Novel Role of the CXC Chemokine Receptor 3 in Inflammatory Response to Arterial Injury

Involvement of mTORC1


Abstract—Atherosclerosis, restenosis, and posttransplant atherosclerosis are characterized by endothelial damage, infiltration of inflammatory cells, and proliferation of smooth muscle cells. The CXCR3-activating chemokines interferon-γ inducible protein 10 (IP10) and MIG (monokine induced by interferon-γ) have been implicated in vascular repair and remodeling. The underlying molecular mechanisms, however, remain elusive. Here, we show that wire-mediated arterial injury induced local and systemic expression of IP10 and MIG, resulting in enhanced recruitment of CXCR3+ leukocytes and hematopoietic progenitor cells. This was accompanied by profound activation of mammalian target of rapamycin complex (mTORC1), increased reactive oxygen species production, apoptosis, and intimal hyperplasia. Genetic and pharmacological inactivation of CXCR3 signaling not only suppressed recruitment of inflammatory cells but also abolished mTORC1 activation, reduced reactive oxygen species generation, and blocked apoptosis of vascular cells, resulting in significant reduction of intimal hyperplasia in vivo. In vitro, stimulation of T cells with IP10 directly activated mTORC1 and induced generation of reactive oxygen species and apoptosis in an mTORC1-dependent manner. These results strongly indicate that CXCR3-dependent activation of mTORC1 directly links stimulation of the Th1 immune system with the proliferative response of intimal cells in vascular remodeling. (Circ Res. 2009;104:189-200.)

Key Words: intimal hyperplasia ■ inflammation ■ apoptosis ■ chemokines ■ mTORC1

The impact of inflammation in atherosclerosis, the most important cause of morbidity in the Western world, has been well known for a long time. Injury of the endothelium by proatherogenic factors such as free radicals, hypertension, and diabetes mellitus or by mechanical stress during percutaneous coronary intervention induces an early inflammatory reaction, resulting in infiltration of T cells and monocytes into the injured vessel. This defined recruitment of inflammatory cells is essential for development of atherosclerosis and vascular remodeling in response to arterial injury. Infiltration of inflammatory cells is accurately regulated by various members of the CC as well as CXC family of chemokines and their corresponding receptors. The essential role of chemokines for vascular remodeling is highlighted by recent animal data suggesting that blocking chemokine/chemokine receptor interactions interferes with vascular remodeling. Among other chemokine receptors, activation of the CXC chemokine receptor (CXCR3) by interferon (IFN)-γ inducible protein 10 (IP10) and MIG (monokine induced by IFN-γ) is critically involved in the recruitment of CXCR3-positive T helper type 1 lymphocytes (Th1 cells) in atheromas. Likewise, blockade or depletion of CXCR3 severely attenuates recruitment of Th1 cells to the sites of inflammation and significantly reduces early steps of atherogenesis. However, to date, the role of CXCR3 activation in the vascular response to arterial injury has not been investigated.

The molecular mechanisms linking the Th1 immune system with the proliferative response of intimal cells in vascular remodeling are unknown. Activation of chemokine receptors has not only been linked to chemoattraction and antiangiogenic effects but also to generation of reactive oxygen species (ROS) and induction of apoptosis. There is also some evidence that chemokines play a role in activation of the mammalian target of rapamycin complex (mTORC1). For example, CCL5-mediated migration of activated CD4⁺ T cells depends on the activation of mTORC1. The mTORC1 kinase plays a fundamental role in the regulation of cell viability, translation initiation, and cell cycle progression by altering the phosphorylation state of downstream targets such as p70 S6 kinase (p70S6K). Recent evidence suggests
that mTORC1 also is essential for vascular remodeling because pharmacological mTORC1 inhibitors, such as sirolimus or everolimus, are able to prevent the development of atherosclerosis and intimal hyperplasia in patients and animal models.18–20 These data make mTORC1 an attractive target to integrate T-helper signaling and the proliferative response of vascular cells after injury.

Here, we show that activation of mTORC1 is required for IP10-mediated induction of ROS and apoptosis of T cells. In vivo, stimulation of CXCR3 by IP10 or MIG involves recruitment of CXCR3+ cells to the site of injury, activation of mTORC1, enhanced formation of ROS, and induction of apoptosis in vascular cells. This contributes to pronounced vessel remodeling in a model of wire-induced arterial injury.

Materials and Methods
An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Model of Endothelial Denudation by Wire-Mediated Arterial Injury
Surgery was carried out as described previously.21 At the time points indicated, mice were euthanized, the femoral artery was dissected, and 5.0 mm of the vessel was excised beginning at the insertion of the previously used side branch. Cross-sections (2 μm) were stained with Elastica–van Gieson for morphometric analysis or were used for immunohistochemical analysis.

