Activation of Endothelial Toll-Like Receptor 3 Impairs Endothelial Function

Sebastian Zimmer, Martin Steinmetz, Tobias Asdonk, Inga Motz, Christoph Coch, Evelyn Hartmann, Winfried Barchet, Sven Wassmann, Gunther Hartmann, Georg Nickenig

Rationale: Endothelial dysfunction and atherosclerosis are chronic inflammatory diseases characterized by activation of the innate and acquired immune system. Specialized protein receptors of the innate immune system recognize products of microorganisms and endogenous ligands such as nucleic acids. Toll-like receptor 3 (TLR3), for example, detects long double-stranded RNA and is abundantly expressed in endothelial cells. Whether innate immunity contributes to atherogenic mechanisms in endothelial cells is poorly understood.

Objective: We sought to determine the effects of TLR3 activation in endothelial cells.

Methods and Results: We first investigated whether stimulation of TLR3 influences endothelial biology in mice. Intravenous injection of polyinosine polycytidylic acid, a synthetic double-stranded RNA analog and TLR3 ligand, impaired endothelium-dependent vasodilation, increased vascular production of reactive oxygen species, and reduced reendothelialization after carotid artery injury in wild-type mice compared with controls but had no effect in TLR3<sup>−/−</sup> animals. TLR3 stimulation not only induced endothelial dysfunction but also enhanced the formation of atherosclerotic plaques in apolipoprotein E–deficient mice. In vitro incubation of endothelial cells with polyinosine polycytidylic acid induced production of the proinflammatory cytokines interleukin-8 and interferon-γ–induced protein 10, increased formation of reactive oxygen species, diminished proliferation, and increased apoptosis, which suggests that endothelial cells are able to directly detect and respond to TLR3 ligands. Neutralization of interleukin-8 and interferon-γ–induced protein 10 antagonizes the observed negative effects of polyinosine polycytidylic acid. We found elevated levels of circulating endothelial progenitor cells in polyinosine polycytidylic acid–treated mice, although they displayed increased endothelial dysfunction. Stimulation of TLR3 in cultured endothelial progenitor cells, however, led to increased formation of reactive oxygen species, increased apoptosis, and reduced migration. Injection of endothelial progenitor cells that had been incubated with polynosine polycytidylic acid ex vivo hindered reendothelialization after carotid artery injury. Therefore, endothelial progenitor cell function was affected by TLR3 stimulation. Finally, apolipoprotein E–deficient/TLR3-deficient mice exhibited improved endothelial function compared with apolipoprotein E–deficient/TLR3<sup>+/+</sup> littermates.

Conclusions: Immunorecognition of long double-stranded RNA by endothelial cells may be an important mechanism involved in endothelial cell activation and development of endothelial dysfunction. (Circ Res. 2011; 108:00-00.)

Key Words: endothelium • immune system • inflammation • molecular biology

Endothelial dysfunction and atherosclerosis are chronic inflammatory diseases characterized by an accumulation of immune cells in the vascular wall. Activation of the immune system is evident in all stages of atherogenesis and involves both innate and acquired immune responses. The mechanisms of acquired immunity have been studied extensively, but the contribution of innate immunity and its involvement in disease development are largely unknown. Unlike acquired immunity, the innate immune system relies on a limited number of highly conserved receptors to detect “foreign” pathogens. These so-called pattern-recognition receptors are expressed by multiple cell types and specialize in the detection of pathogen-associated molecular patterns common to entire classes of pathogens. This provides the host with an immediate response to invading pathogens, independent of immunogenic experience.
A set of pattern-recognition receptors is specialized for the immunorecognition of viral nucleic acids. For instance, long double-stranded RNA is detected by toll-like receptor (TLR) 3 and MDA5 (melanoma differentiation–associated gene 5)2–5; CpG motifs in DNA by TLR9; double-stranded DNA by DAI (DNA-dependent activator of interferon-regulatory factors)7 and AIM2 (absent in melanoma 2)8; single-stranded RNA containing certain sequence motifs by TLR7/8; or blunt-end double-stranded RNA containing a triphosphate at the 5’ end by RIG-I (retinoic acid inducible gene 1).10,11 Activation of these nucleic acid–detecting receptors induces a complex signaling cascade that results in the production of type I interferons and inflammation.12

Endothelial function is essential for vascular integrity. The endothelium provides a barrier, regulates vascular tension, and is involved in angiogenesis and blood clotting. Local and systemic inflammation, however, can impair endothelial function and lead to cellular damage.13 Damaged endothelial cells are renewed by healthy adjacent cells or by circulating progenitor cells. Both endothelial cells and endothelial progenitor cells (EPCs) are affected by inflammation, but to what extent they contribute to or are involved in inflammation is not yet known.14

Here, we hypothesize that immunorecognition of double-stranded RNA by TLR3 influences endothelial integrity by inducing proinflammatory and proatherosclerotic mechanisms. We therefore studied the consequences of TLR3 stimulation on endothelial biology in mice, cultured endothelial cells, and EPCs.

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Animals and Procedures

Eight- to 12-week-old C57Bl/6J wild-type mice, TLR3-deficient mice (TLR3−/−; C57BL/6J genetic background), apolipoprotein E (ApoE)–deficient mice (ApoE−/−; C57BL/6J genetic background), and ApoE−/−/TLR3−/− mice (C57BL/6J genetic background) were used for the present study. For endothelial function tests, vascular reactive oxygen species (ROS) production analysis, and quantification of circulating and spleen-derived EPCs, wild-type and TLR3−/− mice were injected intravenously with either 100 µg of polynosine polycytidylic acid (polyIC) suspended in 200 µL of 0.9% NaCl solution or vehicle every 48 hours for 7 days. For investigation of atherosclerotic plaque development, 10-week-old ApoE−/− mice received a high-fat, cholesterol-rich diet that contained 21% fat, 19.5% casein, and 1.25% cholesterol for a total of 7 weeks and were injected intravenously with 100 µg of polyIC or vehicle once in week 5 and then every other day during the last 2 weeks of diet. For endothelial function tests in TLR3-deficient ApoE−/− mice, ApoE−/−/TLR3−/− and ApoE−/−/TLR3+/− were fed the same high-fat, cholesterol-rich diet for 10 weeks. Aortic segments, spleen, blood, and carotid arteries were collected and processed immediately after the animals were euthanized.

