Int J Clin Exp Pathol 2013;6(12):2813-2823
www.ijcep.com /ISSN:1936-2625/IJCEP1309032

Original Article

TLR2-deficiency of cKit$^+$ bone marrow cells is associated with augmented potency to stimulate angiogenic processes

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Received September 11, 2013; Accepted October 21, 2013; Epub November 15, 2013; Published December 1, 2013

Abstract: Objective: Toll-like receptor 2 (TLR2)-deficiency is associated with the preservation of vascular function and TLR2-deficient (TLR2$^{-/-}$) mice exhibit increased neovascularization following induction of hindlimb ischemia. Hematopoietic stem cells play an important role in ischemia-induced angiogenesis and we now investigated whether the effects observed in TLR2$^{-/-}$ mice may be attributed to TLR2 deficiency on bone marrow-derived stem cells. Approach and Results: cKit-positive (cKit$^+$) bone marrow cells (BMC) were isolated from wild type (WT) and TLR2$^{-/-}$ mice employing MACS-bead technology. Co-incubation of TLR2$^{-/-}$cKit$^+$ BMC with mature endothelial cells (ECs) resulted in increased tube formation of ECs on matrigel, augmented sprouting in a 3D-collagen matrix and increased migratory capacity compared to co-incubation with WT cKit$^+$ BMC. In an *in vivo* matrigel plug assay, TLR2$^{-/-}$cKit$^+$ BMC exhibited enhanced formation of capillary-like networks. In a murine model of hindlimb ischemia, administration of TLR2$^{-/-}$ cKit$^+$ BMC to WT mice augmented capillary density and reperfusion of ischemic M. gastrocnemius muscle tissue to the level of TLR2$^{-/-}$ mice. Western Blot analysis revealed comparable expression of CXCR4 on TLR2$^{-/-}$cKit$^+$ BMC but increased activation of the PI3K downstream signaling molecule protein kinase B (PKB/AKT) compared to WT cKit$^+$ cells. Conclusions: The absence of TLR2 on cKit$^+$ BMC is associated with augmented potency to support angiogenic processes *in vitro* and *in vivo*. Functional inhibition of TLR2 may therefore provide a novel tool to enhance stem cell function for the treatment of vascular diseases.

Keywords: TLR2, cKit, hematopoietic stem cells, angiogenesis, ischemia

Introduction

Toll-like receptors (TLRs) are pattern recognition receptors that can induce innate immune responses. Activation of TLRs by exogenous ligands such as pathogens or endogenous danger-associated molecular patterns (DAMPs) results in the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and the promotion of a pro-inflammatory milieu [1]. Recently, Toll-like receptor 2 (TLR2) has been shown to be involved not only in the activation of inflammatory cells at sites of tissue injury or local infection, but also in mediating increased inflammatory cell egress from the bone marrow [2]. During infection or application of TLR2 ligands, activation of TLR2 on hematopoietic stem cells triggers their differentiation towards the myeloid cell lineage [3], resulting in increased numbers of circulating immune cells such as macrophages [4]. Although augmented generation of cells belonging to first line host defense is mandatory to fight acute infection, TLR2-mediated modulation of stem- and progenitor cell fate inside or outside of the bone marrow may imbalance the provision of the number or potency of regular stem cell progeny. For example, TLR2 agonists have been shown to impair proliferation and maturation of neuronal and glial progenitor cells [5, 6]. In turn, blockade of TLR2 reduced the dental pulp stem cell suppressing effect of pathogens in the oral cavity [7]. In the bone marrow, TLR2-signaling in stem cells has been proposed to

result in the depletion of endothelial progenitor cell pools required for vascular regeneration and these effects were absent in TLR2-deficient ($TLR2^{-/-}$) mice [8].

TLR2 deficiency and functional blockade have been associated with the preservation of vascular function. Prevention from excess TLR2-mediated activation of inflammatory cascades protected the vasculature from ischemia and reperfusion injury [9], atherosclerosis lesion progression [10, 11] and endothelial injury in response to cerebral [12, 13], myocardial [14] and skeletal muscle ischemia [15]. Although evidence points towards endothelial TLR2 in mediating at least part of these vasculoprotective effects, the contribution of the absence of TLR2 signaling in stem- and progenitor cells that can participate in vascular regenerative processes has not been determined so far.

In the present study, we compared the potency of cKit-positive ($cKit^+$) bone marrow-derived stem cells (BMCs) from $TLR2^{-/-}$ mice to those isolated from WT mice to augment endothelial cell functions relevant to angiogenic processes. $cKit^+$ cells were co-incubated with mature endothelial cells and investigated for their capacity to modulate endothelial tube formation, endothelial sprouting and migration *in vitro*. Furthermore, the capillary-like tube formation activity of $TLR2^{-/-}cKit^+$ BMC was compared to WT $cKit^+$ BMC *in vivo* and their potency to modulate neovascularization processes in response to ischemia was analyzed employing a second *in vivo* mouse model, the murine model of hindlimb ischemia. Moreover, both $TLR2^{-/-}cKit^+$ and WT $cKit^+$ BMC were analyzed for their expression of CXCR4 and activation of the CXCR4 downstream signaling molecule protein kinase B (PKB)/AKT relevant to progenitor cell homing [16].

