Human Cytomegalovirus–Platelet Interaction Triggers Toll-Like Receptor 2–Dependent Proinflammatory and Proangiogenic Responses

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Objective—Human cytomegalovirus (HCMV) is a widespread pathogen that correlates with various clinical complications, including atherosclerosis. HCMV is released into the circulation during primary infection and periodic viral reactivation, allowing virus–platelet interactions. Platelets are important in the onset and development of atherosclerosis, but the consequences of platelet–HCMV interactions are unclear.

Approach and Results—We studied the effects of HCMV–platelet interactions in blood from healthy donors using the purified clinical HCMV isolate VR1814. We demonstrated that HCMV bound to a Toll-like receptor (TLR) 2–positive platelet subpopulation, which resulted in signal transduction, degranulation, and release of proinflammatory CD40L and interleukin-1β and proangiogenic vascular endothelial–derived growth factor. In mice, murine CMV activated wild-type but not TLR2-deficient platelets. Very widespread from murine CMV–stimulated wild-type platelets also activated TLR2-deficient platelets, indicating that activated platelets generated soluble mediators that triggered further platelet activation, independent of TLR2 expression. Inhibitor studies, using ADP receptor antagonists and apyrase, revealed that ADP release is important to trigger secondary platelet activation in response to HCMV. HCMV–activated platelets rapidly bound to and activated neutrophils, supporting their adhesion and transmigration through endothelial monolayers. In an in vivo model, murine CMV induced systemic upregulation of platelet–leukocyte aggregates and plasma vascular endothelial–derived growth factor in mice and showed a tendency to enhance neutrophil extravasation in a TLR2-dependent fashion.

Conclusions—HCMV is a well-adapted pathogen that does not induce immediate thrombotic events. However, HCMV–platelet interactions lead to proinflammatory and proangiogenic responses, which exacerbate tissue damage and contribute to atherogenesis. Therefore, platelets might contribute to the effects of HCMV in accelerating atherosclerosis.

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Key Words: atherosclerosis □ blood platelets □ cytomegalovirus □ toll-like receptors

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duman cytomegalovirus (HCMV), a member of the β-herpesvirus family, is a widespread, well-adapted pathogen that infects a majority of the world’s population. After primary infection that is generally subclinical in the immunocompetent host, HCMV establishes latency and lifelong persistence.1 In response to inflammatory stress, latently infected monocytes differentiate into macrophages, enabling reactivation and replication of HCMV.2,3 Although this reactivation is asymptomatic in immunocompetent individuals, HCMV has been associated with a spectrum of clinical pathologies including atherosclerosis, restenosis after coronary angioplasty, and transplant vascular sclerosis.1

During primary HCMV infection or HCMV reactivation, whole virus, virus particles, and free viral DNA circulate in the blood,4 allowing HCMV–platelet interactions. Platelets are blood cells that fulfill a central function in hemostasis by facilitating the cessation of bleeding but also play a pivotal role in the development, as well as in the lethal consequences, of atherosclerosis. On activation, platelets assist and modulate inflammatory reactions and immune responses by direct interaction with leukocytes and endothelial cells and via release of soluble inflammatory mediators that mediate leukocyte activation and enhance their recruitment.5 Platelets also express surface receptors that can detect bacteria and viruses, allowing
them to quickly relay activating signals to the innate and adaptive immune compartment to orchestrate leukocyte migration through tissues or to govern repair processes.\(^6,7\) Platelets are known to bind HCMV,\(^8\) but the consequences of this interaction and the receptors involved are unknown. HCMV can interact with a plethora of receptors, including integrins and growth factor receptors.\(^9\) HCMV is also sensed by Toll-like receptors, a family of pattern recognition molecules that can engage a variety of microbial products and subsequently trigger an inflammatory response.\(^5\) Interestingly, the ligands for host TLRs are viral envelope glycoproteins, which also play key roles in the HCMV entry pathway.\(^10\) Therefore, TLR2, TLR7, and TLR9 form the central basis of the membrane detection machinery for HCMV.\(^11,12\) Although TLRs were initially thought to be restricted to cells of the innate immune system, it has been revealed recently that the majority of TLRs are also expressed on the surface of platelets.\(^13\)–\(^15\) Interestingly, not all platelets express TLRs: 55% to 60% are positive for TLR2, 10% to 40% are positive for TLR4, and 1% to 16% are positive for TLR9.\(^14\)–\(^16\) It was speculated that platelet expression of TLR might be due to the adsorption of free TLR in the plasma. However, megakaryocytes are also positive for TLRs,\(^14,17\) and platelet TLR1/2, TLR4, and TLR9 have been proven to be functional because they trigger intracellular signaling, resulting in platelet activation.\(^18\)–\(^21\)