Results

To investigate whether activation of the innate immune system and specifically TLR3 might be involved in the inflammatory vascular development of atherosclerosis, C57Bl/6J mice received an intravenous injection of vehicle or 100 µg of polyIC, a synthetic long double-stranded RNA analog, every other day for 7 days. All mice showed no external signs of side effects; body weight, and food and water intake remained unchanged (data not shown). Because TLR3 is expressed in endothelial cells of human atherosclerotic plaques (Online Figure I) and endothelial dysfunction is an essential step of atherogenesis, we first assessed endothelial function in aortic segments of polyIC- and vehicle-treated mice using organ chamber experiments. Mice treated with vehicle displayed normal endothelium-dependent vasodilation, whereas TLR3 stimulation significantly impaired endothelial function (Figure 1A; maximum relaxation: vehicle 19.5 ± 6.5% versus polyIC 45.8 ± 7.7%, n = 9 to 10). Endothelium-independent vasodilation (Figure 1B; n = 9 to 10) and KCl- and phenylephrine-induced vasoconstriction (data not shown) were not affected by polyIC treatment. Another main mediator of vascular dysfunction is oxidative stress. Total vascular ROS production was therefore determined in aortic rings with L-012–enhanced chemiluminescence. PolyIC-treated mice displayed significantly increased aortic ROS formation compared with controls (Figure 1C; vehicle 100 ± 16.8% versus polyIC 189 ± 26.8%, n = 9 to 10). Increased ROS release can be caused by uncoupling of endothelial nitric oxide synthase 3, but endothelium-dependent vasodilation was reversed after the addition of N6-nitro-L-arginine methyl ester, which indicates that no functionally relevant endothelial nitric oxide synthase 3 uncoupling was present in aortic tissue of these mice, and there was no difference between vehicle- and polyIC-treated mice (data not shown). Dihydroethidium-enhanced microscopy was used to study which cells were responsible for the increased ROS formation. Although both the intima and media showed increased radial production, the effect on endothelial cells was predominant (Online Figure II).

To determine whether these biological effects of polyIC were indeed mediated by TLR3 stimulation, we analyzed TLR3-deficient (TLR3−/−) mice. TLR3−/− mice were treated with polyIC every other day for 7 days, as described above. PolyIC had no effect on endothelium-dependent vasodilation (Figure 1D; n = 10), endothelium-independent vasodilation (Figure 1E; n = 10), or aortic ROS production (Figure 1F; n = 10). Therefore, polyIC-induced endothelial dysfunction was a specific result of TLR3 stimulation. TLR2 and TLR4 have been associated with atherosclerosis development and known to affect endothelial cell biology. We therefore investigated TLR2/4 mRNA expression in aortic tissue from wild-type and TLR3−/− mice to determine whether TLR3 deficiency affected TLR2/4 expression. Aortic...
TLR2 and TLR4 mRNA expression was similar in wild-type and TLR3/H11002/H11002 mice (Online Figure III).

Vascular damage develops in response to reoccurring endothelial lesions, and vascular health is therefore dependent on the regenerating capacity of the endothelium. Wild-type and TLR3/H11002/H11002 mice were treated with vehicle or polyIC via intravenous injection every other day for a total of 10 days and were subjected to carotid artery injury on day 5. A 4-mm segment proximal to the bifurcation was denuded, and the rate of reendothelialization was assessed after staining with Evans blue. PolyIC treatment significantly inhibited reendothelialization in wild-type mice but had no effect in TLR3/H11002/H11002 mice (Figures 2A and 2B; wild-type: vehicle 27.5±3.0% versus polyIC 55.2±4.6%; TLR3/H11002/H11002: vehicle 28.8±2.7% versus polyIC 30.0±3.8%; n=5 to 7). Together, these data suggest that the immune response to TLR3 stimulation is associated with three fundamental endothelial mediators of atherosclerosis, namely, endothelial dysfunction, increased vascular ROS production, and reduced reendothelialization.

The effects of TLR3 stimulation on atherosclerotic plaque formation were therefore studied in ApoE/H11002/H11002 mice. Ten-week-old ApoE/H11002/H11002 mice received a high-fat, cholesterol-rich diet for 10 weeks. PolyIC treatment significantly inhibited reendothelialization in wild-type but not TLR3/H11002/H11002 mice, which suggests that TLR3 stimulation hinders endothelial repair. *P<0.05 compared with vehicle-treated wild-type mice. A, PolyIC treatment significantly impaired reendothelialization in wild-type but not TLR3/H11002/H11002 mice, which suggests that TLR3 stimulation hinders endothelial repair. *P<0.05 compared with vehicle-treated wild-type mice. B, Representative carotid arteries. Black bars indicate the remaining denuded area.

Figure 1. PolyIC treatment impairs endothelial function but has no effect in TLR3/H11002/H11002 mice. To determine whether TLR3 activation affects endothelial function in vivo, C57Bl6 mice received intravenous injections with vehicle or 100 μg of polyIC every 48 hours for 7 days. A, Stimulation of TLR3 is associated with significantly impaired maximum endothelium-dependent vasodilation, whereas endothelium-independent vasodilation is not affected (B), which together indicates direct endothelial dysfunction. C, Total ROS production was also significantly increased in aortic segments of mice treated with polyIC, which highlights the increased vascular oxidative stress. Next, TLR3/H11002/H11002 mice were injected with vehicle or 100 μg of polyIC as described above to examine whether the observed polyIC effects in wild-type mice were mediated via TLR3. Endothelium-dependent (D), endothelium-independent (E), and aortic ROS production (F) were not affected by polyIC treatment, which suggests a specific TLR3-induced effect in wild-type mice. *P<0.05 compared with vehicle-treated wild-type mice.