Materials and methods

Isolation of cKit⁺ bone marrow-derived cells

$cKit^+$ cells were isolated from 8-10 week old WT (C57BL/6J) or $TLR2^{-/-}$ mice (B6.129-$Tlr2^{tm1Kir}$/J) as described previously [17]. Mouse bone marrow was isolated from femur and tibia and cell suspensions were incubated with magnetic microbeads coated with anti-cKit monoclonal antibody (Miltenyi Biotec, Bergisch Gladbach, Germany). MS columns® and the MiniMacs® cell separator system were employed to obtain $cKit^+$ cell fractions. For fluorescence labeling of $cKit^+$ cells, cells were incubated with 2.5 µg/mL CellTracker™ CM-DiI (Invitrogen, Karlsruhe, Germany).

Cell culture of endothelial cells

Human Umbilical Vein Endothelial Cells (HUVECs) were purchased from PromoCell, Germany, and cultivated in endothelial growth medium (EndoPrime Kit, PAA, United Kingdom) supplemented with 10% fetal calf serum on gelatin-coated dishes (Attachment Factor, Gibco, Germany). Cells were harvested by trypsinization (0.05%, Gibco, Germany) and used from passage 2 to 5.

Matrigel angiogenesis assay

As described previously [18], $1x10^4$ HUVECs were incubated alone or in the presence of either $3x10^3$ WT or $TLR2^{-/-}cKit^+$ BMC in duplicate in 100 µL endothelial growth medium including reagents or vehicle for 8 hours in 96-well plates precoated with 70 µL Matrigel Basement Membrane Matrix (BD Bioscience, USA). Tubular HUVEC structures and $cKit^+$ cells were photographed using a fluorescence microscope (Leica, Germany) employing 100x magnification at 8 random high power fields (HPF) per variant. Tubular length was assessed per high-power field employing ImageProPlus Software, CA, USA. Per independent experiment, mean values of all variants were expressed as relative to control set to 1.0.

Spheroid angiogenesis assay

$3.2x10^4$ HUVECs were suspended either alone or together with $8x10^3$ WT or $TLR2^{-/-}cKit^+$ cells in 10 mL endothelial basal medium containing 20% methylcellulose solution (dissolved in M199 medium; Sigma, Germany) and incubated in round-bottom 96-well plates (100 µL per well) for 24 hours to form spheroids as previously described [18]. Type I rat tail collagen (BD Biosciences, MA, USA) was diluted 1:1 with 0.1% acetic acid, mixed with 10X M199 medium, and neutralized with 0.2 N NaOH immediately before use. Spheroids were harvested, centrifuged and resuspended in methylcellulose solution supplemented with 5% FCS and mixed (1:1) with collagen working solution. Spheroid suspensions were then distributed into pre-warmed 24-well plates by addition of 1 mL to each well and incubated at 37°C for 30

min. After solidification of the collagen, 300 µL of medium were added to each well and incubated for 24 hours at 37°C. Pictures of 10 spheroids at random fields were taken employing a fluorescence microscope and the mean cumulative sprout length and the number of labeled cKit$^+$ cells was determined by ImageProPlus software.

Migration assay

HUVECs were grown on 6-well plates until confluence. A 10 µL pipette tip was employed to scratch over well plates twice vertically and horizontally for obtaining four 90° crosses on each well. Wells were gently washed and 2 mL of medium containing 5x10^4 WT or TLR2$^{-/-}$cKit$^+$ cells were added in duplicate per variant. All scratched-crosses were photographed every other hour until a total of 6 hours of incubation and scratch wounds were analyzed employing ImageProPlus software.

Matrigel plug assay

Animal experiments were approved by the governmental ethical board for animal research in Mecklenburg-Vorpommern (7221.3-1.1-108/12 and 7221.3-1.2-038/12) and are in accordance with German law on animal protection and the Guide for the Care and Use of Laboratory Animals. Animals were bred and housed at the Institute for Experimental Surgery, Central Animal Care Facility, Rostock University, Rostock, Germany. Eight to ten week-old male wild-type (WT; C57BL/6J) and TLR2 knock-out (TLR2$^{-/-}$; B6.129-Tlr2^{tm1Kir}/J) mice were subjected to intraperitoneal anesthesia by injection of 8 mg/kg xylazine hydrochloride (cp-pharma; Burgdorf, Germany) and 12 mg/kg ketamine hydrochloride (Pharmanovo GmbH; Hannover, Germany). 2x10^6 WT or TLR2$^{-/-}$cKit$^+$ cells were resuspended in 500 µL of Matrigel and subjected to subcutaneous injection. Plugs were harvested following three weeks of incubation, fixed and counterstained with DAPI for visualization of cell nuclei. In some mice, 100 µL of fluorescein griffonia (bandeiraea) simplicifolia lectin I (Vector Laboratories, Burlingame, CA, USA) were applied by left-ventricular injection in anesthetized mice for analysis of plug perfusion. 10 min later, mice were euthanized, plugs were harvested and investigated employing a fluorescence microscope and lengths of capillary networks were quantified per HPF employing ImageProPlus software.