It this study, we show that HCMV induces platelet activation via binding to a TLR2-positive platelet subpopulation, which in turn triggers further platelet activation via release of platelet granule content (eg, ADP), which results in proinflammatory and proangiogenic responses. Thus, HCMV–platelet interactions are likely to exacerbate tissue damage and contribute to atherogenesis.

### Materials and Methods

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

### Results

**HCMV Bound Platelets via TLR2**

HCMV–receptor interactions seem to be cell-type specific. To determine which platelet receptors bind HCMV, we added purified, ultraviolet-inactivated virus strain VR1814 to whole blood and measured virus–cell interactions. 1.9% of blood cells and 1.8% of all platelets bound HCMV (Figure 1). Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) and TLR2 have been reported to bind HCMV, and as platelets are known to express both receptors, we analyzed whether either were involved in platelet–HCMV interactions. 1.1% of all blood cells were positive for DC-SIGN, whereas 52.4% of the HCMV-positive cells were DC-SIGN positive (Figure 1). 0.7% of platelets were positive for DC-SIGN, whereas the HCMV-positive platelets of 32.1% were DC-SIGN positive (Figure 1). Blood cells of 2.1% were positive for TLR2, whereas the HCMV-positive cells of 90.5% were also TLR2 positive. Platelets of 1.9% were positive for TLR2, whereas the HCMV-positive platelets of 91.4% were positive for TLR2 (Figure 1). These results imply that HCMV predominantly binds to TLR2-positive platelets.

**HCMV Induced Platelet Activation via TLR2**

We then determined whether HCMV–platelet interactions induced platelet activation. As depicted in Figure 2A, the lowest concentration of HCMV sufficient to induce a significant increase in platelet surface expression of P-selectin was 1×10⁹ HCMV particles/mL. Platelet activation in response to HCMV was a rapid process because a significant increase in platelet surface expression of P-selectin could be already observed after 5 minutes of stimulation and a plateau was reached at 10 minutes of incubation (Figure 2B). HCMV-induced surface expression of P-selectin was completely abolished in the presence of a TLR2-blocking antibody or the phosphoinositide 3-kinase inhibitor LY294002 (Figure 2B). In line with these results, HCMV-treated platelets also had increased AKT, p38, and extracellular signal-regulated kinase (ERK) phosphorylation, with less pronounced effects in platelets that were treated with a blocking TLR2 antibody (Figure 2C). Although only 1% to 3% of platelets were positive for TLR2 and bound HCMV, 12% to 22% became activated in response to HCMV (Figure 2B).
Analysis of murine TLR2−/− platelets revealed that platelets lacking TLR2 were unable to respond to murine CMV (MCMV), although platelet supernatant from wild-type mice was able to trigger platelet activation even in TLR2−/− mice (Figure 2D). This indicated that in response to CMV platelets release mediators that result in further platelet activation. We then tested whether ADP/ATP release was responsible for the observed effects. Indeed, we found that apyrase or ADP receptor antagonists abrogated HCMV-mediated platelet activation (Figure 2E).