Figure 2. Activation of TLR3 hinders reendothelialization. Endothelial repair is essential for vascular health and is reduced in patients with atherosclerosis. To test the cumulative effects of TLR3 stimulation on endothelial repair, wild-type and TLR3/H11002/H11002 mice were injected with 100 μg of polyIC every other day for 10 days and were subjected to carotid artery injury (4-mm electric denudation) on day 5. Reendothelialization was assessed after staining with Evans blue. A, PolyIC treatment significantly impaired reendothelialization in wild-type but not TLR3/H11002/H11002 mice, which suggests that TLR3 stimulation hinders endothelial repair. *P<0.05 compared with vehicle-treated wild-type mice. B, Representative carotid arteries. Black bars indicate the remaining denuded area.
diet for a total of 7 weeks and were injected intravenously with 100 µg of polyIC or vehicle once in week 5 and then every other day during the last 2 weeks of diet. Similar to wild-type mice, endothelium-dependent vasodilation was significantly impaired by polyIC treatment in ApoE−/− mice (Figure 3A; maximum relaxation: vehicle 79.6 ± 6.8% versus polyIC 50.6 ± 5.7%, n = 9), whereas endothelium-independent vasodilation was not affected (Figure 3B; n = 9). Vascular ROS formation was also significantly increased by TLR3 stimulation (Figure 3C; vehicle 100 ± 6.8% versus polyIC 180 ± 37.9%, n = 9). ApoE−/− mice that received vehicle developed only minimal atherosclerotic lesions above the valve leaflets (Figure 3E; n = 9). In contrast, those mice treated with polyIC showed extensive plaques throughout the aortic arch (Figures 3D and 3E; vehicle 29.6 ± 4.0% versus polyIC 67.1 ± 2.5%, n = 9). Thus, TLR3 stimulation potentiates the development of atherosclerosis.

These results pose the question of whether endothelial cells, as direct targets, are able to detect and respond to TLR3 ligands. Consequently, we studied in vitro cytokine production of endothelial cells on stimulation with polyIC. Endothelial cells were incubated with 4 ng/mL polyIC or vehicle for 24 hours. The cytokine concentration in the supernatant medium was then analyzed by enzyme-linked immunosorbent assay. TLR3 activation increased the concentration of the proinflammatory cytokines interleukin-8 (IL-8) and interferon-γ-induced protein 10 (IP-10) but not that of IL-6 (Figure 4A; IL-8: vehicle 3248 ± 251 pg/mL versus polyIC 5685 ± 946 pg/mL; IP-10: vehicle 63 ± 29 pg/mL versus polyIC 1222 ± 835 pg/mL; IL-6: vehicle 867 ± 70 pg/mL versus polyIC 1104 ± 154 pg/mL; n = 4 to 6). TLR3 expression in endothelial cells was also investigated after stimulation with polyIC. TLR3 activation led to a significant upregulation of TLR3 mRNA (Figure 4B; vehicle 1.0 ± 0.1 2−ΔΔCt versus polyIC 40.9 ± 5.4 2−ΔΔCt, n = 4). These results suggest that endothelial cells are activated by polyIC through TLR3.

Although inflammatory cytokines can be atherogenic, activation of TLR3 may also directly affect endothelial cell biology and thus support vascular lesion development. Our in vivo experiments showed increased ROS formation and reduced reendothelialization, both signs of endothelial dysfunction. We therefore investigated TLR3-induced ROS production in endothelial cells. Incubation with polyIC led to a significant increase in oxidative stress with L012-enhanced chemiluminescence (Figure 4C; vehicle 100% versus polyIC 133.4 ± 4.5%, n = 5) and as measured by 2′,7′-dichlorofluorescein fluorescence microscopy (Online Figure IV, A and B; vehicle 1259 ± 16.2 versus polyIC 1368 ± 27.5 mean fluorescence intensity, n = 9). Endothelial rejuvenation is dependent in part on proliferation, apoptosis, and migration of resident endothelial cells, and because TLR3 stimulation impaired in vivo reendothelialization, we sought to determine which
endothelial properties were affected. First, aortic endothelial outgrowth assays were performed from wild-type mice treated with vehicle or 100 μg of polyIC/mL cell culture medium for 7 days. In all mice that received vehicle injections, an abundant cellular outgrowth was noted (Online Figure V, a & A; n = 6). Surprisingly, no cellular outgrowth was evident in any of the polyIC-treated mice (Online Figure V, b and B; n = 6). Therefore, we studied the individual effects in cultured endothelial cells. Here, TLR3 stimulation significantly inhibited proliferation (Figure 4D; vehicle 55.4 ± 2.5% versus polyIC 30.6 ± 1.2% of cells stained positive for bromodeoxyuridine, n = 4 to 5), induced apoptosis (Figure 4E; vehicle 100 ± 3.5% versus polyIC 136.2 ± 6.9% of control, n = 5), and impaired migration (Figure 4F; vehicle 29.5 ± 10.1% versus polyIC 55.5 ± 5.7% remaining cell-free area of total scratch area, n = 5). To examine whether countering oxidative stress and proinflammatory cytokines can negate the negative effects of polyIC, we performed endothelial scratch assays. Preincubation of endothelial cells with the superoxide scavenger tiron had no effect on in vitro reendothelialization; however, preincubation with either an anti-IP-10 neutralizing antibody, an anti-IL-8 neutralizing antibody, or the combination of both completely antagonized the negative effects of polyIC (Figure 4F; tiron: vehicle 23.6 ± 4.1% versus polyIC 54.7 ± 7.9%; anti-IP-10: vehicle 23.6 ± 11.0% versus polyIC 14.4 ± 4.4%; anti-IL-8: vehicle 19.6 ± 9.6% versus vehicle 14.0 ± 10.3%; anti-IP-10 and anti-IL-8: vehicle 10.0 ± 6.4% versus polyIC 9.9 ± 4.4% remaining cell-free area of total scratch area; n = 5). These in vitro results propose a significant role for immunorecognition of double-stranded RNA in human endothelial biology.

The endothelium is not a static tissue, and circulating EPCs contribute to the restoration of damaged endothelial cells. The rejuvenation capacity of these EPCs has been linked specifically to the number and function of available EPCs. We therefore studied EPC numbers in response to TLR3 stimulation in vivo. Wild-type, TLR3−/−, and ApoE−/− mice were treated as detailed above, and blood was collected. Although polyIC treatment was associated with impaired endothelial function, TLR3 stimulation led to a significant increase of scal1/flk1-positive cells in peripheral blood of wild-type and ApoE−/− mice (Figure 5A; wild-type: vehicle 100 ± 7.9% versus polyIC 165 ± 14.4% of control; ApoE−/−: vehicle 104 ± 3.3% versus polyIC 131 ± 11.4% of control; n = 9 to 10) but not of TLR3−/− mice (Figure 5A; TLR3−/−: 100 ± 6.4% versus polyIC 105 ± 3.3% of control, n = 10). Not all EPCs circulate constantly, and the spleen is known as a functional reservoir for EPCs. Hence, the spleens of all mice were collected, and mononuclear cells were isolated by density gradient and cultured according to previously developed protocols. Stimulation of TLR3 significantly increased the number of spleen EPC colony-forming units in wild-type but not TLR3−/− or ApoE−/− mice (Figure 5B; wild-type: vehicle 100 ± 3.6% versus polyIC 317 ± 91.8% of control; TLR3−/−: 100 ± 17.8% versus polyIC 94 ± 10.3% of control; ApoE−/−: vehicle 98 ± 23.7% versus polyIC 104 ± 35.9% of control; n = 9 to 10).