Murine hindlimb ischemia model

Unilateral hindlimb ischemia was induced as previously described [18-20]. In brief, the right femoral artery (immediately distal to the branch of the deep femoral artery) as well as the distal portion of the saphenous artery were permanently ligated employing a 7-0 polypropylene suture (Prolene™, Ethicon, Germany) and the ligated femoral artery was then removed. Wounds were carefully sutured using 6-0 sutures (Prolene™). 24 hours after the induction of ischemia, mice were subjected to intracardiac injection of 3x10^6 labeled WT or TLR2$^{-/-}$cKit$^+$ cells or vehicle.

Infrared thermal imaging (thermography)

Thermal imaging was performed as previously described [20]. In brief, before and immediately after surgical ligation of the femoral artery and during follow-up on post-operative day (POD) 1, 5, 10 and 21, mice were anesthetized as described above and placed on a 37°C heating pad for 6 min following 3 min on a table surface at room temperature and infrared imaging was performed employing a ThermaCAM B20HS camera, FLIR Systems, Wilsonville, OR, USA. Images were analyzed using FLIR QuickReport 1.2 software by determination of the temperature at the middle of the pad of both the operated and non-operated hindlimb. The difference in temperature (°C) between both pads of each animal was analyzed using GraphPadPrism software.

Immunohistochemistry

For harvest of M. gastrocnemius muscle tissue on POD 21, mice were euthanized and capillary density in the gastrocnemius muscle was assessed on 5 µm-thick, acetone-fixed frozen sections after staining with ABs against CD31 (1:50 dilution; SantaCruz, USA) followed by Cy3-labeled secondary ABs (Molecular Probes, USA). Cell nuclei were counterstained with DAPI. The number of CD31-immunopositive cells per muscle fiber was manually counted on 8 random microscope fields per section (200x magnification).

Western blot analysis

cKit$^+$ cells were resuspended in lysis buffer containing fresh protease and phosphatase inhibitors. After incubation for 20 min on ice, cell

