of electric injury and wire injury of the left common carotid artery is indicated by a red dashed line. B, Scheme of the en face prepared injured carotid artery. The red boxes illustrate the border areas between injured and uninjured endothelium where endothelial cell proliferation was investigated. C, Scheme of the injured carotid artery. The red box indicates the site for morphological analysis in cross sections of the injured carotid artery.
Supplemental Figure II: Other TLR-ligands do not promote reendothelialization following vascular injury. Reendothelialization was quantified in Evans blue-stained en face prepared carotid arteries 3 days after vascular injury. PBS (control) or Pam$_2$CSK$_4$ (10 µg), Pam$_3$CSK$_4$ (10 µg), LPS (10 µg) and MALP-2 (10 µg) were injected i.v. 2 hours after injury. Representative pictures are shown. Blue staining indicates endothelial denudation. Initial injury is indicated by a bracket. TLRs recognizing the respective ligand are given in red. Scale bar = 1 mm, *P<0.05 vs. control, n=4-5.
Supplemental Figure III: MALP-2 does not induce neointima formation following vascular electric injury. **A**, Representative H&E stained cross sections of the injured carotid artery at day 3, 14 and 28 after vascular injury with i.v. injection of PBS (control) or MALP-2 (10 µg) are shown. Scale bar = 50 µm. **B**, Representative CD31 stained (green) cross sections of the injured carotid artery at day 3, 14 and 28 after vascular injury with i.v. injection of PBS (control) or MALP-2 (10 µg) are shown. Nuclei are shown in blue. Scale bar=50 µm, n=3-4.
Supplemental Figure IV: MALP-2 does not induce platelet aggregation. Platelet aggregation of human platelet rich plasma was measured with an aggregometer in the presence of 10, 100 and 1000 µg/mL MALP-2. 10 µM ADP was used as control. Maximal platelet aggregation is given in %. A representative protocol is shown.
Supplemental Figure V: MALP-2 increases numbers of monocytes and granulocytes in peripheral blood. **A**, Monocytes **B**, Granulocytes and **C**, T-cells cells were quantified by FACS analysis 24 and 48 hours after i.v. injection of MALP-2 (10 µg). Representative plots with gate settings for the respective markers are shown. *P<0.05, **P<0.01 vs. PBS, n=3-4.
Supplemental Figure VI

Supplemental Figure VI: MALP-2 increases numbers of CFU in peripheral blood. A, CFU were quantified on methylcellulose 24 hours after repeated injection of MALP-2 (10 µg). G-CSF (4 µg) injection was used as positive control. B, CFU were quantified on methylcellulose 24, 48 and 72 hours after a single i.v. injection of MALP-2 (10 µg). *P<0.05, **P<0.01 vs. PBS, n=4-6.
Supplemental Figure VII: Effect of MALP-2 on plasma levels of cytokines and growth factors. A, Protein array analyses of cytokines and growth factors in plasma of mice 24 hours after i.v. injection of PBS or MALP-2 (10 µg), C=control. B, G-CSF levels in plasma of mice 1 hour after injection of PBS and after i.v. MALP-2 injection (10 µg) to the indicated time points were measured by ELISA. *P<0.05 vs. PBS, n=3-4.
Supplemental Material and Methods

Experimental Animals

Male C57BL/6N or FVB/N wild type mice (Charles River Laboratories, Sulzfeld, Germany) at the age of 10 to 12 weeks were used for all experiments. Animals were housed in the animal facility at the Hannover Medical School during experiments. Animal experiments were approved by the governmental animal ethics committee and performed according to the guidelines of the Federation of European Laboratory Animal Science Associations.

