HMGB-1 induces c-kit\(^+\) cell microvascular rolling and adhesion via both toll-like receptor-2 and toll-like receptor-4 of endothelial cells

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Abstract

High-mobility group box 1 (HMGB-1) is a strong chemo-attractive signal for both inflammatory and stem cells. The aim of this study is to evaluate the mechanisms regulating HMGB-1–mediated adhesion and rolling of c-kit\(^+\) cells and assess whether toll-like receptor-2 (TLR-2) and toll-like receptor-4 (TLR-4) of endothelial cells or c-kit\(^+\) cells are implicated in the activation of downstream migration signals to peripheral c-kit\(^+\) cells. Effects of HMGB-1 on the c-kit\(^+\) cells/endothelial interaction were evaluated by a cremaster muscle model in wild-type (WT), TLR-2 \((-/-)\) and Tlr4 (LPS-del) mice. The mRNA and protein expression levels of endothelial nitric oxide synthase were determined by quantitative real-time PCR and immunofluorescence staining. Induction of crucial adhesion molecules for rolling and adhesion of stem cells and leukocytes were monitored in vivo and in vitro. Following local HMGB-1 administration, a significant increase in cell rolling was detected (32.4 ± 7.1\% in ‘WT’ versus 9.9 ± 3.2\% in ‘control’, \(P<0.05\)). The number of firmly adherent c-kit\(^+\) cells was more than 13-fold higher than that of the control group (14.6 ± 5.1 cells/mm\(^2\) in ‘WT’ versus 1.1 ± 1.0 cells/mm\(^2\) in ‘control’, \(P<0.05\)). In knock-out animals, the fraction of rolling cells did not differ significantly from control levels. Firm endothelial adhesion was significantly reduced in TLR-2 \((-/-)\) and Tlr4 (LPS-del) mice compared to WT mice (1.5 ± 1.4 cells/mm\(^2\) in ‘TLR-2 \((-/-)\)’ and 2.4 ± 1.4 cells/mm\(^2\) in ‘Tlr4 (LPS-del)’ versus 14.6 ± 5.1 cells/mm\(^2\) in ‘WT’, \(P<0.05\)). TLR-2 \((-/-)\) and Tlr4 (LPS-del) stem cells in WT mice did not show significant reduction in rolling and adhesion compared to WT cells. HMGB-1 mediates c-kit\(^+\) cell recruitment via endothelial TLR-2 and TLR-4.

Keywords: high-mobility group box 1 • c-kit\(^+\) • intravital microscopy • cremaster muscle

Introduction

The importance of chemokines and local inflammation for stem cell trafficking to injured tissue has been shown in the recent past [1]. HMGB-1, also called amphotericin, is a highly conserved, ubiquitously expressed, nuclear protein [2], which is released in the extracellular space by necrotic but not apoptotic cells. Moreover, HMGB-1 is secreted by cells after inflammatory activation and acts in the extracellular space as a chemoattractant for inflammatory cells, smooth muscle cells and stem cells [3, 4]. The receptor for advanced glycation end-products (RAGE) and members of the TLR family are known cellular receptors of HMGB-1. Recently, HMGB-1 has been shown to enhance integrin-dependent homing of endothelial progenitor cells [5]. HMGB-1 is also capable to induce mesangioblast migration through an endothelial...
monolayer in vitro, and may act in vivo as a signal to attract both mesangioblast and bone marrow derived stem cells [6]. On the other hand, HMGB-1 in endothelial cells induces the up-regulation of adhesion molecules VCAM-1 and ICAM-1, which are required for integrin-mediated adhesion and subsequent transmigration of inflammatory cells [7]. Stem cell adhesion and homing rely on similar mechanisms. Bone marrow derived stem cells and endothelial progenitor cells require β1-integrin and β2-integrin for their transmigration through the endothelial barrier [8]. In a murine model of myocardial infarction, exogenously administrated HMGB-1 led to recovery of left ventricular function through regeneration of cardiomyocytes from resident cardiac c-kit+ stem cells [9], suggesting that the presence HMGB-1 may have physiological relevance in the setting of ischaemic cardiovascular pathologies.

C-kit, as a murine hemangioblast marker, is continued to be expressed in their adulthood [10]. These cells, including endothelial progenitor cell (EPC) and haematopoietic stem cell (HSC), have been implicated in contributing to angiogenesis, organ repair and tissue remodelling by migrating from the bone marrow into the peripheral circulation. Therefore, it is highly important to examine c-kit+ cell rolling and adhesion in the microvascular endothelium [11, 12].