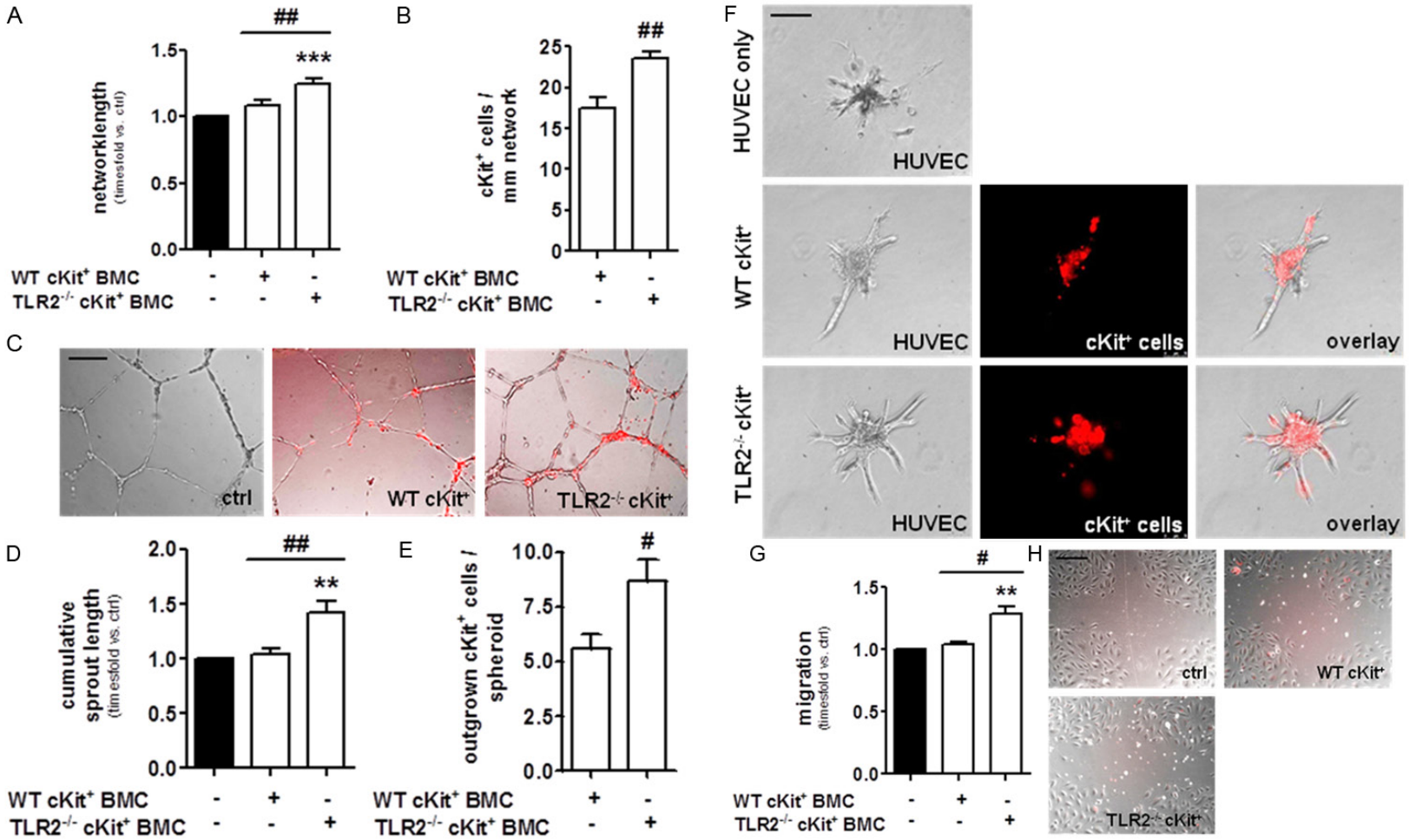

Figure 1. Bone marrow-derived cKit$^+$ cells from TLR2$^{-/-}$ mice more potently promote the angiogenic function of endothelial cells *in vitro*. (A) HUVEC showed increased capillary-like tube formation in the presence of TLR2$^{-/-}$cKit$^+$ cells compared to WT cKit$^+$ cells or at basal level (ctrl). ***P<0.001 *vs*. HUVECs alone, ##P<0.01 *vs*. WT cKit$^+$ cells. Quantitative summary of n=6 independent experiments. (B) Increased numbers of TLR2$^{-/-}$cKit$^+$ cells integrated into endothelial cell tubules provided by HUVECs compared to WT cKit$^+$ cells. ##P<0.01, n=6. (C) Representative tubular networks provided by HUVECs (translucent) after 8 hours of incubation in the presence of either WT or TLR2$^{-/-}$cKit$^+$ cells (labeled in red). (D) In the spheroid angiogenesis-assay, co-incubation of HUVECs with TLR2$^{-/-}$cKit$^+$ cells resulted in increased outgrowth of endothelial cells from collagen-embedded HUVEC spheroids and (E) enhanced migration of TLR2$^{-/-}$cKit$^+$ cells along HUVEC sprouts in comparison to co-incubation with WT cKit$^+$ cells. **P<0.01 *vs*. HUVECs at basal level, #P<0.05 and ##P<0.01, quantitative summary of n=6 independent experiments. (F) Representative spheroids exhibiting HUVEC tubular sprouts (translucent) and migrated cKit$^+$ cells (red) following 24 h of incubation in a collagen matrix. (G) TLR2$^{-/-}$cKit$^+$ cells more potently promoted the migration of HUVECs in a scratch wound assay compared to WT cKit$^+$ cells or HUVECs at basal level (ctrl). Quantitative summary of n=5 independent experiments. **P<0.01 *vs*. ctrl, #P<0.05 *vs*. WT cKit$^+$ cells. (H) Representative pictures of scratch-wound closures following 4 hours of incubation. Pictures of co-incubation with cKit$^+$ cells show overlays of brightfield and red fluorescence microscopic pictures. Bars represent 50 µm.

lysates were cleared by centrifugation and equal amounts of protein were loaded and fractionated by electrophoresis on 10-12% SDS polyacrylamide gels together with molecular weight standards and then transferred to nitrocellulose membranes (Immobilon transfer membranes, Millipore Corporation, USA). Membranes were blocked in 2.5% BSA (in TBS/0.1% Tween-20) for 2 hours at RT prior to incubation with primary ABs overnight at 4°C. Visualization of protein bands was achieved using a HRP-conjugated secondary donkey anti-rabbit (1:1000; Cell Signaling Technology, USA) or anti-mouse IgG AB (1:2500; Sigma, USA) for 1 hour at RT, followed by detection of HRP with enhanced chemiluminescent substrate (Pierve ECL2, Thermo Scientific, USA) and autoradiography. Densitometry was performed employing Quantity One 4.6.6.0 software (Bio-Rad, USA). Antibodies against PKB/AKT and phospho-PKB/AKT (S473) were purchased from R&D Systems, USA, against CXCR4 from BD Biosciences and against beta-actin from Sigma, Germany.

Statistical analysis

Results are presented as mean ± SEM. All statistical analyses were performed employing One-way ANOVA followed by Bonferroni's comparison for three or more variables, Students t-test was applied for the comparison of two variables. A *P* value of less than 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism software 4.01 (GraphPad Software Inc., San Diego, CA, USA).

Results

TLR2$^{-/-}$cKit^{+} BMC more potently support endothelial cell angiogenesis in vitro compared to WT cKit^{+} BMC

In order to compare the potency of TLR2$^{-/-}$cKit^{+} BMC to stimulate mature endothelial cell angiogenic function to the effects exerted by WT cKit^{+} BMC, we first co-incubated either TLR2$^{-/-}$ cKit^{+} BMC or WT cKit^{+} BMC with endothelial cells on matrigel. Following 8 hours of incubation, endothelial cells co-incubated with TLR2$^{-/-}$ cKit^{+} BMC exhibited increased tubular network formation compared to those co-incubated with WT cKit^{+} BMC (**Figure 1A**, **1C**). Analysis of the number of cKit^{+} cells incorporated into the capillary-like network formations of HUVECs revealed that more TLR2$^{-/-}$cKit^{+} BMC had integrated into HUVEC networks compared to WT cKit^{+} cells (**Figure 1B**). Investigations employing the spheroid angiogenesis assay revealed that the presence of TLR2$^{-/-}$cKit^{+} BMC resulted in increased sprouting activity of HUVECs in a 3D-collagen matrix compared to HUVECs incubated in the presence of WT cKit^{+} BMC (**Figure 1D**, **1F**). Moreover, TLR2$^{-/-}$cKit^{+} BMC revealed enhanced activity to migrate along outgrown HUVEC sprouts compared to WT cKit^{+} BMC (**Figure 1E**). In a scratch wound assay, the presence of TLR2$^{-/-}$cKit^{+} BMC stimulated HUVECs to faster close a scratch wound in an otherwise confluent HUVEC cell layer in contrast to the presence of WT cKit^{+} BMC (**Figure 1G**, **1H**), that did not significantly alter the migration process of HUVECs. Hence, we gathered evidence that TLR2$^{-/-}$cKit^{+} BMC exert beneficial influence on the angiogenic potency of mature endothelial cells and stimulated angiogenic processes more potently than WT cKit^{+} BMC.