Although elevated numbers of circulating EPCs are generally associated with improved endothelial function, we found increased numbers of EPCs in polyIC-treated mice with dysfunctional endothelium. This may have been caused by a direct effect of TLR3 stimulation in EPCs. EPC function was thus tested in response to polyIC treatment in vitro. Produc-
ApoE type of EPCs) was increased significantly in wild-type and sca1/flk1-positive particles (circulating angiogenic cells, a sub-

type of EPCs) was increased significantly in wild-type and TLR3<sup>−/−</sup> mice that had been incubated ex vivo with vehicle or polyIC after carotid artery injury. Ex vivo incubation with polyIC of EPCs derived from wild-type mice significantly impaired reendothelialization after carotid artery injury (Figures 7A and 7B; wild-type EPCs: vehicle 0.42±0.03 mm<sup>2</sup> versus polyIC 1.02±0.04 mm<sup>2</sup>, n=6). Injection of wild-type mice with ex vivo polyIC-incubated EPCs derived from TLR3<sup>−/−</sup> mice had no effect on reendothelialization compared with controls (Figures 7A and 7B; TLR3<sup>−/−</sup>-EPCs: vehicle 0.48±0.05 mm<sup>2</sup> versus polyIC 0.50±0.06 mm<sup>2</sup>, n=6). Therefore, EPC function was directly impaired by TLR3 stimulation and contributed to endothelial dysfunction.

Finally, to investigate whether TLR3 activation innately contributes to endothelial dysfunction in atherogenesis independent of external stimulation, we studied ApoE/TLR3 double-knockout mice. ApoE<sup>−/−</sup> and ApoE<sup>−/−</sup>/TLR3<sup>−/−</sup> littermates received a high-fat, cholesterol-rich diet for 10 weeks. Endothelium-dependent vasodilation in ApoE<sup>−/−</sup>/TLR3<sup>−/−</sup> mice was improved compared with ApoE<sup>−/−</sup> mice (Figure 8A; maximum relaxation: ApoE<sup>−/−</sup> 36.7±4.4% versus ApoE<sup>−/−</sup>/TLR3<sup>−/−</sup> 76.7±10.4%, n=6). No difference in endothelium-independent vasodilation (Figure 8B) or KCl- or phenylephrine-induced vasoconstriction (data not shown) was noted. These results suggest that TLR3 is involved in atherogenesis independent of synthetic stimulation.

Discussion

The main contenders in vascular inflammation have been identified, but the rules by which they act remain unclear. Recent evidence from the literature suggests that vascular inflammation is not a mere macrophage crusade but rather an elaborate campaign that involves both resident smooth muscle and endothelial cells, as well as circulating cells. Here, we show that endothelial cells are immune competent and can actively participate in innate immunity. Stimulation of the specific pattern-recognition receptor TLR3 on endothelial cells induced inflammatory and atherogenic mechanisms in endothelial cells in vitro and was associated with damaging

![Figure 5. TLR3 activation increases the number of EPCs.](http://circres.ahajournals.org/)

The spleen represents a reservoir for EPCs, and early-outgrowth EPCs can be cultured from isolated spleen-derived mononuclear cells. B. The number of EPC colony-forming units (CFU) was increased significantly in wild-type but not TLR3<sup>−/−</sup> or ApoE<sup>−/−</sup> mice after TLR3 stimulation. "P<0.05 compared with vehicle-treated wild-type mice; "P<0.05 compared with vehicle-treated ApoE<sup>−/−</sup> mice.

![Figure 6. PolyIC directly affects EPCs in vitro.](http://circres.ahajournals.org/)

Although elevated numbers of circulating angiogenic cells are generally associated with improved endothelial function, we found increased numbers of circulating angiogenic cells in polyIC-treated mice with dysfunctional endothelium. Circulating angiogenic cell function in response to TLR3 stimulation was therefore tested in vitro. Incubation of cultured circulating angiogenic cells with 4 ng of polyIC per 1 mL of cell culture medium for 24 hours significantly increased production of ROS (A) and elevated apoptosis (B). In endothelial scratch assays, incubation with polyIC significantly inhibited the migration of late-outgrowth EPCs to a similar degree as cultured endothelial cells. "P<0.05.
processes in vivo. The results of the present study support a concept in which a vicious circle of cellular damage, endogenous RNA release, and TLR3 activation contributes to the development of endothelial dysfunction.

We found an increased production of the proinflammatory cytokines IP-10 and IL-8 by endothelial cells on exposure to the TLR3 stimulus polyIC. IP-10, also known as chemokine (C-X-C motif) ligand 10 (CXCL10), is a proinflammatory cytokine secreted by monocytes, endothelial cells, and fibroblasts in response to exogenous type I interferons or TLR ligands that induce type I interferon, including TLR3. IP-10 binds to CXCR3 and has been associated directly with the development of atherosclerosis. Heller et al even demonstrated that formation of atherosclerotic plaques is significantly inhibited in ApoE \(^{-/-}\)/IP-10 \(^{-/-}\) mice compared with ApoE \(^{-/-}\) control mice. IP-10 on endothelial cells is involved in the transendothelial migration/recruitment of leukocytes into the vascular wall. IL-8 (CXCL8) is produced by endothelial cells, smooth muscle cells, and macrophages in the vessel wall. It binds to the G-protein–coupled serpentine receptors CXCR1 and CXCR2 and has chemotactic and mitogenic effects. Expression of IL-8 is amplified in atherosclerotic plaques, and circulating IL-8 has been proposed as a biomarker for coronary artery disease. Thus, increased IP-10 and IL-8 secretion through TLR3 stimulation could have detrimental effects on vascular health.