Study Design
Two independent mouse models were used. In a first approach, we compared BALB/c wild-type (WT) mice and BALB/c CXCR3−/− deficient mice (CXCR3−/−). In a second approach, 129S1/SvImJ mice were treated daily with either neutralizing polyclonal anti-IP10/anti-MIG antibody cocktail (anti-IP10/anti-MIG polyclonal antibody [pAb]) or with control IgG 2 days before injury until the day of euthanasia. To exclude strain-related specific features, based on the BALB/c genetic background in our vascular injury model, we decided to use mice based on 129S1/SvImJ genetic background in the second approach.

Cell Culture
The human T-cell lymphoma cell line Jurkat (DSMZ) and the murine T-lymphocyte HT-2 clone A5E cell line based on a BALB/c background (American Type Culture Collection) were seeded on 96-well plates at a density of 10,000 cells per well. All cells were kept in medium for 24 hours with or without sirolimus (100 ng/mL) or everolimus (100 ng/mL) before subsequent stimulation with human IP10 (0.01 μg/mL) or murine IP10 (0.01 μg/mL), respectively, for 2 hours.

Immunohistochemical Analysis
Immunostaining used the streptavidin–alkaline phosphatase technique for smooth muscle actin (Dako) and streptavidin–horseradish peroxidase technique for CD3 (Serotech), CD45 (BD Bioscience), and CD31 (Santa Cruz Biotechnology). The Vectastaining-ABC-kit (Vector Laboratories) was used for c-kit (Santa Cruz Biotechnology) and caspase-3 (R&D Systems) detection. For CXCR3 coimmunostaining, we used a monoclonal antimouse CXCR3 antibody (R&D Systems) and combined it with the antibodies described above. Double-immunofluorescence staining for CD4/CD103 or CD8/CD103 was performed using PE-CD4, PE-CD8, and fluorescein isothiocyanate–CD103 (all eBioscience). For phospho-p70S6K detection, monoclonal phospho-p70S6K (Cell Signaling) was used. Binding of the primary antibodies was detected with compatible fluorescein-conjugated secondary Alexa Fluor antibodies (Invitrogen). For the detection of apoptotic cells, the In Situ Cell Death Detection Kit, Fluorescin (Roche) was used according to the specifications of the manufacturer.

Statistical Analysis
Data are reported as means±SEM. Statistical significance between groups was determined using t test or nonparametric Mann–Whitney test. Values of P<0.05 were considered statistically significant.

For real-time RT-PCR evaluation, the statistical differences in ratio were calculated by using the convergence interval.

Results

IP10 and MIG Induce mTORC1 Activation, Enhanced ROS Formation, and Apoptosis in T Cells
We first investigated the effect of IP10 on activation of mTORC1 in human Jurkat T cells, as well as in a murine T-cell line HT-2, by assaying phosphorylation of p70S6K (p*p70S6K), the major downstream target of mTORC1. Western blots analysis of IP10-stimulated T cells demonstrated increased phosphorylation and thereby activation of p70S6K (p*p70S6K) in both cell lines. Enhanced activation of p70S6K was completely abolished by the pharmacological mTORC1 inhibitors sirolimus and everolimus (Figure 1a and 1c).

Next, we analyzed whether activation of mTORC1 is required for mediating functional effects of IP10 on T cells such as formation of ROS and induction of apoptosis. Stimulation of T cells with IP10 resulted in significant generation of ROS and profoundly induced apoptosis (Figure 1b and 1d) in both cell lines. Strikingly, IP10-induced ROS formation and apoptosis were completely blocked by sirolimus and everolimus (Figure 1b and 1d). These data strongly suggest an essential role for mTORC1 in mediating the detrimental effects of IP10 on T cells and prompted us to analyze the effect of CXCR3 activation in vivo.

Enhanced Expression of IP10, MIG, and CXCR3 on Vessel Injury
To obtain the first evidence on the in vivo role of IP10 and MIG in arterial damage, we analyzed expression of the chemokines 14 days after arterial injury of the mouse femoral artery. In this model, intimal hyperplasia was already evident after 7 days and further progresses within 14 days after vascular injury (Figure 2a and 2b).22 Vascular injury significantly induced mRNA expression of IP10, MIG, and CXCR3 in the injured artery compared to noninjured controls (Figure 2c). Moreover, we also observed pronounced protein expression of IP10 and MIG in serum samples 7 and 14 days after wire injury (Figure 2d and 2e), indicating that both chemokines are also systemically upregulated on vessel injury.