In vivo, TLR2$^{-/-}$cKit^{+} BMC exhibit increased capacity to form capillary-like networks

The potency of TLR2$^{-/-}$cKit^{+} BMC to form capillary-like tubular networks themselves was next investigated employing the *in vivo* matrigel plug assay. Three weeks following subcutaneous injection and incubation of either TLR2$^{-/-}$cKit^{+} BMC or WT cKit^{+} BMC suspended in matrigel, longer coherent capillary-like network structures were detected in matrigel plugs in mice that had received TLR2$^{-/-}$cKit^{+} BMC compared to WT cKit^{+} BMC (**Figure 2A**, **2B**). Tubular structures formed by both TLR2$^{-/-}$cKit^{+} and WT cKit^{+} cells were not functional blood vessels as they did not stain positive for lectin upon systemic perfusion of animals prior to plug harvest (not shown).

Ischemia-induced neovascularization is augmented in TLR2$^{-/-}$ and WT mice that received TLR2$^{-/-}$cKit^{+} BMC

Ischemia is one of the most potent inductors of angiogenic processes [21] and bone marrow-derived stem- and progenitor cells participate in the process of neovascularization in ischemic tissues [22]. We have previously shown that TLR2$^{-/-}$ mice exhibit an increased angiogenic response in a mouse model of hindlimb ischemia compared to WT counterparts [20]. In

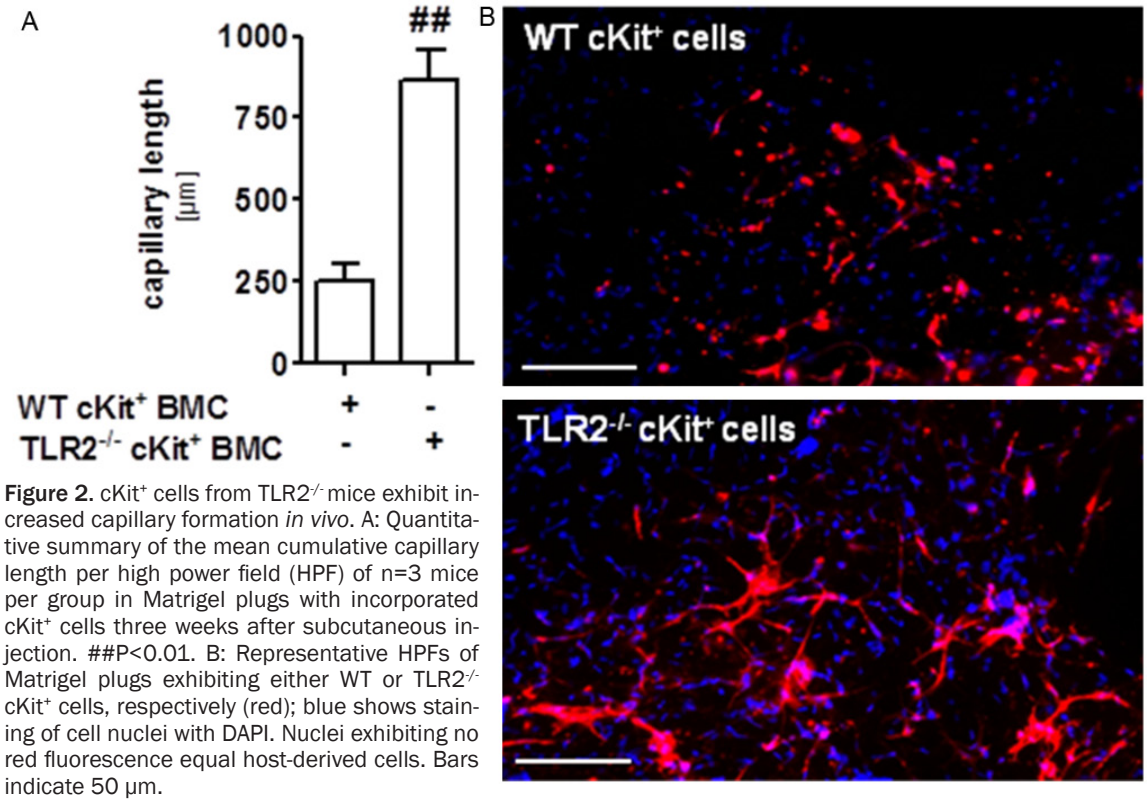

Figure 2. cKit$^+$ cells from TLR2$^{-/-}$ mice exhibit increased capillary formation *in vivo*. A: Quantitative summary of the mean cumulative capillary length per high power field (HPF) of n=3 mice per group in Matrigel plugs with incorporated cKit$^+$ cells three weeks after subcutaneous injection. ##P<0.01. B: Representative HPFs of Matrigel plugs exhibiting either WT or TLR2$^{-/-}$ cKit$^+$ cells, respectively (red); blue shows staining of cell nuclei with DAPI. Nuclei exhibiting no red fluorescence equal host-derived cells. Bars indicate 50 µm.