Vascular Injury Models

Electric injury – C57BL/6N Mice were anesthetized by i.p. injection of ketamine (400 mg/kg) and xylazine (5 mg/kg). Carotid artery electric injury was performed as described previously. In brief, the left common carotid artery was injured with a bipolar microregulator (ICC50, ERBE-Elektromedizin GmbH, Tuebingen, Germany) below the bifurcation (Figure I in the online-only Data-Supplement). An electric current of 2 W was applied for 2 seconds to each millimeter of carotid artery over a total length of 4 mm with the use of a size marker parallel to the carotid artery. MALP-2 (10 µg), Pam2CSK4 (10 µg), Pam3CSK4 (10 µg) or LPS (10 µg) in a total volume of 200 µL PBS was injected i.v. 2 hours after injury. The same volume of PBS was injected into control mice. For some experiments, 200 µL of PBS containing neutralizing antibodies against TLR2 and TLR6 (1 µg each) was injected into the tail vein 30 min before MALP-2 injection. Appropriate amount of isotype-matching IgG was used for control experiments. Three days after carotid injury, mice were euthanized by cervical dislocation under deep isoflurane anaesthesia and reendothelialization was evaluated by staining the denuded area with 50 µL of a 5% Evans blue solution via injection into the heart. Pictures of the en face prepared injured left common carotid artery were taken with the help of a microscope (DM4000B, Leica Microsystems, Wetzlar, Germany) and a digital camera (DFC 320, Leica Microsystems) at 25x magnification. The
reendothelialized area was calculated as difference between the blue-stained area and the initially injured area by computer-assisted morphometric analysis (ImageJ, NIH, Bethesda, MD) and presented as percentage reendothelialization. Of note, this model has been shown to allow reproducible and consistent quantification of reendothelialization.\(^1\)

**Wire injury** – FVB/N mice were anesthetized by i.p. injection of ketamine (400 mg/kg) and xylazine (5 mg/kg). Carotid artery wire injury was performed as described by Lindner et al.\(^2\) Through middle line neck incision on ventral side LCCA including bifurcation were exposed clean from surrounding tissue. 5-0 surgical suture were looped around LCCA proximal to aortic arch and left internal carotid artery (LICA) for temporary control of blood flow. Two ligatures (6-0 suture) were placed around left external carotid artery (LECA) and distal one was tied off. A incision hole were made in the LECA and a flexible wire (0.36 mm, HI-TORQUE\(^\circledR\) Guide wire, #22339H, Abbott Laboratories, Abbott Park, Illinois) was introduce into LCCA, passed 3 times to and fro with rotation. The wire was removed and LECA was then tied off proximally. The skin incision was closed with surgical suture. MALP-2 (10 µg) in a total volume of 200 µL PBS was injected i.v. 2 hours after injury. The same volume of PBS was injected into control mice. All surgical procedure was performed under dissection microscope. 28 days after wire carotid injury mice were euthanized by cervical dislocation under deep isoflurane anaesthesia. Left and right common carotid arteries were harvested and embedded in Tissue Tek OCT (Sakura Finetek, Staufen, Germany) and 5 µm serial cryostat sections (CM3050S, Leica Microsystems) were made starting from bifurcation point towards arch till 2500 µm. Morphometric analysis was performed on total six sections per sample two each at a distance of 500 µm apart were fixed in ice cold acetone and stained with H&E followed by computer-assisted morphometric analysis (ImageJ) to calculate neointima area.