In this study, we aimed to investigate HMGB-1 ability to regulate c-kit+ cell–endothelium interactions in the cremaster muscle microcirculation, using intravital fluorescence microscopy. We found that HMGB-1–mediated peripheral c-kit+ cell recruitment did not depend on the presence of TLRs 2 or 4 of stem cells but depended on both, TLR-2 and TLR-4 of endothelial cells. In addition, we evaluated whether HMGB-1 affects adhesion molecule redistribution in endothelial cells in vitro.

**Materials and methods**

**Experimental design**

All animal experiments were carried out according to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1996) and were permitted by the local animal care and use committee. Lipopolysaccharide (LPS, Endotoxin), representing a known pro-inflammatory molecule and TLR-4 agonist, was obtained from Sigma-Aldrich (St. Louis, Missouri, USA). The macrophage-activating lipopeptide 2 kD (MALP-2), representing a selective TLR-2 agonist, was obtained from Enzo Life Sciences (Hoboken, New Jersey, USA). The macrophage-activating lipopeptide 2 kD (MALP-2) is a synthetic lipopeptide derived from the outer membrane of LPS (Escherichia coli serotype 0111:B4). The analyses of microcirculation also included the measurement of vessel diameter and wall shear rates based on the Newtonian definition $\gamma = 8 \times v/d$, where $\gamma$ represents the red blood cell velocity divided by 1.6 according to the Baker-Wayland factor [14] and $\gamma$ represents the single vessel diameter (Table S1).

**Intravital fluorescence microscopy: c-kit+ cell behaviour at the interface of venular endothelium following HMGB-1 stimulus**

Male WT mice, TLR-2 (−/−) mice and Tlr4 (LPS-del) were anaesthetized with ketamine (75 mg/kg) and xylazine (25 mg/kg). An arterial catheter was inserted retrograde into the left femoral artery to establish the injection route for fluorescent reagents and labelled c-kit+ cells. The right cremaster muscle was dissected and prepared for intravital fluorescence microscopic analysis as previously described [15, 16]. For intravital microscopy, c-kit+ and c-kit+ TLR-2 (−/−) and Tlr4 (LPS-del) cells were fluorescently labelled with the CFDA SE (carboxy-fluorescein diacetate, succinimidyl ester; A2 Molecular Probes, Carlsbad, CA) according to manufacturer’s protocol. To observe the inflammatory response to HMGB-1, circulating endogenous leukocytes (ELs) of recipient origin were coloured by intravenous injection of 0.1 ml of 1% rhodamine 6G (Sigma-Aldrich). Fluorescently-labelled dextran was injected to visualize the blood flow. Rhodamine 6G (background for CFDA SE-labeled c-kit+ cells) or fluorescein isothiocyanate (background for rhodamine-labelled EL) were used as background. As intravital microscopic system, an Axiostar fluorescence microscope (Carl Zeiss, Jena, Germany) was modified for epi-illumination and connected to a charge-coupled device (CCD) video camera. Six post-capillary venules were randomly chosen before cell injection to define the areas of cellular adherence analysis. With respect to the groups, the cremaster muscles were exposed to HMGB-1, heated HMGB-1, LPS or MALP-2, 15 min. before the first c-kit+ cell injection. In HMGB-1 and Control’ groups, CFDA SE–labelled c-kit+ cells, c-kit+ TLR-2 (−/−) or Tlr4 (LPS-del) cells were applied at $0.4 \times 10^5$ cells per injection, with a total of five consecutive injections. In ‘LPS’ and ‘MALP-2’ groups, only WT c-kit+ cells were injected. C-kit+ cell behaviour along the endothelial lining was considered as ‘rolling’ when a more than 50% reduction of cell velocity in combination with the typical cellular ‘stick and release’ motion was present. The rolling cells were expressed as percentage of all passing cells along a predefined venular distance during 1 min. of observation. All the cells that showed random brief tethering phenomena were not considered as rolling cells. Firm
adhesion was recorded when cells did not move for more than 30 sec. The number of firmly adherent c-kit+ cells was calculated in relation to the endothelial surface of the predefined venules (diameter × length × π) and was expressed as adherent cells/mm² endothelial surface. Intravital fluorescence microscopy analysis was carried out by two researchers, who were blinded to the treatment.