order to address whether these effects are exerted by TLR2-deficiency on the endothelium itself or can be, at least in part, also attributed to missing TLR2 signaling in hematopoietic stem- and progenitor cells, WT mice were subjected to intracardiac delivery of either TLR2$^{-/-}$ cKit$^+$ BMC or WT cKit$^+$ BMC and compared to both WT and TLR2$^{-/-}$ mice receiving only vehicle 24 hours after the induction of ischemia. Evaluation of the capillary density in all treatment groups 21 days after surgery revealed that the injection of TLR2$^{-/-}$cKit$^+$ BMC into WT mice resulted in significantly augmented density of CD31/DAPI double-positive endothelial cells in M. gastrocnemius muscle tissue compared to the delivery of WT cKit$^+$ cells to WT mice (**Figure 3A**, **3B**). These effects obtained by treatment of WT mice with TLR2$^{-/-}$cKit$^+$ BMC were comparable in magnitude to those obtained in mice exhibiting a whole-body knock-out of TLR2. Both TLR2$^{-/-}$cKit$^+$ BMC-treated WT and TLR2$^{-/-}$ mice exhibited significantly more pronounced angiogenic response to ischemia compared to WT mice treated with vehicle alone. The recovery of blood flow was furthermore monitored employing thermal imaging. Analogous to increased vascular density, we observed an increase in temperature of formerly ischemic, i.e. chilled hindlimbs in both TLR2$^{-/-}$cKit$^+$ BMC-treated WT and TLR2$^{-/-}$ mice on post operative day (POD) 21 displayed as a decrease in temperature difference between both pads of the same animal (**Figure 3C**, **3D**). In contrast, both WT cKit$^+$ BMC- and vehicle-treated WT mice did not exhibit a relevant change in temperature on POD 21 compared to immediately following induction of ischemia. Thus, TLR2-deficiency was associated with increased neovascularization capacity and augmented restoration of blood flow in ischemic hindlimbs and these beneficial effects could be encompassed by application of TLR2$^{-/-}$cKit$^+$ BMC to WT mice.

TLR2$^{-/-}$cKit$^+$ BMC exhibit equal protein expression of CXCR4 but increased basal activation of the PI3K downstream signaling molecule AKT

Homing of hematopoietic cells to sites of ischemia is an important mechanism that contributes to neovascularization processes [22]. During ischemia, increased levels of the cytokine stromal-cell derived factor-1 (SDF-1) are expressed by tissues and SDF-1 augments the recruitment of CXCR4-positive BMC from the