**Material, Reagents, Antibodies**

All cell culture plates were purchased from TTP (St. Louis, MO). MALP-2 was synthesized and purified as described,\(^3\) dissolved in 30% 2-propanol/water to a 1 mg/mL stock solution and diluted
MALP-2 promotes reendothelialization in cell culture medium for in vitro applications and in PBS for in vivo applications, respectively. G-CSF (Granocyte) was purchased from Chugai Pharma (London, UK). Pam$_2$CSK$_4$ and Pam$_3$CSK$_4$ were purchased from InvivoGen (San Diego, CA) and LPS from Sigma-Aldrich (Taufkirchen, Germany). Fibronectin and collagen I were purchased from BD Bioscience (Franklin Lakes, NJ) and Sigma-Aldrich (Taufkirchen, Germany), respectively. Following primary and secondary antibodies were used in this study: rat monoclonal anti-CD31 (#550274, BD Pharmingen, Heidelberg, Germany), mouse monoclonal anti-TLR2 (sc-21759, Santa Cruz biotechnology, Santa Cruz, CA), rabbit polyclonal anti-TLR6 (sc-30001, Santa Cruz), mouse monoclonal anti-GAPDH (sc-32233, Santa Cruz), chicken Alexa Fluor® 488-conjugated anti-rat (Invitrogen, Darmstadt, Germany), sheep peroxidase-conjugated polyclonal anti-mouse (GE Healthcare, Buckinghamshire, UK), donkey peroxidase-conjugated polyclonal anti-rabbit (GE Healthcare) and normal mouse or rabbit IgG (Santa Cruz) as isotype control. Nuclear staining for immunofluorescence microscopy was conducted with either TO-PRO®-3 iodide or Hoechst 33342 (both Invitrogen).

**Morphometry and Immunostaining**

OCT-embedded common carotid arteries were cut systematically in serial 6 µm cross sections using a cryotome (Leica CM3050S, Leica Microsystems, Wetzlar, Germany). Analyses were carried out in the injured left common carotid artery, whereas the contralateral not injured right carotid artery served as control (Figure I in the online-only Data-Supplement). For morphometric analysis, sections were stained with hematoxylin and eosin (H&E). For immunofluorescence analysis, sections were stained with an antibody against CD31 (1:100) and visualized using an appropriate Alexa Fluor® 488-conjugated secondary antibody (1:500). Negative controls using IgG controls matching in species and concentration were run in parallel. Pictures were taken with the help of an inverted microscope (AxioObserver.Z1, Carl Zeiss) and a digital camera (AxioCam MRe, Carl Zeiss) at 200x magnification.
Cell Culture

Smooth muscle cells were isolated from the aorta of C57BL/6N mice by an enzymatic dispersion method as described before. Cells were cultured on collagen-coated 75 cm² flasks (Nunc, Langenselbold, Germany) in DMEM (Biochrom, Berlin, Germany) containing 1.0 g/L glucose supplemented with 10% FCS and 1% penicillin/streptomycin, and cells at passages 2–5 were used for successive experiments. Endothelial cells were isolated from the lung of C57BL/6N mice using an enzymatic dispersion method with the help of anti-CD144 antibodies (VE-cadherin, BD Pharmingen) and Dynabeads (Dynal, Invitrogen) following the manufacturer’s protocol. Cells were cultured on fibronectin-coated 25 cm² flasks (Nunc) in DMEM/F12 (Gibco, Invitrogen, Carlsbad, CA) containing 20% FCS and 1% penicillin/streptomycin plus endothelial cell growth supplement (C-30120, Promocell, Heidelberg, Germany). Cells at passages 2–5 were used for successive experiments.

Platelet Preparation and Aggregometry

Blood from healthy donors was collected in tubes containing 0.1 mM sodium citrate. Platelet-rich plasma (PRP) was obtained by centrifugation at 300 g for 10 min at room temperature. Transmission of 500 µL PRP containing 10, 100, 1000 ng/mL MALP-2 or 10 µM ADP was recorded in a 2 channel aggregometer (APACT, Greiner, Frickenhausen, Germany) over 6 minutes and expressed as percentage aggregation after adjustment of the system to 100% transmission with plasma.

Cell Proliferation Assay

Proliferation of endothelial cells in vivo was measured by incorporation of the nucleoside analog 5-ethynyl-2’-deoxyuridine (EdU) using the Click-iT® EdU Alexa Fluor® 555 Imaging Kit (Invitrogen) as described previously. Briefly, the explanted carotid segments were fixed in 3.7% PFA/PBS.
overnight at 4°C, permeabilized with 0.5% Triton X-100/PBS and hybridized with Alexa Fluor® 555 azide in the reaction cocktail as described in the manufacturer’s protocol. For co-localization studies the segments were subsequently incubated with antibodies against CD31 (1:50) after blocking of unspecific binding with 5% normal goat serum. Following incubation overnight at 4°C visualization was performed with appropriate Alexa Fluor® 488-conjugated secondary antibodies (1:100), nuclei were stained with TO-PRO®-3 iodide (1:200). Whole mount carotid segments were mounted on glass slides and imaged on a confocal laser scanning microscope (Leica DM IRB with a TCS SP2 AOBS scan head).