Recruitment of CXCR3+ T Cells, Endothelial Cells, and Hematopoietic Progenitor Cells to the Site of Arterial Injury
To investigate whether enhanced expression of IP10, MIG, and CXCR3 is associated with increased recruitment of CXCR3+ positive cells to the site of injury, we analyzed expression of CXCR3 after vascular injury by immunofluorescence. Notably, we observed pronounced infiltration of CXCR3+ positive cells into the damaged vessel within 14 days after injury (Figure 3a). Because expression of CXCR3 is not restricted to T cells but has been identified on several cell types of the hematopoietic lineage, includ-
ing endothelial cells (ECs) and hematopoietic progenitor cells (HPCs), we characterized the recruited CXCR3 cells in further detail. Femoral arteries were coimmunostained at day 0, 7, and 14 after arterial injury for CXCR3 and several markers for the major cellular components of intimal tissue including CD3 (T cell), c-kit (HPC), CD31 (EC), and -actin (smooth muscle cells [SMC]) (Figure 3a and 3b). Infiltration of CD3 and c-kit cells was massively induced, whereas CD31 ECs were lost on arterial damage. Counting of double-positive CXCR3 and CD3 or c-kit cells revealed that a large portion of intimal CXCR3 cells corresponded to CD3 T cells (34.0%) and c-kit HPCs (27.6%). CXCR3 cells represented a lower extent CD31 ECs (25.7%). Enhanced expression of -actin in the injured vessel corresponds to the increased formation of neointima. Notably, -actin SMCs did not express CXCR3.

These results indicate that enhanced expression of CXCR3 is caused by increased infiltration of CXCR3 T cells but also to recruited HPCs, suggesting that the impact of the CXCR3 axis is not restricted to immune-mediated effects during vascular remodeling.

**CXCR3 Deficiency Diminishes Intimal Hyperplasia and Adventitial Neovascularization**

To analyze the role of IP10 and MIG-mediated activation of CXCR3 during vascular remodeling, we performed the vascular injury experiments in CXCR3 mice. Strikingly, deletion of CXCR3 resulted in pronounced reduction of intimal hyperplasia and lumen loss compared to WT mice 14 days after vascular injury (Figure 4a and 4b). In a second approach, we blocked the 2 major ligands of CXCR3, IP10 and MIG, by a neutralizing polyclonal anti-IP10/anti-MIG antibody cocktail. Similar to the CXCR3 mice, we found significantly reduced intimal hyperplasia and lumen loss in...
the anti-IP10/anti-MIG pAb–treated group compared to the control IgG–treated group (Figure 4c and 4d).

Because neovascularization has an impact on neointima formation after vascular intervention,23 we performed immunohistochemical staining of adventitial neovascularization. Using an antibody against endothelial CD31, we found strong adventitial neovascularization after vascular injury, which was less prominent in CXCR3-deficient mice (Figure II in the online data supplement). However, we did not detect a positive correlation between the extent of neovascularization and intimal hyperplasia (data not shown).

Reduced Recruitment of T Cells and HPCs in CXCR3-Deficient Mice Corresponds to Reduced Inflammation at the Site of Injury

To obtain insight into the underlying mechanisms of CXCR3-mediated effects on vascular remodeling, we first elucidated the influence of CXCR3 deficiency on the cellular composition of intimal tissue after vascular injury. Recruitment of CD3+ T cells was significantly reduced in CXCR3−/− mice compared to WT controls. This was accompanied by a massive decrease in the recruitment of CD45+ leukocytes. We also found significantly diminished recruitment of HPCs to the site of arterial injury in CXCR3−/− compared to WT mice (Figure 5a and 5b). Likewise, immunohistochemical evaluation of the anti-IP10/anti-MIG pAb–treated mice displayed the same phenotype as CXCR3−/− mice (Figure 5c and 5d).

To gain insight into specific T-cell subpopulations recruited to the lesion site, we analyzed potential indicators for the development of regulatory T cells such as CD4/CD8 and CD103 by immunofluorescence. As shown in Figure 6, comparable amounts of CD4 and CD8 T cells were recruited to the lesion. Coinmunostaining demonstrated that 40% of intimal CD4 cells were also CD103+, whereas 55% of CD8 cells coexpressed CD103. In contrast, in CXCR3−/− mice, we found a significantly decreased recruitment of all analyzed T-cell subsets (Figure 6).

IP10 and MIG Induce Activation of mTORC1 After Vascular Injury

To analyze the role of mTORC1 in mediating the effects of CXCR3 activation during vascular remodeling, we investigated activation of mTORC1 after arterial injury. Vascular injury caused profound activation of the mTORC1 kinase pathway, as demonstrated by an increased phosphorylation of p70S6K in the vessel wall (Figure 7). Most strikingly, deletion of CXCR3 almost abolished phosphorylation of p70S6K (Figure 7a). Profound activation of mTORC1 was also observed in control IgG–treated 129S1/SvImJ mice and was almost completely blocked by neutralizing of IP10 and MIG (Figure 7b).
These results clearly demonstrate that engagement of the CXCR3 chemokines IP10 and MIG not only governs recruitment of CXCR3$^+$ cells to the site of vascular injury but also represents the critical step for activation of mTORC1 in vascular cells during vessel remodeling.