Quantitative real-time PCR analysis

For analysis of mRNA levels, cremaster muscles (n = 6, for each group) were removed and carefully dissected along the central axis. The two half were separated and one of them was snap-frozen in liquid nitrogen. Total RNA was extracted following the instructions of the TRIZOL® Reagent (Invitrogen, Carlsbad, CA, USA) including DNase treatment. Primer sets for real-time PCR (Applied Biosystems, Foster City, CA, USA) are summarized in Table S2. Amplification and detection were carried out with the StepOnePlus™ Real-Time PCR System (Applied Biosystems) in TaqMan Universal Master Mix (Applied Biosystems), according to the instructions of the producer (Applied Biosystems). Reactions were repeated at least three times using the following program: 1 cycle of 50°C for 2 min., 1 cycle of 95°C for 10 min. and 40 cycles of 95°C for 15 sec. and 60°C for 1 min. DNA extracts were analysed in at least triplicate and negative controls were included in each assay. Cycle thresholds (CT) for individual reactions were determined with StepOne™ Software 2.0 (Applied Biosystems) and the target genes were normalized against GAPDH with the formula: ΔCT = CT target − CT GAPDH. Calculated ΔCT of triplicates was averaged and ΔΔCT were obtained using 'Control' group as calibrator sample (formula: ΔΔCT = ΔCT sample − ΔCT calibrator sample). The changes in gene expression after HMGB-1 treatment were expressed as fold differences (2−ΔΔCT).

Immunohistochemistry

After sacrifice of recipients, the cremaster muscles were removed and divided in two pieces for downstream analyses. From these, one piece was fixed in 4% formaldehyde and embedded in paraffin for immunohistochemistry (at least n = 5, for each group). After deparaffinization and protein retrieving, cremaster tissue sections (5 μm thick) were incubated with mouse antimouse endothelial nitric oxide synthase (eNOS) monoclonal antibody overnight (Becton Dickinson, Heidelberg, Germany). On the second day, an Alexa 488 conjugated donkey antimouse secondary antibody (Becton Dickinson) and a goat anti-hamster Alexa 488 conjugated secondary antibody (Molecular Probes) were utilized in immunofluorescence analysis of β1-integrin, P-selectin and ICAM-1 motility on SVEC membrane following HMGB-1 preconditioning

It has been shown that HMGB receptor is present on microvascular endothelial cells [7]. To investigate endothelial activation mediated by HMGB-1 mouse endothelial cells (SVEC) were pre-treated with the chemokine to induce a prompt cellular response with an increase of adhesion molecule valency/avidity. The increased proteins affinity and membrane redistribution might be implicated in the cellular migratory activity. HMGB-1 effect was compared with activities of other well-known chemokines such as TNF-α and stromal derived factor 1α (SDF-1α).

To mimic the endothelial response to HMGB-1 in vitro, simian virus 40-transformed mouse endothelial cell (SVEC) were incubated in the presence of active or heated HMGB-1 (100 ng/ml). Tumour necrosis factor α (TNF-α; 200 U/ml; R&D Systems, Minneapolis, MN, USA) or SDF-1α (100 ng/ml; R&D Systems) were utilized as positive controls. Slides preparation and P-selectin staining were obtained utilizing the protocols previously described used for c-kit+ cells. For β1-integrin and ICAM-1 detection, hamster antimouse β1-integrin monoclonal, hamster antimouse ICAM-1 antibodies (Becton Dickinson) and a goat anti-hamster Alexa 488 conjugated secondary antibody (Molecular Probes) were utilized in sequence. Nuclei were detected by TOPRO 3 (Invitrogen) and probes were investigated with TCS SPE confocal microscopy (Leica Microsystems). The numbers of SVEC cells presenting β-integrin, P-selectin or ICAM-1 polarization were counted in 20 randomly chosen high-power fields (HPFs, 630×). Results were expressed as cells per HPF. All morphometric analyses were performed by two investigators who were blinded to the treatment.

Isolation and fluorescent-activated cell sorter analysis of c-kit+ cells from murine bone marrow

c-kit+ cells were isolated from mouse (C57BL/6J; B6.129-Tlr21tm1kJr/C3H/HeJ; 8 weeks old) bone marrow using magnetic microbeads coated with anti-c-kit monoclonal antibody, Miltenyi Biotec MS columns® and the MiniMacs® cell separation system (Miltenyi Biotec, Bergisch Gladbach, Germany). Cell separation cycle was repeated two times to obtain higher cell number. To perform flow cytometry analysis, cells were incubated for 10 min. at 4°C with monoclonal antimouse CD117 conjugated with PE (clone 3G1; 1:10; Miltenyi Biotec, Bergisch Gladbach, Germany), mouse antimouse TLR-2 antibody (Santa Cruz, Santa Cruz, CA, USA) and goat antimouse TLR-4 antibody (Abcam, Cambridge, MA, USA). After primary antibody reaction, cells were washed and resuspended with antimouse and anti-goat Alexa 488 secondary antibodies (Invitrogen). Cells were washed with phosphate-buffered saline (PBS)/2 mM EDTA and analysed by an FACS LSR II flow cytometer (Becton Dickinson). Dead cells were excluded with propidium iodide staining. Analysis of data was carried out with BD FACS Diva software. At least 10,000 events per sample were recorded to create side scatters plots against logarithmic fluorescence intensity. The purity of the enriched c-kit+ cell population tested with flow cytometry was consistently higher than 95% (Fig. S1).