Not only inflammation induced by cytokines but also direct effects of TLR3 activation in endothelial cells can contribute to atherogenesis. We have shown that endothelial production of ROS is increased by TLR3 stimulation. The rapid formation of ROS by immune cells, as in the oxidative burst of neutrophils and macrophages, is an important mechanism of encountering pathogens. ROS release can lead to cellular dysfunction and damage, and it is well established that ROS contribute to all stages of endothelial dysfunction and atherosclerosis development. The TLR3-stimulated ROS formation by endothelial cells in the present study is consistent with TLR3-induced ROS induction in neutrophils and macrophages.

Additionally, we found increased apoptosis and reduced proliferation of endothelial cells in response to polyIC. Together, these polyIC effects on endothelial cells could be responsible for the compromised endothelial function and regenerating capacity in vivo, as reflected by the impaired endothelium-dependent vasodilation and impaired reendothelialization in the present experiments. A different aspect of endothelial function, namely, hemostasis balance, in response to polyIC treatment has been studied by Shibamiya and colleagues. In their model, polyIC upregulated tissue factor and downregulated thrombomodulin expression by endothelial cells. In vivo, polyIC elevated D-dimer levels, which indicates increased coagulation and fibrinolysis. This further...
ther corroborates the critical role of TLR3 in endothelial cell function.

Although polyIC treatment is associated with an increased number of circulating EPCs, reendothelialization is reduced. This seemingly contradictory finding could be explained by 2 mechanisms. First, TLR3 stimulation could directly impair EPC function. We found elevated apoptosis and increased ROS formation by EPCs in response to polyIC. Second, the TLR3-induced increase in EPC numbers may be the consequence of severe endothelial damage, and EPC numbers may simply not be sufficient to keep up with the increased repair demand.

The primary function of TLR3 is thought to be the detection of viral RNA. Although there is evidence of viral products in atherosclerotic plaques, it seems unlikely that recurrent or chronic viral infections are a major contributor to atherosclerotic development. Recent studies demonstrated that short RNA sequences similar to those used for RNA interference (small interfering RNA, or siRNA) are sufficient to stimulate TLR3.22 Although small interfering RNA has no full TLR3 ligand activity (unpublished data) and may have additional effects on microRNA (RNA-induced silencing complex consumption), small interfering RNA is not comparable to the well-established TLR3 ligand polyIC used in the present study; still, the observation by Cho et al that small interfering RNA inhibits neovascularization through activation of TLR3 is well in agreement with our findings. The cellular damage that leads to this endogenous TLR3 ligand release is found in all stages of atherosclerosis development and may therefore be of significant impact.

Other TLRs have also been associated with the development of atherosclerosis. TLR4, a transmembrane receptor for lipopolysaccharide, for example, is expressed in low levels in healthy arterial walls but is upregulated in atherosclerotic plaques. Furthermore, it is concentrated in the shoulder region, where a plaque is most likely to rupture.23 TLR4 plaques. Furthermore, it is concentrated in the shoulder healthy arterial walls but is upregulated in atherosclerotic

region, where a plaque is most likely to rupture.23 TLR4 plaques. Furthermore, it is concentrated in the shoulder healthy arterial walls but is upregulated in atherosclerotic lipopolysaccharide, for example, is expressed in low levels in

Other TLRs have also been associated with the development of atherosclerosis. TLR4, a transmembrane receptor for lipopolysaccharide, for example, is expressed in low levels in healthy arterial walls but is upregulated in atherosclerotic plaques. Furthermore, it is concentrated in the shoulder region, where a plaque is most likely to rupture.23 TLR4 stimulation induces production of proinflammatory cytokines and adhesion molecules by endothelial cells and mediates the release of matrix metalloproteinase by macrophages.24 Interestingly, mice deficient in TLR4 and MyD88 (myeloid differentiation factor 88) show reduced atherosclerosis development.25 TLR2 is also expressed in human atherosclerotic plaques. Stimulation of TLR2 significantly enhances neointima formation and promotes atherosclerotic plaque development.26 In contrast to TLR3, these receptors are specialized in detecting products of bacterial membranes, which, although sometimes found in atherosclerotic plaques, are ubiquitously present. Endogenous TLR3 ligands, however, are released constantly during cellular damage and could be important for all stages of atherogenesis.

Together, the present data provide novel insights into the functional role of innate immunity in endothelial biology and support the potential involvement of TLR3 in the pathomechanisms of atherosclerosis. TLR3 is expressed by endothelial cells, and endogenous RNA ligands are readily available through continuous cellular damage. Because TLR3 severely compromises endothelial function and promotes atherosclerotic plaque development, we propose that inhibition of TLR3 function may diminish TLR3-dependent endo-

thelial dysfunction and thus provide a potentially novel target for the treatment of atherosclerosis.

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Disclosures
None.

References


Novelty and Significance

What Is Known?

- Pattern-recognition receptors contribute to vascular inflammation and atherogenesis.
- The Toll-like receptor 3 (TLR3) is activated by viral long double-stranded RNA and endogenous RNA released by cellular apoptosis and necrosis, and it induces a proinflammatory cellular response.
- Short single-stranded RNA, such as those used in RNA inhibition, stimulate TLR3 in endothelial cells and inhibit neangiogenesis.

What New Information Does This Article Contribute?

- Specific TLR3 stimulation impairs endothelial function, hinders reendothelialization, increases vascular oxidative stress, and promotes the development of atherosclerotic plaques in mice.
- The innate immune response induced by TLR3 activation results in mobilization of endothelial progenitor cells, but their vascular protective functional capacity is reduced.
- TLR3 deficiency improves endothelial function in ApoE−/− mice.