Proliferation of endothelial cells and smooth muscle cells in vitro was measured by incorporation of the nucleoside analog 5-bromo-2'-deoxyuridine (BrdU). 10,000 endothelial cells were seeded in fibronectin-coated 96-well plates in DME/F-12 containing endothelial growth supplements and 20% FCS and cultured for 24 hours. 10,000 smooth muscle cells were seeded in collagen-coated 96-well plates in DMEM containing 10% FCS and cultured for 24 hours. Subsequently, cells were starved in serum free medium without any supplements over night and after changing the medium for additional 24 hours in the presence of 1.0 µg/mL MALP-2. BrdU was added to the cell culture for the last 4 hours of the experiment. At the end of the experiments, pictures from the cells were taken with the help of an inverted cell culture microscope (AxioObserver.Z1, Carl Zeiss, Wetzlar, Germany) and a digital camera (AxioCam MRc, Carl Zeiss) at 50x magnification. After cell lyses, the amount of BrdU incorporation was determined with a commercial colorimetric quantification kit (Roche) according to the manufacturer’s protocol by measuring the absorbance at 450 nm with a plate reader (µQuant; Bio-Tek Instruments).

**Cell Scratch Wound Healing Assay**

25,000 endothelial cells were seeded in fibronectin-coated 96-well plates in DME/F-12 containing endothelial growth supplements and 20% FCS. 25,000 smooth muscle cells were seeded in collagen-coated 96-well plates in DMEM containing 10% FCS. Cells were cultured for 24 hours to
obtain a confluent cell monolayer. Subsequently, a horizontal scratch on the cell-covered surface of
the cell culture dish was made with the help of a yellow pipette tip and cells were cultured in serum
free medium without any supplements in the presence of 1.0 µg/mL MALP-2. Pictures from the
cells were taken at baseline directly after scratching as well as 24 hours, 48 hours and 72 hours after
scratching with the help of an inverted microscope (AxioObserver.Z1, Carl Zeiss) and a digital
camera (AxioCam MRc, Carl Zeiss) at 50x magnification. The percentage of the recovered area vs.
baseline for each time point was determined using the image analysis software ImageJ (NIH,
Bethesda, MD).

**Protein Array and Enzyme-linked Immunosorbend Assay (ELISA)**

For protein array analyses isolated primary endothelial and smooth muscle cells were cultured to
complete confluence in fibronectin-coated 6-well plates in DME/F-12 containing endothelial
growth supplements and 20% FCS and in collagen-coated 6-well plates in DMEM containing 10%
FCS, respectively. Cells were starved in serum free medium without any supplements overnight and
stimulated with 1 µg/mL MALP-2 for 24 hours. Moreover, the left carotid artery immediately after
injury and the not injured right carotid artery were isolated and cut in ~1 mm segments. 4-5 carotid
segments were placed per well of a 96-well plate and cultured in DMEM/F-12 without any
supplements and subsequently stimulated ex vivo with 1 µg/mL MALP-2 for 24 hours. In addition,
plasma was collected from C57BL/6N mice 1 day after i.v. injection of 10 µg MALP-2 or PBS.
Supernatants and plasma samples were analyzed with the help of a commercial protein array
specific for selected cytokines and growth factors (RayBiotech, Norcross GA) according to the
manufacturer’s instructions. In brief, membranes were blocked for 30 minutes with blocking buffer
and incubated with 2 mL of supernatants (1:2 diluted with blocking buffer) overnight at 4°C. After
washing, biotin-conjugated antibody cocktail was added and again incubated overnight at 4°C,
followed by 2-hour incubation with streptavidin-conjugated peroxidase at room temperature.
Membranes were incubated with peroxidase substrate and exposed to enhanced chemiluminescence
MALP12 promotes reendothelialization

films (Hyperfilm ECL; GE Healthcare). Films were digitalized and quantified densitometrically using an image analysis system (GeneGenius, Syngene, Cambridge, UK) and the software Quantity One (BioRad, Munich, Germany).