Statistical analyses

All statistical analyses were performed using Sigma Stat software version 3.0 (SPSS Inc., Chicago, IL, USA). Results are showed as mean ± S.E.M. Comparisons of the groups were executed using the one-way ANOVA, which applies post-hoc multiple Holm–Sidak analysis. In case the data failed normality testing, the non-parametric Kruskal–Wallis or post-hoc multiple Dunn tests were employed. P < 0.05 was considered statistically significant.
Results

**In vivo interaction of c-kit^+ cells with the endothelium was predominant in post-capillary venules**

Overall, interaction between injected c-kit^+ cells and the vascular endothelium was a rare event and occurred almost exclusively in post-capillary venules (diameter: 40–80 μm). c-kit^+ cell contact to endothelial lining aroused to a different extent in the respective experimental groups. In the observed venules, rolling and firmly adherent c-kit^+ cells in response to superfusion of HMGB-1 could be clearly counted (Fig. 1A).

**HMGB-1 alone enhances venular rolling of c-kit^+ cells in vivo**

*In vivo* rolling c-kit^+ cells were quantified using intravital fluorescence microscopy. There was a more than 50% reduction of
velocity along the endothelial lining. In the heated HMGB-1-superfused cremaster muscle (‘Control’), the fraction of rolling c-kit+ cells was 9.9 ± 3.2%. Following local HMGB-1 administration, a more than three-fold increase in c-kit+ cell rolling was detected (32.4 ± 7.1% in ‘HMGB-1’ versus 9.9 ± 3.2% in ‘Control’, P < 0.05; Fig. 1B). Additional local treatment with SDF-1α had no further impact on the percentage of rolling c-kit+ cells (data not shown).

**LPS and MALP-2 did not lead to significant rolling of c-kit+ cells**

To clarify the effect of TLR-2 and TLR-4 on c-kit+ cell rolling after specific activation, we treated the cremaster muscle either with LPS or MALP-2. Intravital microscopy data did not show a significant variation of cellular rolling in ‘LPS’ and ‘MALP-2’ groups when compared to ‘Control’ (c-kit+ cell rolling: 11.8 ± 2.3% in ‘LPS’ and 8.1 ± 2.0% in ‘MALP-2’ versus 9.9 ± 3.2% in ‘Control’, P = 0.37; Fig. 1B).

**HMGB-1 enhances firm endothelial adhesion of c-kit+ cells in vivo**

The number of firmly adherent c-kit+ cells after cremaster muscle superfusion with heated HMGB-1 (‘Control’) was 1.1 ± 1.0 cells/mm2. In response to treatment with the active chemokine, the number of firmly adherent c-kit+ cells was significantly higher than in respective control group (c-kit+ cell adhesion: 14.6 ± 5.1 cells/mm2 in ‘HMGB-1’ versus 1.1 ± 1.0 cells/mm2 in ‘Control’, P < 0.05). In fact, stem cell adhesion after tissue stimulation with HMGB-1 exceeded that of the untreated control mice more than 13-fold (Fig. 1C). Once more, after combined treatment of the cremaster muscle tissue with HMGB-1 and SDF-1α, the number of adherent c-kit+ cells was not further enhanced (data not shown).

**LPS and MALP-2 induce moderate adhesion of c-kit+ cells**

We tested whether TLR-2 and TLR-4 targeted stimulation on the cremaster muscle could influence c-kit+ cell adhesion. Following topical administration of LPS or MALP-2, we observed by intravital microscopy an increase of c-kit+ cell adhesion in ‘LPS’ and ‘MALP-2’ groups when compared to ‘Control’ (c-kit+ cell adhesion: 4.4 ± 2.3 cells/mm² in ‘LPS’ and 7.5 ± 3.3 cells/mm² in ‘MALP-2’ versus 1.1 ± 1.0 cells/mm² in ‘Control’, P = 0.27; Fig. 1C). However, the levels of adherent cells in these groups were not comparable with the amount of firm adhesion observed in ‘HMGB-1’ group.