Atherosclerosis is the leading cause of death in the Western world; however, the mechanisms underlying atherosclerotic lesion formation remain poorly understood, and the contribution of innate immunity to atherogenesis is largely unknown. TLR3 is a pattern-recognition receptor abundantly expressed in endothelial cells and is specialized in the immunorecognition of viral and endogenous RNA. TLR3 ligands are released by vascular cell damage and are thus readily available throughout the stages of atherogenesis. Our data demonstrate that stimulation of TLR3 induces inflammatory and atherogenic mechanisms in endothelial cells in vitro and promotes atherosclerotic plaque development by impairing endothelial function in vivo. Because TLR3 activation so severely compromises endothelial function, we propose that inhibition of TLR3 function may diminish TLR3-dependent endothelial dysfunction and thus provide a potentially novel target for the treatment of atherosclerosis.
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Detailed Methods:

Animals and procedures

8-12 week-old C57BL/6J wild-type mice, toll like receptor 3-deficient mice (TLR3-/-, C57BL/6J genetic background), Apolipoprotein E-deficient mice (ApoE-/-, C57BL/6J genetic background) from Charles River and ApoE-/- TLR3-/- (C57BL/6J genetic background) were used for this study. All animals were maintained in a 22°C room with a 12-hour light/dark cycle, and received food and drinking water ad libitum. For endothelial function tests, vascular ROS production analysis, and quantification of circulating and spleen derived endothelial progenitor cells wild-type and TLR3-/- mice were injected intravenously with either 100 μg polyinosine polycytidylic acid (polyIC, from Sigma Aldrich) suspended in 200 μl 0.9% NaCl solution or vehicle every 48 hours for seven days. For investigation of atherosclerotic plaque development, 10-week-old ApoE-/- mice received a high-fat, cholesterol-rich diet that contained 21% fat, 19.5% casein, and 1.25% cholesterol (Ssniff) for a total of 7 weeks and were injected intravenously with 100μg polyIC or vehicle once in week 5, and then every other day during the last two weeks of diet. For investigation of atherosclerotic plaque development, 10-week-old ApoE-/- mice received a high-fat, cholesterol-rich diet for 10 weeks. Aortic segments, spleen, blood and carotid arteries were collected and processed immediately after sacrifice. All animal experiments were performed in accordance with institutional guidelines and the German animal protection law.

Aortic ring preparations and tension recording

Vasodilation and vasoconstriction of isolated aortic ring segments were determined in organ baths filled with oxygenated modified Tyrode buffer (37°C), as previously described ¹⁻³. Adventitial tissue was carefully removed, and 3-mm segments of the thoracic aorta were investigated. A resting tension of 10 mN was maintained throughout the experiment. Drugs were added in increasing concentrations in order to obtain cumulative concentration-response curves: KCl 20 and 40 mmol/l, phenylephrine 1 nmol/l -10 μmol/l, carbachol 10 nmol/l -100 μmol/l (assessment of endothelium-dependent vasodilation after precontraction with phenylephrine), and nitroglycerin 1 nmol/l -10 μmol/l (assessment of endothelium-independent vasodilation after precontraction with phenylephrine). The drug concentration was increased when vasoconstriction or -relaxation was completed. To assess potential eNOS uncoupling, L-NAME (100 μmol/l) was added at the end of the carbachol protocol. Drugs were washed out before the next substance was added.

Measurement of reactive oxygen species

ROS release in intact aortic segments was determined by L-012 chemiluminescence, as previously described ¹. Aortas were carefully excised and placed in chilled, modified Krebs-HEPES buffer. Connective tissue was removed and aortas were cut into 2 mm segments. Chemiluminescence of aortic segments was assessed in scintillation vials containing Krebs-HEPES buffer with 100 μmol/l L-012 over 15 min in a scintillation counter (Lumat LB 9501, Berthold) in 1 min intervals. The vessel segments were then dried and dry weight was determined. ROS release is calculated as relative chemiluminescence per mg aortic tissue. For measuring cellular ROS production endothelial cells were incubated with polyIC or
vehicle for 4 hours, trypsinated, pelleted, resuspended in Krebs-HEPES buffer with 100 μmol/l L-012 and immediately place in the scintillation counter. Intracellular ROS production in endothelial cells was measured by 2', 7'-dichlorofluorescein (DCF; 10 μmol/L) fluorescence microscopy, as described previously 4. The relative fluorescence intensity is the average value of all experiments. For the detection of vascular ROS production, 5μm cryosectioned slides of the aortic bulbus were incubated with 2μM dihydroethidium in Krebs-HEPES buffer at 37°C for 30min and the examined under a Zeiss Axiovert 200M microscope using AxioVision software.

**Cellular aortic outgrowth**

Wild-type mice were treated with vehicle or 100μg polyIC every other day for seven days, as described above. The descending aorta was excised, fatty and connective tissue removed and then cut into 5mm segments. The short segments were opened longitudinally and placed face-down on a fibronectin-coated dish. They were then incubated in endothelial cell growth medium (PromoCell) at 37°C and 5% CO₂ for 120 hours. Images were acquired with a Zeiss Axiovert 200M microscope and the area of cellular outgrowth measured using AxioVision software.

**Carotid artery injury**

Carotid artery injury was performed on day 5 of polyIC treatment prior to the third polyIC injection. All mice were anesthetized with intraperitoneal injections of 150 mg/kg body weight ketaminehydrochloride (Ketanest, Pharmacia) and 0.1 mg/kg body weight xylazinehydrochloride (Rompun 2%, Bayer). A small incision from the cranial apex of the sternum to just below the mandible was made. After careful preparation of an approximately 6 mm long segment proximal of the bifurcation, the common carotid artery was electrically denuded. A 4 mm long lesion was made by applying two serial 5 second bursts of 2 Watt using a 2 mm wide forcep. The skin was then sutured and the mice allowed to recover in individual cages before returning to their litters. On day 10 of polyIC treatment, 50 μl Evan’s blue solution was injected intravenously and allowed to circulate for 5 min. The mice were then sacrificed and both common carotid arteries fully excised. The arteries were rinsed in 0.9 % NaCl solution and the residual connective tissue carefully removed. Images were taken, and the total lesion area (4 mm) and remaining denuded area (stained blue) measured using AxioVision version 4.5.0 software (Zeiss). Re-endothelialisation is expressed by illustrating the remaining denuded area.

**Histological analysis of atherosclerotic plaques**

Hearts with ascending aortas were embedded in Tissue Tek OCT embedding medium (Miles), snap-frozen, and stored at −80°C. Samples were sectioned on a Leica cryostat (6 μm), starting at the apex and progressing through the aortic valve area into the ascending aorta and the aortic arch, and were placed on slides. For the detection of atherosclerotic lesions, aortic cryosections were fixed with 3.7% formaldehyde for 1h, rinsed with deionised water, stained with oil red O working solution (0.5%) for 30 min, and were rinsed again. Hematoxylin staining was performed according to standard protocols. All sections were examined under a Zeiss Axiovert 200M microscope using AxioVision version 4.5.0 software. For quantification of atherosclerotic plaque formation in the aortic root, lipid-staining area and total area of serial histological sections were measured. Atherosclerosis data are expressed as lipid-staining area in percent of total surface area. The investigators who performed the histological analyses were blinded to the treatment of the respective animal group.
Cell culture

HCAEC (Lonza) and HUVEC (PromoCell) were cultured on 6 cm dishes in endothelial cell growth medium (PromoCell). Experiments were performed with cells of passages 5 to 8 when grown to 70-80% confluence. For stimulation of TLR3, HCAEC, HUVEC and EPC were incubated with 4 ng polynosine polycytidylic acid (Sigma)/μl cell culture medium or vehicle.