IP10 and MIG Induce ROS Generation and Apoptosis of Vascular Cells After Arterial Injury

Because our in vitro data indicated that enhanced activation of mTORC1 is essential for increased ROS formation and induction of apoptosis in T cells (Figure 1), we analyzed whether enhanced mTORC1 activation also corresponds to high levels of ROS and apoptosis in the injured vessel. Whereas noninjured vessels of WT and CXCR3$^{-/-}$ mice displayed low levels of ROS and apoptosis in the injured vessel. Whereas noninjured vessels of WT and CXCR3$^{-/-}$ mice displayed low levels of ROS, as shown by low-intensity in situ dihydroethidine fluorescence, wire injury of WT mice markedly increased ROS generation. This increase was completely abolished in CXCR3$^{-/-}$ mice (Figure 8a). Moreover, we found a strong apoptotic reaction in the media of the injured artery of WT mice (Figure 8b and 8c). Inhibition of the CXCR3 axis either in CXCR3-deficient mice or in mice treated with neutralizing polyclonal anti-IP10/anti-MIG antibody cocktail dramatically reduced vascular apoptosis, as shown by a reduction in terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL)$^+$ and caspase-3$^+$ medial cells after vascular injury (Figure 8b and 8c and supplemental Figure Ia through Id).

Because CXCR3 is not expressed on vascular SMCs (Figure 3), IP10 and MIG did not directly induce ROS generation or apoptosis in SMCs (Figure 8d). To investigate the role of the CXCR3 axis for SMC activation, we performed experiments demonstrating that the cell-free supernatant of IP10-stimulated T cells induced ROS generation and apoptosis in SMCs (Figure 8e). Treatment of SMCs with...
sirolimus or everolimus completely abolished supernatant-induced ROS formation and induction of apoptosis (Figure 8e). To further elucidate which stimuli of the cell-free T-cell supernatant was responsible for the mTORC1-dependent activation of ROS and apoptosis in SMCs, we added the free radical scavenger N-acetylcysteine (NAC) to the supernatant. Under these antioxidative conditions, we observed a significant inhibition of ROS generation and apoptosis of supernatant-treated SMCs (Figure 8e).

**Discussion**

Here, we show that activation of the CXCR3 axis is essential for vascular remodeling in an in vivo mouse model of arterial injury. Enhanced intimal proliferation involves recruitment of inflammatory CXCR3$^+$ T cells, leukocytes, and HPCs to the site of injury, profound activation of mTORC1, induction of ROS, and pronounced apoptosis of vascular cells.

**The CXCR3 Chemokines IP10 and MIG Are Critical for Vascular Inflammation and Recruitment of HPCs After Vascular Injury**

Emerging evidence suggests a functional role for the T-cell chemoattractants IP10 and MIG, as well as their receptor CXCR3, in chronic inflammatory diseases. In our model of vascular injury, IP10 and MIG govern the early recruitment of CXCR3$^+$ T cells to the injured vessel and subsequent intimal hyperplasia. The intrinsic role of IP10 and MIG in vessel remodeling is indicated by local and systemic upregulation of both chemokines early after arterial injury, which is associated with the recruitment of CXCR3$^+$ T cells.

The presence of T cells in atherosclerotic lesions and intimal hyperplasia is well established, although its explicit role remains controversial. Our results appear to differ from those of Hansson et al and Remskar et al, who reported that depletion of T cells resulted in an increase of intimal thickening in response to injury. However, we hypothesize that the CXCR3 deficiency, rather, may lead to an
Figure 5. CXCR3 deficiency reduces recruitment of T cells, leukocytes, and HPCs. a and b, Immunohistochemical analysis of the cellular composition of intimal tissue from WT and CXCR3−/− mice 14 days after femoral artery injury. a, Representative sections of immunohistochemical stainings. Scale bar=100 μm. b, Quantitative analysis of immunohistochemistry for T cells (CD3), leukocytes (CD45), HPCs (c-kit), and SMCs (α-actin) (means±SEM; n=8). *P<0.05 vs WT mice. c and d, Immunohistochemical analysis of intimal tissue from mice treated with control IgG or anti-IP10/anti-MIG pAb after arterial injury. c, Representative sections of immunohistochemical stainings. Scale bar=100 μm. d, Quantitative analysis of immunohistochemistry (means±SEM; n=8). *P<0.05 vs control IgG–treated group.
Figure 6. CXCR3 deficiency decreases recruitment of CD4⁺CD103⁻ and CD8⁺CD103⁻ regulatory T cells. a, Double immunofluorescence staining of frozen arteries sections from WT and CXCR3⁻/⁻ mice 7 days after arterial injury for CD4 (red) and CD103 (green). Shown are representative images. Arrows demonstrate colocalization of CD4 with CD103. Scale bar = 100 μm. b, Quantification of the amount of CD4⁺ cells, CD4⁺CD103⁻ cells, and CD4⁺CD103⁻ cells (means ± SEM; n=8). *P<0.05 vs WT mice. c, Double immunofluorescence staining for CD8 (red) and CD103 (green). Shown are representative images. Arrows demonstrate colocalization of CD8 with CD103. Scale bar = 100 μm. d, Quantification of the amount of CD8⁺ cells, CD8⁺CD103⁻ cells, and CD8⁺CD103⁻ cells (means ± SEM; n=8). *P<0.05 vs WT mice.
immune modulatory effect in contrast to a complete inhibition of the Th1 immune response. In this context, our data are in strong congruency to previous findings in a mouse model of allograft rejection. Accordingly, CXCR3 deficiency was associated with a significant reduction in recruitment of leukocytes and all analyzed T-cell subsets, leading to a strong inhibition of intimal hyperplasia in a model of acute and chronic allograft rejection, as well as in our mouse model of vascular injury.