**HMGB did not induce WT c-kit rolling and firm adhesion in TLR-2 and TLR-4 knockout mice**

To further validate the role of TLR-2 and TLR-4 in HMGB-1–mediated endothelial adhesion behaviour of c-kit+ cells, we studied stem cell endothelial interactions in TLR-2 (–/–) and Tlr4 (LPS-del) mice, after HMGB-1 stimulation in vivo. The fraction of rolling cells did not differ significantly from wild-type control levels in TLR-2 (–/–) and Tlr4 (LPS-del) mice after local treatment with HMGB-1 (c-kit+ cell rolling: 8.8 ± 3.9% in ‘TLR-2ko’ and 3.1 ± 1.6% in ‘TLR-4ko’ versus 9.9 ± 3.2% in ‘Control’, P = 0.3). In contrast to WT mice, the local treatment with HMGB-1 in TLR-2 (–/–) and Tlr4 (LPS-del) did not induce rolling (Fig. 2A). The number of firmly adherent c-kit+ cells was clearly reduced in TLR-2 (–/–) and Tlr4 (LPS-del) animals after local treatment with HMGB-1 compared to WT mice after HMGB-1 stimulation (c-kit cell adhesion: 1.5 ± 1.4 cells/mm² in ‘TLR-2ko’ and 2.4 ± 1.4 cells/mm² in ‘TLR-4ko’ versus 14.6 ± 5.1 cells/mm² in ‘HMGB-1’, P < 0.05; Fig. 2B). It is reasonable to speculate HMGB-1 mediates c-kit+ cell recruitment via both the TLR-2 and TLR-4 signalling of endothelial cells.

**HMGB induces rolling and firm adhesion of c-kit+ cells from TLR-2 and TLR-4 knockout in WT microvasculature**

Because TLR is presented on both endothelial cell and stem cell surface, it is important to clarify whether the reported effect of HMGB-1 on c-kit+ cell–endothelial cell interaction is additionally mediated via TLR on the stem cell side. We, therefore, investigated rolling and adhesion capacity of c-kit+ cells from TLR-2 and TLR-4 knockout mouse in WT microvasculature. HMGB-1 induces rigorous cell migration. The number of rolling TLR-2 (–/–) c-kit+ and Tlr4 (LPS-del) c-kit+ cells was significantly increased compared to untreated ‘Control’ (c-kit+ cell rolling: 18.6 ± 1.2% in ‘TLR-2ko cell’ and 18.2 ± 2.2% in ‘TLR-4ko cell’ versus 9.9 ± 3.2% in ‘Control’, P < 0.01 and P < 0.05). There was no significant difference on rolling cell number in ‘TLR-2ko cell’ and ‘TLR-4ko cell’ groups compared to ‘HMGB-1’group (Fig. 2C). The number of firmly adherent TLR-2 (–/–) and Tlr4 (LPS-del) c-kit+ cells was significantly increased after topical HMGB-1 application compared to ‘Control’ mice (c-kit+ cell adhesion; 16.3 ± 5.2 cells/mm² in ‘TLR-2ko cell’ and 11.7 ± 3.6 cells/mm² in ‘TLR-4ko cell’ versus 1.1 ± 1.0 cells/mm² in ‘Control’, P < 0.05; Fig. 2D). There was no significant difference on adherent cell number in ‘TLR-2ko cell’ and ‘TLR-4ko cell’ groups compared to ‘HMGB-1’group (P = 0.84 and 0.82, respectively). This data suggest that mobilization and recruitment of c-kit+ cell by HMGB-1 do not require the presence of TLR-2/4 on c-kit+ cell membrane.

**HMGB-1 mediated up-regulation of eNOS and c-kit expression is TLR-2 dependent**

We examined whether HMGB-1 treatment altered eNOS and c-kit expression in the cremaster muscle by real-time PCR and
Immunostaining. Quantitative real-time PCR revealed that eNOS mRNA was significantly up-regulated in HMGB-1 superfused muscles (P < 0.05; Fig. 3A) compared with ‘Control’. These data were further supported by immunostaining of eNOS, which identified a clear eNOS expression pattern in HMGB-1–treated cremaster muscles: eNOS was detected in the endothelial cells bordering the vessel lumen (Fig. 3B). Furthermore, the mRNA level of c-kit was considerably increased after HMGB-1 superfusion compared to the heated chemokine (Fig. 3A).

To uncover the role of TLRs in HMGB-1–mediated c-kit+ cell interaction with the endothelium, we verified eNOS and c-kit mRNA expression in the knockout animals (‘TLR-2ko’ and TLR-4ko’ groups). Interestingly, the lack of TLR-2 abolished completely the positive regulation of eNOS and c-kit triggered by HMGB-1 (Fig. 3A). In the absence of TLR-4, there was no significant change of the two genes compared to ‘HMGB-1’ (P > 0.05; Fig. 3A).