Cytokine quantification

HCAEC were stimulated with polyIC for 24 hours and the concentration of IL-6, IL-8 and IP-10 in the supernatant medium then determined by ELISA. Commercially available kits for human IL-6, IL-8 and IP-10 (R&D Systems) were used according to the manufactures protocols.

Real-time PCR

For analysis of gene expression in cultured HCAEC, cells were lysed using a 10G needle and homogenized with a motorized homogenizer. RNA was isolated with peqGOLD RNA-Pure (peqLAB Biotechnology). RNA concentration and quality was verified with a spectrophotometer. Then, 1 μg of the isolated total RNA was reversely transcribed using Omniscript RT Kit (Qiagen) according to the manufactures protocol. The single-stranded cDNA was amplified by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) with the TaqMan system (ABI-7500 fast PCR System) using SYBR-Green dye. For TLR3, the primers 5’-CCT GGT TTG TTA ATT GGA TTA ACG A-3’ and 5’-TGA GGT GAA GTG TTG CAA AGG-3’, for TLR2 5’- AAG GCA TTA AGT CTC CGG AAT TAT C-3’ and 5’-TCG CTT AAG TGA AGA GTC AGG TGA T-3’, for TLR4 5’-CCT CTG CCT TCA CTA CAG AGA CTT T-3’ and 5’-TGT GGA AGC CTT CCT GGA TG-3’, and for 18s rRNA 5’- TTG ATT AAC TCC CTG CCC TTT GT-3’ and 5’-CGA TTC TCC GAG GGC CTC ACT A-3’ were used. For quantification, TLR2/3/4 mRNA expression was normalized to endogenous 18s rRNA.

Apoptosis and proliferation

To determine HCAEC and EPC apoptosis, the Cell Death Detection ELISA Kit (Roche) was used according to the manufacturers instructions. Briefly, HCAEC or EPC were cultured and stimulated as mentioned previously for 24 hours. Cells were pelleted, lysed and transferred to a streptavidin-coated microplate well. Anti-histone (biotin-labelled) and anti-DNA (peroxidase-conjugated) antibodies were added leading to the binding of nucleosome complexes to streptavidin. Samples were incubated with peroxidase substrate (ABTS) and the coloured product was measured spectrophotometrically. Apoptosis was calculated in enrichment factor, which equals the millimass unit of sample (dying or dead cells) distributed to millimass unit of corresponding control (viable cells); mU absorbance (10^{-3}). The results are shown in relative proportion to the control group. Proliferation of HCAEC was assessed by detecting the incorporation of bromodeoxyuridine (BrdU) after a 24 hour co-incubation with BrdU and polyIC. BrdU positive nuclei were determined using anti-BrdU antibody (Abcam) and total number of cells using DAPI staining.

NADPH oxidase activity
NADPH oxidase activity was measured by a lucigenin-enhanced chemiluminescence assay in buffer B containing phosphate 50 mmol/l (pH 7.0), EGTA 1 mmol/l, protease inhibitors (Complete, Roche), sucrose 150 mmol/l, lucigenin 0.005 mmol/l, and NADPH 0.1 mmol/l. Endothelial cells were incubated with vehicle or polyIC for 4h, mechanically scratched from the cell culture dish and suspended in ice-cold buffer B lacking lucigenin and substrate. Total protein concentration was adjusted to 1 mg/ml. Aliquots of the protein sample (100 µl) were measured over 10 minutes in quadruplicates using NADPH as substrate in a scintillation counter (Berthold Lumat LB 9501) in 1-minute intervals.

Flow cytometry

Murine blood samples were analyzed as previously described 5. Following red cell lysis, the viable lymphocyte population was analyzed for sca-1-FITC (Becton Dickinson) and flk-1-PE (Becton Dickinson). Isotype identical antibodies and unstained samples served as controls in every experiment (Becton Dickinson). Cell fluorescence was measured immediately after staining using a FACS Calibur instrument (Becton Dickinson). Data were analyzed using CellQuest software (Becton Dickinson). Units of all measured components are specific events obtained after measuring 20,000 events in a pre-specified lymphocyte gate during FACS analysis.

EPC cell culture

EPC were isolated from murine spleen and cultured in accordance with previously published methods 6. Briefly, mononuclear cells (MNC) were isolated using Ficoll density gradient (Biocoll, Biochrom) and seeded into 24-well tissue culture plates pre-coated with human fibronectin (Sigma) at 1x10^6 (acLDL/lectin staining) or 4x10^6 (CFU-Hill) cells/ml in endothelial basal medium (EBM) and supplements as recommended by the manufacturer (Promocell). After 7 days of culture, fibronectin-adherent cells were washed and incubated with 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine (Dil)-labelled acetylated low-density lipoprotein (acLDL; CellSystems) and stained with FITC-labeled Ulex europaeus agglutinin (lectin; Sigma). Cells double-positive for acLDL and lectin staining were judged to be EPC 5. At least sixteen high-power field fluoroscopy microscopic scans of each well were analyzed, and mean values were calculated. For CFU-Hill, cells were isolated and cultured as described previously. After 2 days of initial culture, 1x10^6 cells derived from the supernatant were re-seeded per well and cultured for additional 7 days in EBM on human fibronectin pre-coated wells 7, 8. The number of colonies was manually counted using light microscopy at the end of the incubation period.