In addition to enhanced infiltration of lymphocytes, the CXCR3 chemokines IP10 and MIG are directly involved in the recruitment of HPCs to the injured vessel. CXCR3 is known to be expressed on CD34+ HPCs and regulates adhesion and aggregation of these cells. As shown by double-immunofluorescence staining, c-kit+ HPCs expressed the chemokine receptor CXCR3 and were recruited to the site of the injured vessel. Likewise, blocking the CXCR3 axis significantly reduced the portion of c-kit+ cells in the intimal tissue after vascular injury. Bone marrow–derived HPCs give rise to substantial numbers of neointimal ECs and SMCs after endothelial denudation with controversial effects on intimal hyperplasia. However, Sahara et al showed in wire injury experiments with c-kit−/sca-1−/lin− HPC-engrafted mice that medial and neointimal SMCs comprised up to 60% of HPCs, whereas no ECs originated from this cell fraction. Likewise, we found a strong correlation between recruitment of c-kit+ cells and intimal hyperplasia in our model (data not shown), whereas the extent of recruited c-kit+ cells and the degree of reendothelialization were negatively correlated (data not shown). This allows us to conclude that inhibition of recruitment of c-kit+ cells in CXCR3-deficient mice is an important factor in reduction of intimal hyperplasia but has no adverse effect on reendothelialization.

Previously, the CXC chemokine stromal cell–derived factor-1α and its receptor CXCR4 have been described as a central signaling axis regulating the homing of circulating HPCs to the injured vessel wall. Our data establish the CXCR3 axis as a second major player in the recruitment of HPCs to the site of vessel injury during vascular repair and remodeling.

**CXCR3-Dependent Activation of mTORC1 Is a Key Event in ROS Generation and Induction of Apoptosis After Vascular Injury**

Beyond recruitment of inflammatory T cells and HPCs, IP10 and MIG play an essential role in the activation of mTORC1 in vascular cells, increased ROS formation, and sustained induction of apoptosis after arterial injury.

Signaling by the highly conserved mTORC1 regulates cell growth and apoptosis and activates the specific downstream effector p70S6K by phosphorylation. Here, we demonstrate that IP10 directly induces activation of mTORC1 in T cells, as revealed by enhanced phosphorylation of p70S6K. Our in vitro data also suggest that increased ROS formation and apoptosis are causally linked to activation of mTORC1 because both could be effectively prevented by the mTORC1 inhibitors sirolimus and everolimus. Accordingly, induction of ROS and apoptosis was almost abolished in the absence of mTORC1 activation in CXCR3-deficient mice and on neutralization of the CXCR3 ligands IP10 and MIG in vivo. These results strongly indicate that CXCR3-dependent activation of mTORC1 directly links stimulation of the Th1 immune system with the proliferative response of intimal cells in vascular remodeling. In accordance with this notion, Wang et al recently demonstrated IFN-γ-induced activation of the mTORC1 complex in immunodeficient mice after adenoviral transfer of human IFN-γ. However, our in vitro data with T cells do not explain how the CXCR3 axis induces apoptosis of intimal SMCs in vivo.

![Figure 7. CXCR3-dependent activation of mTORC1 after arterial injury. mTORC1 activation in injured arteries was measured by immunofluorescence staining against p70S6 kinase 14 days after vascular injury in WT and CXCR3-deficient mice (a) and in mice treated with control IgG or neutralizing anti-IP10/anti-MIG pAb (b). Shown are representative images. Scale bar=100 μm. Statistical quantification of p70S6 kinase+ intimal cells after vascular injury: means±SEM; n=8; *P<0.05 vs control.](http://circres.ahajournals.org/)