**HMGB-1–mediated up-regulation of P-selectin expression is TLR-2 dependent**

To clarify the effects of HMGB-1 on the adhesion capacity of endothelial and circulating cells, quantitative real-time PCR was performed and mRNA levels of P-selectin were quantified. As shown in Figure 4, the mRNA level of P-selectin was up-regulated in response to HMGB-1 cremaster superfusion (P < 0.01). Thus, cell adhesive properties were promptly increased after local HMGB-1 application. In the TLR-2 knockout mice, P-selectin expression was drastically down-regulated (P < 0.01; Fig. 4), suggesting a crucial role of the receptor in HMGB-1–mediated cell adhesion competence.

**Inflammatory response after low-dosage HMGB-1 superfusion of the cremaster muscle**

Given the potential role of HMGB-1 as mediator of inflammatory response, we carefully evaluated the feasible dosage of the chemokine (dose/response test) by intravital fluorescence microscopy (data not shown). After choosing the favourable amount of protein (400 ng/ml), the expression of crucial markers of inflammation such as β1-integrin and leukocyte common marker CD45 was assessed by real-time PCR. HMGB-1 superfusion induced a moderate increase in EL adhesion (Fig. 5A). However, the levels of β1-integrin and CD45 mRNAs did not change significantly in response to HMGB-1 signal (Fig. 5B). In ‘TLR-2ko’ group, expression levels of the two analysed mRNAs were significantly reduced (Fig. 5B).
Stimulation with HMGB-1 induces β1-integrin, P-selectin and ICAM-1 polarization in mouse endothelial cells

To analyse the effect of HMGB-1 on the expression and redistribution of β1-integrin, P-selectin and ICAM-1 on endothelial plasmalemma, SVEC cells were preconditioned and observed by confocal microscopy. In HMGB-1–treated endothelial cells, we detected an evident polarization of membrane proteins compared to untreated controls (Fig. 6). HMGB-1 signal induced a prompt response of SVEC cells, which showed significant membrane redistribution of β1-integrin (10 ± 2 cells/HPF, \( P < 0.05 \)), P-selectin (8 ± 4 cells/HPF, \( P < 0.05 \)) and ICAM-1 (30 ± 4 cells/HPF, \( P < 0.01 \); Fig. 6). To clarify whether this effect is specific for HMGB-1, endothelial cells were additionally stimulated with TNF-α, another well-known mediator of inflammation induced β1-integrin, P-selectin and ICAM-1 polarization in similar extent to HMGB-1 (Fig. 6). In contrast, pre-treatment of mouse
endothelial cells with SDF-1α-induced endothelial ICAM-1 but not β1-integrin and P-selectin polarization in a significant extent (Fig. 6).

Discussion

The primary intention of our study was to investigate the influence of HMGB-1, TLR-2 and TLR-4 on the preliminary phases of c-kit+ cell diapedesis and transmigration in vivo. In addition, we aimed to further characterize the effect of HMGB-1 on the expression of adhesion molecules, which are involved in cellular tethering, rolling and adhesion on the vascular wall in vivo. We describe for the first time the HMGB-1–dependent interactions of c-kit+ cells with the microvascular endothelium, using intravital microscopy. HMGB-1 preconditioning of mouse cremaster muscles triggered a significant increase in c-kit+ cell rolling and adhesion in vivo, which represent the first steps for cellular extravasation. Moreover, the chemokine generated a drastic up-regulation of eNOS and c-kit signals in the cremaster muscle after superfusion. The presence of both HMGB-1 receptors TLR-2 and TLR-4 on vascular endothelial cell was mandatory for HMGB-1–enhanced affinity between c-kit+ cells and the endothelial lining. Furthermore, TLR-2 was essential for the HMGB-1–mediated expression of eNOS, c-kit and P-selectin in the cremaster muscle after treatment. On the other hand, there was no difference of HMGB-1–mediated effects on c-kit+ isolated from TLR-2 (−/−) and Tlr4 (LPS-del) animals compared to ‘control’ cells. In vitro, HMGB-1 stimuli were determinant for the reorganization of important adhesion molecules on the plasmalemma of endothelial cells.