Endothelial scratch assay

Endothelial scratch assays were performed as previously described 5. Briefly, endothelial cells and late EPC were grown to confluence, incubated with 4ng polyIC/ml cell culture medium for 18h, scratched with a sterile pipette, and then a marked position photographed every 2 hours for 6 hours. The remaining cell free area was measured and correlated (in percent) to the initial scratched area. For pretreatment with antioxidants and neutralizing antibodies, endothelial cells were incubated with either Tiron 50µM/ml (4,5-Dihydroxy-1,3-benzenedisulfonic acid disodium salt, Sigma Aldrich), anti-human IP-10 neutralizing antibody (0.05µg/ml, R&D Systems), or anti-human IL-8 neutralizing antibody (10µg/ml, R&D Systems) for 90min and then treated with 4ng/ml polyIC or vehicle. After 18h of incubation the cell culture plates were scratched and photographed at 0 and 24 hours.
**EPC transfusion study**

The EPC transfusion study was performed in accordance with previously published protocols by Losordo et al.\(^\text{10}\). To enhance the number of circulating infused EPC, splenectomy was performed in all mice 7 days before carotid denudation and EPC transfusion. The spleen was removed with vessel ligation through a lateral incision in the left abdominal wall. Cultured spleen-derived EPC from wild-type and TLR3-/− mice were incubated with vehicle or 4 ng polyIC/ml cell culture medium for 24 hours after the first medium change on day 4. They were then washed and incubated with fresh, polyIC-free medium until day 7. Recipient mice were administered 1x10^6 of EPC by intravenous injection from tail vein just after induction of arterial injury.

**Human coronary arteries**

1-2 cm segments of the left anterior descending artery were collected post mortem during autopsy from 10 patients. The segments were formalin-fixed and embedded in paraffin. 10 μm thick sections were placed on glass slides and stained with hematoxylin and eosin according to standard protocols. For detection of TLR3 expression, immunohistochemical stains were performed. Briefly, slides were deparaffinized, rehydrated and boiled in Tris/EDTA buffer (pH 9.0) for 15 min. They were then washed twice in TBS plus 0.025 % Triton X-100 for 5 min and subsequently incubated in 10 % normal goat serum with 1 % BSA in TBS for 2 hours at room temperature. Next, the primary antibody (rabbit polyclonal TLR3 antibody, ab52429, Abcam) was diluted 1:500 in TBS and the slides allowed to incubated overnight at 4°C. Immunodetection was accomplished using an alkaline phosphatase conjugated goat anti-rabbit secondary antibody (1:2500 dilution, 60 min at 22 °C, Sigma Chemical) and SigmaFast BCIP/NBT chromogen tablet system according to the manufactures protocols. The slides were finally counterstained with hematoxylin.

**Statistical analysis**

Data are presented as mean ± standard error of mean (SEM). For statistical analysis, 2-tailed, unpaired Student's t-test and ANOVA for multiple comparisons were employed where applicable. Post-hoc comparisons were performed with the Neuman-Keuls test. P<0.05 indicates statistical significance.
Supplemental Figures and Figure Legends

Online Figure I:

**TLR3 is expressed in human atherosclerotic plaques.** To examine whether TLR3 is expressed in human atherosclerotic plaques, immunohistochemical stains of paraffin embedded human coronary arteries were performed. A representative immunohistochemical stain shows TLR3 expression in the endothelial lining (black arrows, A) and macrophages in the vessel wall (white arrows, B).
Online Figure II:

**TLR3 stimulation increases endothelial ROS formation.** To determine the cellular ROS source in the vascular wall, we used a modified dihydroethidium enhanced microscopy. A representative image of the descending thoracic aorta from vehicle-treated mice shows normal background ROS formation distributed across the vessel. In polyIC-treated mice there is increased ROS production primarily in the endothelium.
Online Figure III:

**TLR3 deficiency does not affect expression of aortic TLR2 and TLR4.** Toll like receptor 2 and 4 have been associated with atherosclerosis development and known to affect endothelial cell biology. To determine if TLR3 deficiency results in altered TLR2/4 expression, we investigated TLR2/4 mRNA expression in aortic tissue from wild-type and TLR3-/- mice. There is no significant difference in aortic TLR2 or TLR4 mRNA expression between wild-type and TLR3-/- mice.
**Online Figure IV**

**TLR3 activation increases cellular ROS formation.** The production of reactive oxygen species by endothelial cells was examined by 2', 7'-dichlorofluorescein (DCF; 10 μmol/L) fluorescence microscopy. Incubation of endothelial cells with polyIC significantly increased cellular ROS production (**A**). Representative image of DCF fluorescence microscopy (**B**). The NADPH oxidase is a major source of cellular oxidative stress. We therefore investigated NADPH oxidase activity using a lucigenin-enhanced chemiluminescence assay. PolyIC incubation however had no effect on endothelial NADPH oxidase activity (**C**). (*p<0.05*)
Online Figure IV

A  Endothelial ROS Formation

B  Vehicle  polyIC

C  NADPH Oxidase Activity in Endothelial Cells
Online Figure V:

Aortic cellular outgrowth is abolished by TLR3 stimulation. Since TLR3 stimulation impaired *in vivo* re-endothelialisation, we sought to determine which endothelial properties are affected (migration, proliferation, etc.). Wild-type mice were treated with vehicle or 100µg polyIC every other day for seven days. Short segments of the descending thoracic aorta were excised, cleaned from fatty and connective tissue, placed on cell culture dishes coated with collagen type IV, and incubated in endothelial growth medium for five days. In all mice that received vehicle injections an abundant cellular outgrowth was noted (a & A, n=6). Surprisingly, no cellular outgrowth was evident in any of the polyIC-treated mice (b & B, n=6). Representative images of the cellular outgrowth are shown (a & b at 72 hours of incubation, A & B at 120 hours of incubation).

![Aortic Outgrowth Graph](image-url)
Online Table I

Details of the endothelium-dependent vasodilation curves. PolyIC-treatment of wild-type and ApoE/- mice significantly impairs the maximum response to carbachol. In TLR3/- mice intravenous polyIC injection do not affect endothelium-dependent vasodilation, suggesting the observed effects are TLR3-mediated.

<table>
<thead>
<tr>
<th>Endothelium-dependent vasodilation curve</th>
<th>EC 50 Carbachol (log mol/l)</th>
<th>Max. response Developed Tension (% of max. Contraction)</th>
</tr>
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<tbody>
<tr>
<td>Wild-type + Vehicle</td>
<td>-5.9 ± 0.17</td>
<td>16.9 ± 8.0</td>
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<tr>
<td>Wild-type + polyIC</td>
<td>-5.3 ± 0.34</td>
<td>40.0 ± 13.0 *</td>
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<td>ApoE/- + polyIC</td>
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Supplemental References