Schwarz et al CXCR3 in Inflammatory Response to Arterial Injury

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Because CXCR3 was not expressed on vascular SMCs, IP10 and MIG cannot directly induce apoptosis of SMCs. Notably, the cell-free supernatant of IP10-stimulated T cells induced ROS generation and apoptosis in SMCs (Figure 8e). Treatment of SMCs with sirolimus or everolimus completely abolished supernatant-induced ROS formation and apoptosis (Figure 8e). These results indicate that mTORC1 activation is required to mediate the detrimental paracrine effects of T cells on SMCs. Because apoptosis of SMCs was also strongly inhibited by supplementing ROS scavengers, we hypothesize that ROS-mediated activation of mTORC1 contributes to apoptotic cell death. In accordance with this hypothesis, ROS generation has recently been shown to activate mTORC1 in vitro. However, we cannot rule out the possibility that, in addition to ROS, another mediator is involved in T cell–induced mTORC1 activation in SMCs, because the present study demonstrates that neutralization of ROS in the T-cell supernatant did not completely inhibit SMCs apoptosis.
Likewise, Th1 cells release numerous cytokines and mediators, such as IFN-γ, tumor necrosis factor-α, and interleukin-2, and future studies will help to define their role in our model. By all means, our in vitro data provide direct experimental support that CXCR3-mediated ROS production of T cells may induce mTORC1 activation and subsequent apoptosis of vascular SMCs in the injured vessel wall. T cell–induced apoptotic cell death of SMCs has previously been observed in a model of allograft atherosclerosis. In our model, wire-mediated arterial injury induced high levels of apoptotic vascular cells in the media of control mice. Likewise, the amount of apoptotic cells correlated positively with the number of recruited CD3+ T cells to the site of injury, emphasizing the impact of T cell–induced apoptosis in vascular remodeling. Because the number of apoptotic vascular cells was significantly decreased in CXCR3−/− and anti-IP10/anti-MIG pAb–treated mice, these data support the notion that CXCR3 and its ligands are crucial in T cell–mediated apoptosis of vascular cells.

The essential role of mTORC1 in apoptosis of SMC has also been implicated by our study, where we demonstrated that sirolimus or everolimus effectively inhibit apoptosis of SMCs in vivo and in vitro. Activation of mTORC1 has also been documented in intimal cells of atherosclerotic coronary arteries in patients. In clinical trials, sirolimus-coated stents abolished intimal proliferation and lumen loss in atherosclerotic coronary arteries, and mTORC1 inhibitors reduced the incidence of intimal thickening in coronary arteries of cardiac allografts. Recently, a role of mTORC1 in inflammatory reactions and in the recruitment of HPCs to the site of vascular injury has widened the impact of mTORC1 activation in vascular remodeling and healing.

Taken together, the present study demonstrates a fundamental role of the Th1 immune response in repair and regeneration of vascular tissue after vessel injury yielding the CXCR3-dependent activation of mTORC1 as the interface between Th1 immune response and proliferative processes in vascular disease. These data may provide a rationale for the development of novel therapeutic strategies aiming at the inhibition of the CXCR3 axis.

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Disclosures

None.

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Retraction

Novel Role of the CXC Chemokine Receptor 3 in Inflammatory Response to Arterial Injury: Involvement of mTORC1: Retraction

The authors of the following article have requested that it be retracted from publication in *Circulation Research*:


Dr Johannes Schwarz admitted to manipulating in vitro data concerning IP-10–induced effects in T cells and SMCs (Figure 1 and Figure 8e). All coauthors involved in this study, other than Dr Schwarz, had no knowledge of any scientific impropriety related to the collection, analysis, or presentation of these in vitro data in this manuscript. Dr Schwarz apologizes for any adverse consequences that may have resulted from the article’s publication and any inconvenience and wasted effort that this may have caused the scientific community and readers of the journal.

Reference


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Supplement Material

Supplemental Materials and Methods

Animals
Specific pathogen-free 129S1/SvIMJ mice weighing between 25 and 30g were purchased from Charles River (Germany). BALB/c wild-type mice (WT) and BALB/c CXCR3-deficient mice (CXCR3\(^{-/-}\)) were kindly provided by Martin Bek (Münster, Germany). CXCR3\(^{-/-}\) mice were normal in appearance, growth and fertility. Neither a direct vascular or cardiac phenotype is described in the literature. However, CXCR3\(^{-/-}\) mice show profound resistance to acute and chronic allograft rejection by significant reduction in recruitment of CD45 cells, macrophages and CD4/CD8 T cell subsets.\(^1\) Moreover, blockade of CXCR3 promotes renal fibrosis.\(^2\)

All experimental procedures performed on animals met the requirements of German legislation on protection of animals and were approved by the Government of Bavaria/Germany.

Model of endothelial denudation by wire-mediated arterial injury
Surgery was carried out using a microscope (Carl Zeiss, Germany) and microsurgical instruments (FST, Germany) as described previously.\(^3\) At time points indicated, mice were euthanized by dislocation of the cervical spine under inhalation anesthesia using a whole body chamber. The mice were then quickly perfused with ice cold ringer solution and further prepared on ice. The femoral artery was dissected and 5.0mm of the vessel beginning at the insertion of the previously used side branch were excised. Samples were fixed in 4% paraformaldehyde overnight at 4°C, and embedded in paraffin. Cross-sections (2µm) were stained with Elastica-van-Gieson for morphometric analysis or were used for immunohistochemical analysis.
**Antibodies**

Rabbit antisera to IP10 and MIG were produced by Biosynthesis (USA) using a synthetic murine IP10-specific peptide (CIHIDDGPVRMRAIGK) and a synthetic murine MIG-specific peptide (CISTSRGTIHYKSLKDLKQFAPS). These reagents have previously been shown to be specific for IP10 and MIG, respectively. Unspecific IgG control was also purchased from Biosynthesis using serum of non-immunized rabbits. All IgGs were purified by IgG columns, dialyzed and diluted to the same concentration. Mice were injected daily with 0.6mg unspecific IgG s.c. or with an antibody mixture of 0.3mg IP10-IgG and 0.3mg MIG-IgG s.c..