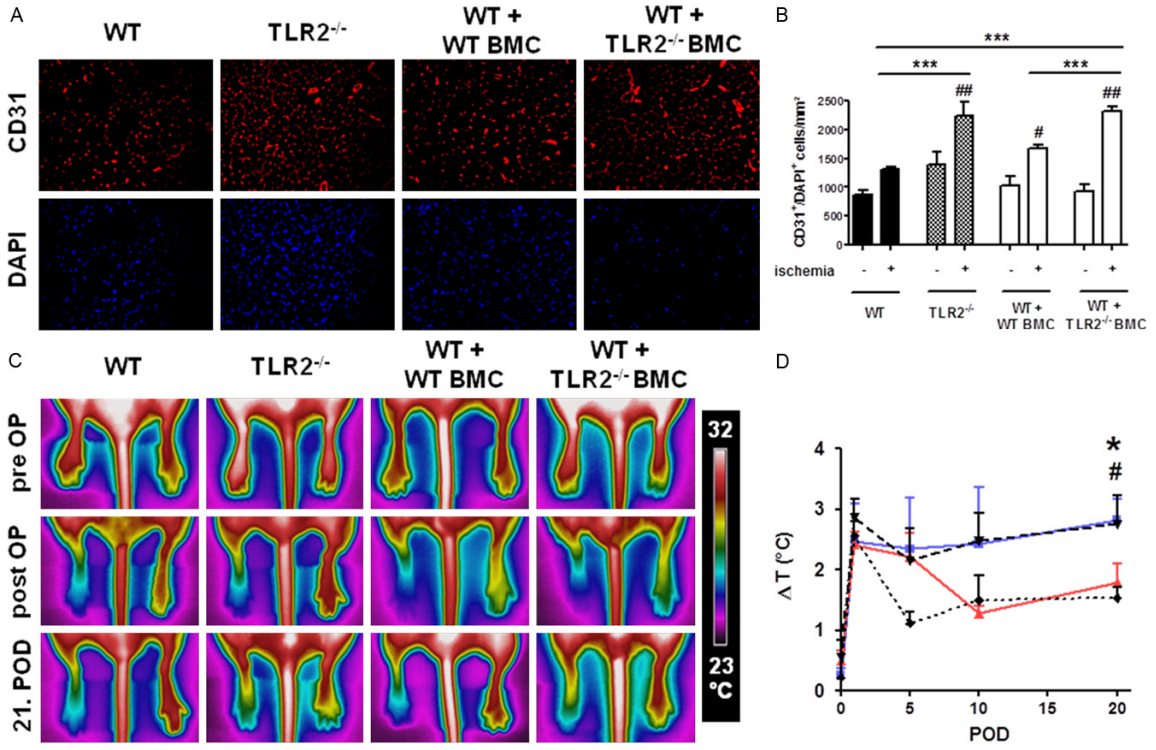

Figure 3. Bone marrow-derived cKit$^+$ cells from TLR2$^{-/-}$ mice exhibit increased capacity to promote neovascularization *in vivo*. A: 21 days following ischemia induction and cKit$^+$ cell administration, WT mice that had received TLR2$^{-/-}$cKit$^+$ bone-marrow derived cells (BMC) exhibited increased vascular density (CD31/DAPI double-positive cells) in M. Gastrocnemius tissue similar to TLR2$^{-/-}$ mice and in contrast to both WT and mice treated with cKit$^+$ WT BMCs. B: Quantitative summary of the CD31/DAPI double-positive cells per mm^2 of n=5-6 mice/group. #P<0.05 and ##P<0.01 *vs*. Non-ischemic control leg of the same animal, ***P<0.001. C: Thermal imaging of mice hindlimbs revealed augmented perfusion in both TLR2$^{-/-}$ and WT mice that had received TLR2$^{-/-}$cKit$^+$ cells. D: Quantitative summary of n=5 mice per group. Black triangle dashed line: WT mice; black rhomb dotted line: TLR2$^{-/-}$; blue square solid line: WT + WT BMC; red triangle solid line: WT + TLR2$^{-/-}$ BMC. *P<0.05 TLR2$^{-/-}$ *vs*. WT mice, #P<0.05 WT mice treated with TLR2$^{-/-}$cKit$^+$ BMC *vs*. WT mice treated with WT cKit$^+$ cells.

circulation [16]. Comparing the amount of CXCR4 protein expression in WT cKit$^+$ and TLR2$^{-/-}$cKit$^+$ BMC however revealed no differences (**Figure 4A**, **4B**). Instead, increased activation (i.e. phosphorylation) of the CXCR4 downstream signaling molecule protein kinase B (PKB)/AKT [23] was detected in TLR2$^{-/-}$cKit$^+$ BMC and may thus account for the increased potency of these cells to support angiogenic processes compared to WT cKit$^+$ cells (**Figure 4C**, **4D**) [24].

Discussion

Endothelial tube formation, migration and the sprouting of endothelial cells into the extracellular matrix are the major constituents of the angiogenic process [21]. Hematopoietic cells home to sites of neovascularization where they exert stimulatory effects on the pre-existing vasculature, thereby augmenting the formation of new capillaries [22]. TLR2 deficiency has been associated with various protective effects on the vasculature and the preservation of vascular function [25, 26]. Studies employing models of transplanting TLR2-deficient bone marrow into WT mice observed a reduction in tissue injury for example after myocardial infarction that was mainly attributed to TLR2 deficiency on leukocytes [27]. In the present study, evidence is provided that extends the spectrum of the beneficial effects of TLR2-deficiency on hematopoietic stem cells to subsets that express cKit and augment the angiogenic capacity of endothelial cells. cKit$^+$ cells isolated from the bone marrow of TLR2-deficient mice promoted *in vitro* capillary tube formation, sprouting as well as the migratory capacity of mature endothelial cells more potently compared to WT cKit$^+$ cells. Since the employment of anti-TLR2 antibodies is currently discussed

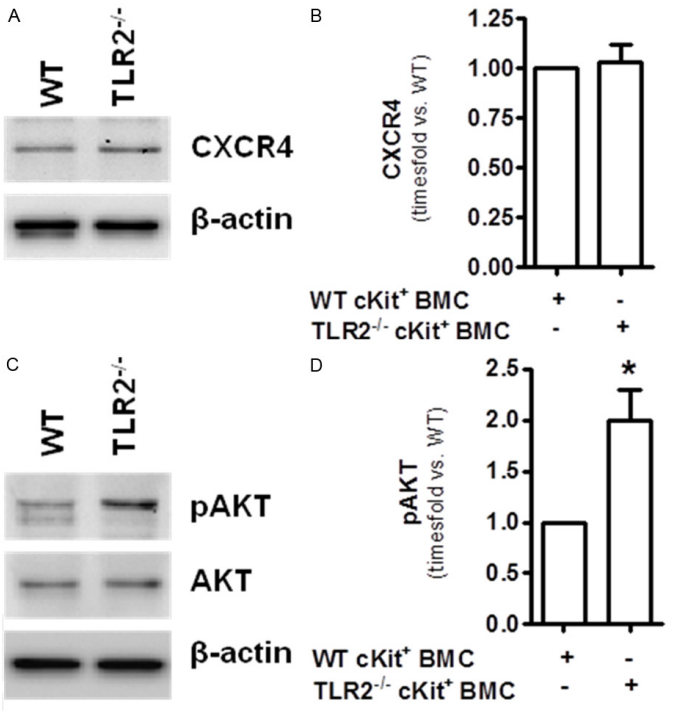

Figure 4. TLR2$^{-/-}$cKit^{+} cells exhibit similar expression of CXCR4 but increased basal phosphorylation of AKT compared to WT cKit^{+} cells. A: Western Blot analysis of both TLR2$^{-/-}$ and WT cKit^{+} cells for CXCR4 protein expression. β-actin indicates equal loading. B: Quantitative densitometric analysis of CXCR4 protein in cells isolated from n=3 mice per group. C: Western Blot analysis for phospho-AKT and AKT. D: Results of the densitometric analysis, *P<0.05, n=3 mice per group.

in the context of the treatment of a variety of vascular diseases [25], these results may point towards a potential therapeutical use of hematopoietic stem cells pre-treated with anti-TLR2 antibodies for therapeutical neovascularization.