Our intravital microscopy study suggests that HMGB-1 is able to activate the cremaster endothelium immediately upon stimulation. The significantly increased rolling and adhesion behaviour of c-kit+ cells indicate that HMGB-1 could have an influence on the activation of endothelial signalling pathways, which may initiate the c-kit+ cell extravasation sequence. To our knowledge there are no other studies that focused on the direct intravital microscopic observation of HMGB-1–mediated c-kit+ cell interaction with the vascular endothelium. Few reports described intravital microscopy analysis of the murine bone marrow [17,18], while the majority of the studies in non-marrow organs have been performed on mature leukocyte behaviour [19,20]. Only a small number of records about peripheral stem cell–endothelium interactions in vivo are available [1,21,22]. In one of these, we illustrated that SDF-1α could significantly increase the adhesion capacity of c-kit+ cells only in presence of additional pro-inflammatory stimulus such as TNF-α [1]. Now, we demonstrate that HMGB-1 alone significantly increased c-kit+ cell commitment to roll and adhere on endothelial lining. This effect might corroborate the important inflammatory effect of HMGB-1 [3,6]. In fact, our current data show a minor increase of adherent ELs after HMGB-1 stimulation while it was not observed with SDF-1α in our previous study [1]. Our results support the view of HMGB-1 as a complex regulator of both inflammatory response and tissue regeneration.

The initiation of c-kit+ cell extravasation mediated by HMGB-1 was confirmed by the results of real-time PCR. Endothelial nitric oxide synthase, which has been shown to have a pivotal role in endothelial progenitor cells mobilization and homing [23, 24], was significantly up-regulated at mRNA and protein level. Moreover, the c-kit+ signal in the tissue was found to be considerably higher in ‘HMGB-1’ group compared to ‘Control’ detected by RT-PCR. These findings were agreeing with the c-kit+ cell homing potential
of HMGB-1 and suggest that its effect might be associated to eNOS activation.

Interestingly, we observed for the first time a central role of TLR-2 and TLR-4 on the rolling and adhesion ability of c-kit⁺ cells. The depletion of TLR-2 and TLR-4 on the vascular endothelium abolished HMGB-1–induced c-kit⁺ cell-endothelial interaction. However, there was no difference between c-kit⁺ cells isolated from WT and TLR-2 (-/-) or Tlr4 (LPS-del) animals regarding their rolling and adhesion behaviour on the endothelium of WT animals. This data reveal a critical function of endothelial TLR-2 and TLR-4 on HMGB-1–mediated c-kit⁺ cell migration. In addition, selective agonists of TLR-2 or TLR-4 alone were not capable to promote endothelial c-kit⁺ cell adhesion, in the same manner as HMGB-1, indicating both receptors contribute to this effect. RT-PCR results suggest that TLR-2 mediates HMGB-1 effects on cellular adhesion via P-Selectin, whereas TLR-4 is likely to influence another adhesion molecule pattern such as VCAM-1 or ICAM-1. Moreover TLR-2 seems to stimulate HMGB-1–dependent moderate local inflammatory reaction, which has been shown to be crucial for c-kit⁺ interaction with the endothelium [1]. With regard to

![Graph A](image)

**Fig. 5** Endogenous leukocyte adhesion on cremaster muscle 15 min. after direct HMGB-1 superfusion. (A) Number (n/mm²) of adherent leukocytes in ‘Control’ and ‘HMGB-1’ groups. (B) Quantitative real-time PCR analysis of the pro-inflammatory signals β1-integrin and CD45 of ‘Control’, ‘HMGB-1’, ‘TLR-2ko’ and ‘TLR-4ko’ groups. The average mRNA expression level of β1-integrin and CD45 and c-kit in ‘Control’ cremasters was arbitrarily given a value of 1 (line) (*P < 0.05 versus ‘Control’).
HMGB-1–mediated inflammation and stem cell mobilization, the exact role of HMGB-1 receptors TLR-2, TLR-4 and RAGE is still poorly understood. A previous study by Chavakis. [5] demonstrates the in vitro effects of HMGB-1 on human EPC adhesion and homing. They found that EPC expressed RAGE and TLR-2 but not TLR-4 on their surface. HMGB-1–induced EPC migration in a RAGE-dependent manner. Neutralizing TLR-2 did not influence the EPC migration significantly. In this study, we found that endothelial TLR-2 and TLR-4 signalling was mandatory for HMGB-1–mediated c-kit<sup>+</sup> cell recruitment. The discrepancy may be due to the fact that different cell populations may display distinct response mechanisms on stimulation with HMGB-1 [5]. van Zoelen et al. were able to show that an HMGB-1 associated increase of TNF-α, interleukin-6 and neutrophil numbers was dependent on TLR-4 and RAGE in the mouse [25]. On the other hand, the group of Yu described that there might be a differential usage of TLR-2 and TLR-4 in HMGB-1 signalling in primary cells or in cell lines [26].