For ELISA measurements and supernatants from the above described experiments from cultured cells, carotid segments and plasma from MALP-2 (10 µg) injected (i.v.) were analyzed for G-CSF, GM-CSF or IL-6 using a commercial ELISA (R&D Systems, Wiesbaden, Germany) according to the manufacturer’s protocol with the help of a plate reader (µQuant; Bio-Tek Instruments, Bad Friedrichshall, Germany).

Colony Forming Unit (CFU) Assay

For the quantification of hematopoietic progenitors peripheral blood was collected from male C57BL/6N mice by heart puncture after mice have been euthanized by cervical dislocation under deep isoflurane anaesthesia. Blood samples were collected at day 1 after repeated injection of MALP-2 (3x10 µg every 24 hours, i.v.) and G-CSF (3x4 µg every 24 hours, i.v.) or at day 1, 2 and 3 after a single injection of MALP-2 (10 µg, i.v.) in a total volume of 200 µl PBS. Erythrocytes were lysed with ammonium chloride buffer (8.29 g/L NH₄Cl, 1.0 g/L KHCO₃, 0.037 g/L EDTA). 1x10⁵ blood cells were mixed with 3 mL methylcellulose (MethoCult™ GF M3434, StemCell Technologies, Vancouver, BC, Canada) and platted in 3.5 cm dishes with grids (Nunc). Cells were incubated at 37°C and 5% CO₂ and colonies were counted after 14 days.

Flow Cytometry

For FACS analysis peripheral blood was collected from male C57BL/6N mice by heart puncture after mice have been euthanized by cervical dislocation under deep isoflurane anaesthesia. EDTA-Blood samples were collected at day 1 and day 2 after i.v. injection of MALP-2 (10 µg) in a total volume of 200 µl PBS. Erythrocytes were lysed with ammonium chloride buffer (8.29 g/L NH₄Cl, 1.0 g/L KHCO₃, 0.037 g/L EDTA). Peripheral mononuclear blood cells were stained with
monoclonal antibodies to determine the frequency/percentage of myeloid cells (Gr-1, CD11b, F4/80) and lymphocytes (B220, CD3; all eBioscience, Frankfurt, Germany). Analysis were performed on a BD FACS LSR II cytometer (BD Biosciences, Heidelberg, Germany) and data were analyzed using FlowJo software (Treestar, Ashland, OR).

**Immunoblot**

Proteins from cellular extracts were separated by denaturing SDS-PAGE (10%), and transferred to a PVDF membrane (GE Healthcare). Transferred proteins were probed with antibodies against TLR2 (1:500) and TLR6 (1:500) and visualized using an appropriate peroxidase-conjugated secondary antibody (1:3,000), ECL solution, and enhanced chemiluminescence films (Hyperfilm ECL; GE Healthcare). Equal protein loading was verified by reprobing the membrane with antibodies against GAPDH (1:2,000). Films were analyzed using an image analysis system (GeneGenius) and the software Quantity One (BioRad).

**Statistical Analysis**

Data are presented as mean±SEM of at least 3 independent experiments. Normality distribution of data was tested by Shapiro–Wilks test. All data were normally distributed and were compared using 2-tailed Student’s t-test for independent samples or one-way ANOVA and a multiple comparison test when more than two groups were examined (Systat Software SigmaStat 3.0, Erkrath, Germany). Differences were considered statistically significant at a value of P<0.05.
Supplemental References