**Study design**

Two independent mouse models were used. In a first approach, we compared CXCR3\(^{+/−}\) (WT) mice (n\(≥\)7) with CXCR3\(^{−/−}\) mice (n\(≥\)7).

In a second approach, 129S1/SvIMJ mice were divided into two different groups (each n\(≥\)7). The control (control IgG) mice were injected daily with 0.6 mg control IgG s.c. starting two days before femoral artery injury and continuing until the day of sacrifice. To block the two CXCR3 chemokines IP10 and MIG, the neutralizing polyclonal anti-IP10/anti-MIG antibody cocktail (anti-IP10/anti-MIG pAb) was injected daily s.c. with a polyclonal antibody mixture of antibody against murine IP10 (0.3mg) and murine MIG (0.3mg) two days before injury until the day of sacrifice.

In both studies, mice were euthanized at three time points (7, 14 days) post femoral injury and non-injured vessels were used as a negative control.

**Real-time RT-PCR**

Preparation of mRNA, cDNA synthesis, and PCR amplification were performed as described previously\(^5\,\text{6}\). After extraction (Dynabeads\textsuperscript{®} Oligo(dT), Invitrogen, USA), RNA was reverse transcribed using superscript II reverse transcriptase (Invitrogen) and CFL5C primer
(Metabion, Germany). PCR (annealing 65°C) was performed with Expanded Long Template PCR system (Roche, Switzerland) with the following primers (CXCR3: # Mm00438259_m1; IP10: #Mm99999072_m1; MIG: # Mm01345157_m1; GAPDH: # Mm99999915_g1; Applied Biosystems, USA). Expression of CXCR3, IP10, and MIG mRNA was normalized to GAPDH in the same sample.

**Cell culture**

Human coronary SMCs (Clonetics, USA) were cultured according to manufacturer’s instruction. Cells were used at passage 4-7 plated into 96 well plates at a density of 10 000 cells/well and stimulated at 80% confluency. The human T cell lymphoma cell line Jurkat (DSMZ, Germany) and the murine T lymphocyte HT-2 clone A5E cell line based on a BALB/c background (ATCC, USA) were also seeded on 96 well plates in the same density. All cells were kept in medium 24 hours with or without sirolimus (100ng/ml, Sigma, USA) or everolimus (100ng/ml, Sigma) before subsequent stimulation with human IP10 (0.01µg/ml, Sigma) or respectively murine IP10 (0.01µg/ml, CRG-2, Sigma) for 2 hours. The ROS scavenger N-acetyl-L-cysteine (NAC, 10mM, Sigma) was added 5 min prior to stimulation with supernatant. Afterwards, analysis of apoptosis was performed according to the manufacture’s instruction (In Situ Cell Death Detection Kit, Fluorescein, TUNEL technology, Roche) using Safire Multi-Detection Monochromometer Microplate Reader (Tecan, Switzerland). For measurement of ROS production, cells were loaded with dichlorodihydrofluorescein diacetate 5µmol/L (Invitrogen) and the dichlorofluorescein (DCF) fluorescence was measured by Safire Multi-Detection Monochromometer Microplate Reader. Finally, cell viability and activity were measured by ROS and TUNEL assay by alamar blue (Invitrogen) absorbance. Each sample was normalized by monitoring alamar blue absorption.
Morphometric Analysis

For morphometric analysis, 10 cross sections from each vessel were used. The first section was adjacent to the side branch used for guide wire insertion and subsequent sections were obtained at a distance of 50µm. Digital microscopic images (10x) of the sections taken with AxioVision 2.0 (Carl Zeiss) were analyzed using ScionImagebeta 4.0 software (Scion Corporation, USA). By measuring the circumference of the lumen, the internal elastic membrane (IEL) and the external elastic membrane (EEL) in all 10 cross sections per vessel, the following parameters were calculated: The medial area comprises the space between IEL and EEL and the intimal area represents the space between IEL and lumen. Non-injured vessels served as controls (n>5).