We have recently shown that incubation of endothelial cells with mono- or polyclonal anti-TLR2 antibodies resulted in the activation of CXCR4 canonical signaling [20]. CXCR4 is expressed on bone marrow-derived precursor cells and mediates the effects of its ligand stromal cell-derived factor-1 (SDF-1), one of the key players in progenitor cell homing [16]. In the present study, CXCR4 was verified to be expressed on cKit^{+} cells but the amount of CXCR4 expression in TLR2$^{-/-}$cKit^{+} was equal to those detected in WT cKit^{+} BMC. However, an increased basal level of activation of the key downstream signal transduction molecule of the SDF-1/CXCR4 system, namely AKT, has been observed in cKit^{+} BMC isolated from TLR2$^{-/-}$ mice compared to those derived from WT mice. Increased expression and activation of AKT in TLR2$^{-/-}$ mice has been observed previously and is associated with the cardio-protective effects of TLR2-deficiency in the context of myocardial ischemia [28]. Moreover, AKT has been shown to be an essential mediator of homing of hematopoietic stem cells that contribute to angiogenic processes and the level of AKT activation correlates with the quality of entrapment of BMC at sites of neovascularization [24]. In this regard, increased basal levels of AKT activation together with our finding that intracardiac delivery of TLR2$^{-/-}$ cKit^{+} cells into mice resulted in increased capillary density following hindlimb ischemia may suggest that TLR2$^{-/-}$cKit^{+} BMC exhibit an increased responsiveness towards chemoattractants throughout the process of homing. However, the upstream activators of AKT in TLR2$^{-/-}$cKit^{+} BMC remain unclear. TLR2-deficient mice have been reported to express higher levels of TLR4 and TLR9 [29] and both TLR4 and TLR9 result in downstream AKT activation [30, 31]. A compensatory upregulation of other TLRs on TLR2-deficient BMC however would challenge the notion that a reduction in TLR signaling accompanied by a diminished activation of inflammatory signaling cascades may represent the underlying mechanism of the beneficial effects of TLR2 functional deficiency for vascular homeostasis. In this context, TLR2 has also been reported to be an important mediator of pro-angiogenic effects in the context of inflammatory processes [32, 33] and disruption of TLR2 signaling may impair tissue regenerative processes such as wound healing due to impaired angiogenic events [34]. Thus, further studies are needed to decipher the exact phenotype of hematopoietic stem and progenitor as well as of cells of the vasculature and the role of inflammatory processes in correspondence to the vasculoprotective phenotype of TLR2-deficient mice.

The stem cell factor receptor cKit is expressed on murine hemangioblasts during embryonic development and on hematopoietic stem cells and endothelial progenitor cells during adulthood [35]. In recent years, the characterization

and potency of hematopoietic progenitors that specifically contribute to vascular regenerative processes have been redefined [36]. Specific combinations of surface antigens may identify different types of progenitor subsets with variable potential to either differentiate into endothelial cells themselves or serve as sources of angiogenic cytokines [37]. In the present study, cKit$^+$ BMC were employed irrespective of further characterization for the expression of specific endothelial progenitor cell marker subsets. In the matrigel plug assay, cKit$^+$ cells formed capillary-like structures and this finding was found to be more pronounced when cKit$^+$ cells isolated from TLR2$^{-/-}$ mice were employed. However, the tubular structures formed by both TLR2$^{-/-}$ and WT cKit$^+$ cells were not associated with functionality as they were not connected to the circulation as indicated by negative staining for lectin upon systemic lectin perfusion. These findings are in line with previous studies reporting that the vast majority of bone marrow derived precursor cells do not contribute to neovascularization by structural contribution to new vessel formation [38]. On the other hand, the supportive role of cKit$^+$ cells in neovascularization processes is undisputed and supported in the present study by the finding of augmented neovascularization in WT mice treated with WT cKit$^+$ BMC in contrast to WT mice having received vehicle alone 24 hours after the induction of hindlimb ischemia.

Further studies are nevertheless required addressing the exact identity and potency of hematopoietic cells that mediate the beneficial effects of TLR2-deficiency on vascular regenerative processes. However, the employment of hematopoietic cells selected by the expression of the marker for differentiation potential cKit may result in the utilization of a cell population that exerts ubiquitous potential. Thus, the present study employing cKit$^+$ cells may also extend the implications of TLR2-deficiency to other implications involving hematopoietic stem cells such as liver, muscle or cardiac tissue regeneration.

Acknowledgements

The authors would like to thank Berit Blendow, Dorothea Frenz, Maren Nerowski and Eva Lorbeer-Rehfeldt (Institute for Experimental Surgery, University of Rostock, Germany) for excellent technical assistance.

Disclosure of conflict of interest

The authors confirm that there are no conflicts of interest.

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