Fig. 6 HMGB-1–induced changes in the expression of SVEC surface adhesion molecules. (A) Representative immunofluorescent staining of β1-integrin in untreated and TNF-α, SDF-1α or HMGB-1–treated SVEC. (B) Number of SVEC showing β1-integrin polarization within the groups. (C) Representative images of P-selectin expression on SVEC plasmalemma in untreated and TNF-α, SDF-1α or HMGB-1–treated cells. (D) Number of SVEC showing P-selectin redistribution after stimulation with different cytokines. (E) Confocal images of ICAM-1 expression patterns on SVEC membrane. (F) Quantity of cells presenting ICAM-1 polarization after preconditioning with TNF-α, SDF-1α or HMGB-1 (*P < 0.05 versus ‘Control’; **P < 0.01 versus ‘Control’, HPF = 630×).
In our investigation of HMGB-1 influence on adhesion molecule expression, we further displayed that the preconditioning of mouse endothelial cell line (SVEC) with HMGB-1 significantly affected the distribution of β1-integrin, P-selectin and ICAM-1 at the plasmalemma. The roles of β1-integrins in the recognition of extracellular matrix molecules has been extensively investigated [27–30]. Their several functions include the contribution to essential activities for the maintenance and functioning of blood vessels such as spreading, retraction, polarization and migration [31,32]. It is probable that the ability of HMGB-1 to reorganize β1-integrin on the endothelial membrane may be involved in the migration and extravasation of c-kit⁺ cells. In addition, HMGB-1 effect on the polarization of P-selectin and ICAM-1 in the endothelial cell line (SVEC) indicates that this chemokine could activate the endothelium eliciting c-kit⁺ homing. The homing of c-kit⁺ cells to sites of neovascularization might share at least some common mechanisms with the homing of leukocytes to sites of inflammation.

An open question is still which other molecules are involved in the mechanisms of endothelial TLR-2 and TLR-4–dependent c-kit⁺ cells adhesion, rolling and penetration. HMGB-1 could activate a series of signalling components through TLR-2 and TLR-4. The activation of TLR-2 and TLR-4 through a MyD88-depending mechanism may lead to nuclear factor–κB activation [33–35]. NF-κB, an important regulator of inflammation, could control the expression of genes encoding the pro-inflammatory cytokines and adhesion molecules (e.g. ICAM, VCAM, E-selectin) [33–35]. The up-regulation of adhesion molecules could increase adhesion of c-kit⁺ cells to stimulated endothelium and eventually promote cell recruitment. In addition, through binding of its receptor, HMGB1 elicited activation of metalloproteases 2 (MMP-2) and MMP-9 and enables the degradation of the extracellular matrix, which may further facilitate the cell penetration and migration [36].

The physiological relevance of HMGB-1 induced stem cell activation in the context of cardiovascular pathologies has been shown before by Limana et al. in an animal model of myocardial infarction. HMGB-1 was shown to induce myocardial regeneration after infarction by enhancement of c-kit⁺ cell proliferation and differentiation [9]. Regarding the relevance of TLRs for stem cell based cardiovascular regeneration processes, recent evidence suggests that TLR-2 might be crucial for mesenchymal stem cell-associated myocardial recovery after ischaemia–reperfusion [37]. With respect of these results and the findings of Chavakis on HMGB-1–induced homing of endothelial progenitor cells [5], the further investigation of HMGB-1–dependent adhesion and migration of c-kit⁺ stem cells in vivo, as performed in the current project, seems conceivable. In addition, we speculated the co-treatment of LPS and MALP-2 may have the identical effect with HMGB-1. To clarify if the co-treatment is capable to mimic the effect of HMGB-1 in vivo, the further investigation should compare the effect of co-treatment with LPS and MALP-2 with HMGB-1.

In conclusion, we demonstrated that HMGB-1 might increase the migration of c-kit⁺ cells in vivo and activate the endothelial cells both in vivo and in vitro. These effects seem to be regulated through TLRs on endothelial but not on c-kit stem cell. Our study highlights the potential effect of HMGB-1 on enhancing stem cell recruitment for tissue regeneration and the essential function of its downstream signalling through TLRs.

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Conflict of interest

The authors confirm that there are no known conflicts of interest associated with this publication.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 FACS analysis of murine bone marrow derived c-kit⁺ cell from WT, TLR-2 (−/−) and Tlr4 (LPS-del) for purity and surface molecule expression.

Table S1 Primers used in real-time PCR

Table S2 Microvascular haemodynamics of murine cremaster muscle

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