Immunohistochemical analysis

For histology and immunohistochemistry, mouse femoral arteries were fixed in 4% paraformaldehyde (pH 7.0) and embedded in paraffin. Serial paraffin sections (2µm) were deparaffinized, dehydrated, and pressure-cooked for 7 minutes in citrate buffer (10mM, pH 6.0) for antigen retrieval, followed by blocking of endogenous peroxidase (3% H₂O₂/dH₂O; 15min). Immunostaining employed the streptavidin-alkaline phosphatase technique (Dako Real Detection System, APAAP, Dako, Denmark) for smooth muscle actin (Dako) and streptavidin-horseradish-peroxidase technique (Dako ChemMate Detection Kit, LSAB, Dako) for CD3 (Serotec, Germany), CD45 (BD Bioscience, USA) and CD31 (Santa Cruz, USA). The Vectastaining-ABC-kit (Vector Laboratories, USA) was used for c-kit (Santa Cruz), and Caspase-3 (R&D Systems) detection. For CXCR3 co-immunostaining we used a monoclonal anti-mouse CXCR3 antibody (R&D Systems) and combined it with the antibodies described above. Double-immunofluorescence staining for CD4-CD103 or CD8-CD103 was performed using PE-anti-mouse-CD4, PE-anti-mouse-CD8 and FITC-anti-mouse-CD103 (all eBioscience, USA). For phospho-p70 S6 kinase detection monoclonal phospho-p70 S6 kinase
(Cell Signaling, USA) was used. Binding of the primary antibodies was detected with compatible fluorescein conjugated secondary Alexa Fluor antibodies (Invitrogen). For the detection of apoptotic cells in the media area the In Situ Cell Death Detection Kit, Fluorescein (TUNEL, technology, Roche) was used according to the manufacturer’s specifications. Nuclei were counterstained with DAPI (Sigma) and covered for examination with mounting media for fluorescence (Vector Laboratories). All immunohistochemical slides were evaluated by counting all positive cells of the respective area, expressed as the number of positive cells per $10^3 \mu m^2$ area or $100 \mu m$ circumference. For quantification of neovascularization in adventitial area, the number of CD31$^+$-cells was counted in four $10000 \mu m^2$ sized high power fields of representative sections and adjusted to the adventitial area. TUNEL staining was evaluated by counting all positive cells of the medial area.

**Western blot analysis**

Western blotting was carried out using standard methods. Briefly, Jurkat-cell or respectively HT-2-cell homogenates were separated on SDS-PAGE, blotted on a nitrocellulose membrane (Amersham Bioscience), and probed with p70 S6 kinase and phospho-p70 S6 kinase specific antibodies (Cell Signaling). Equal loading of wells was ascertained by the use of a monoclonal anti-β-actin (Sigma) antibody. Antibodies were visualized by the enhanced chemiluminescence system (Santa Cruz). Relative quantification of p*P70 S6 kinase was performed by ImageJ measurement of the optical density of bands for p*P70 S6 kinase and normalizing these values to the optical density of the corresponding band for β-actin.

**ELISA**

Blood samples were collected from all animals at day 0, 7, and 14 after vascular injury. The amount of IP10 and MIG in serum was quantified by using immunoassays with anti-mouse CXCL10/IP10 and anti-mouse CXCL9/MIG antibodies (R&D Systems).
Detection of ROS in vivo

Vascular ROS was determined by quantitating formation of dihydroethidine (DHE) as described. Frozen, enzymatically intact 30μm-thick sections of non-injured and injured femoral arteries were incubated at the same time with DHE (10μmol/liter) in PBS for 30 min at 37°C in a humidified chamber protected from light.
Supplemental References


Legends to Supplemental figures

Online Figure I: Injury-induced apoptosis of vascular cells is diminished after blockade of the CXCR3-ligands.

TUNEL analysis (a,b) as well as caspase-3 staining (c,d) was performed in control IgG-treated and anti-IP10/anti-MIG pAb-treated mice 14 days after vascular injury. (a,c)

Representative section; scale bar=100µm. (b,d) Quantification of the immunostaining for TUNEL\(^+\) and caspase-3\(^+\) cells. (Mean±SEM, n>10, *=p<0.05 vs. control IgG-treated group).

Online Figure II: CXCR3-deficiency impairs neovascularization after vascular injury.

(a,b) Immunohistochemical analysis of neovascularization in WT and CXCR3\(^{-/-}\) mice 14 days after femoral artery injury. (a) Representative sections of immunohistological stainings, scale bar=100µm. (b) Quantitative analysis of immunohistochemistry for ECs (CD31)(Mean±SEM, n=8, *=p<0.05 vs. WT mice). (c,b) Immunohistochemical analysis of neovascularization in mice treated with or a neutralizing antibody cocktail against IP10 and MIG (anti-IP10/anti-MIG pAb). (c) Representative sections of immunohistological stainings, scale bar=100µm. (d) Quantitative analysis of immunohistochemistry for ECs (CD31)(Mean±SEM, n=8, *=p<0.05 vs. control IgG-treated group).
Supplement Material

Online Figure II

(a) CD31

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(b) CD31

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(d) CD31

CD31 cells/10^3 µm² adventitial area

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