To further prove that soluble mediators released from HCMV-treated platelets can activate human platelets, we stimulated TLR2-inhibited platelets with the supernatant of HCMV-treated platelets in the presence or absence of apyrase or ADP receptor antagonists (Figure 2F). In line with results from CMV stimulation of murine platelets, the supernatant of HCMV-stimulated platelets and surface expression of CD62P determined by flow cytometry. Means and SDs from 3 independent experiments. C. Western blots of phosphorylated (p-AKT, p-ERK) and total proteins (AKT, ERK) of platelets (3×10⁸/mL) stimulated with HCMV (3×10⁹/mL) for 10 minutes in the presence and absence of a blocking anti-TLR2 antibody (25 μg/mL). One typical Western blot of 3 independent experiments. D. Platelets from wild-type and TLR2−/− mice (3×10⁸/mL) were stimulated with murine CMV (MCMV; 1×10⁹/mL) or supernatant from MCMV-stimulated platelets and surface expression of CD62P determined by flow cytometry. Means and SDs from 3 independent experiments. E. Human platelets (3×10⁹/mL) in the presence and absence of apyrase (10 U/mL) or P2Y1 inhibitor MRS2179 (10 μM/L) or P2Y12 inhibitor MRS2159 (10 μM/L) were stimulated with HCMV (1×10⁹/mL) and surface expression of CD62P determined by flow cytometry. One typical dot plot of 3 independent experiments. F. Platelets were preincubated with anti-TLR2 (25 μg/mL) and then stimulated with supernatants from HCMV-stimulated platelets and surface expression of CD62P determined by flow cytometry. One typical dot plot of 3 independent experiments. *P<0.05. FCS indicates forward scatter.

Platelet Activation by HCMV Did Not Induce Platelet Adhesion or Aggregation
To determine whether HCMV modifies platelet adhesion, we measured platelet adhesion to a collagen-coated surface under high shear stress in whole blood. As shown in Figure 3A, platelet adhesion and aggregate formation were not enhanced by HCMV treatment. We then analyzed the effects of HCMV on platelet aggregation using light transmission aggregometry. As shown in Figure 3B, platelet aggregation was not enhanced in response to HCMV stimulation. Platelet stimulation with HCMV-treated neutrophil supernatant did not result in platelet aggregation either (Figure 3B). We then examined whether platelets provide a more procoagulant surface in response to HCMV stimulation by testing the levels of the procoagulant phosphatidylserine, but no significant increase could be observed (Figure 3C). We then examined whether platelet–fibrinogen binding was affected by HCMV treatment using that HCMV binds to and activates human platelets via a TLR2-dependent mechanism.
fluorescence labeled fibrinogen but could not detect any significant changes (Figure 3D). Based on these data, we conclude that HCMV does not induce procoagulant effects nor can an increase in platelet aggregate formation be observed, suggesting that the action of HCMV on platelets is unlikely to have prothrombotic effects.

Platelets Interact With Different Leukocyte Subsets in Response to HCMV

The initial attachment between platelets and leukocytes is mediated by platelet P-selectin binding to its constitutively expressed leukocyte counter receptor P-selectin glycoprotein ligand-1, resulting in the formation of platelet–leukocyte aggregates. Because HCMV induces surface expression of P-selectin, we analyzed whether this activation could induce the formation of platelet–leukocyte aggregates. The percentage of platelet-positive leukocytes in whole blood significantly increased after HCMV stimulation (Figure 4A). These effects were dependent on P-selectin/P-selectin glycoprotein ligand-1 interaction because a blocking antibody against P-selectin (anti-CD62P) completely abolished the formation of these aggregates (Figure 4A). Moreover, platelets were unable to bind leukocytes in the presence of a TLR2-blocking antibody. To determine whether TLR2 from platelets or leukocytes was responsible for the observed effects, we incubated platelets with anti-TLR2 and added HCMV-activated leukocytes, or vice versa. As depicted in Figure 4A, inhibition of platelet TLR2 was sufficient to reduce platelet–leukocyte aggregate formation to almost basal levels.

Analysis of leukocyte subpopulations interacting with platelets revealed that the strongest increase in platelet–leukocyte aggregates was found with neutrophils, followed by monocytes and lymphocytes (Figure 4B). Analysis of lymphocyte subsets revealed that in response to HCMV only platelet interaction with B cells increased significantly, whereas there was only a trend of increased platelet–T-cell interaction (Figure 4B). Analysis of dendritic cell subpopulations revealed that HCMV-activated platelets interacted with both the myeloid and the plasmacytoid dendritic cells (Figure 4B).

Platelets Enhanced Neutrophil Activation in Response to HCMV

We then examined the effects of HCMV on neutrophil activation in the presence and absence of platelets. HCMV led to CD11b activation (Figure 4D) and enhanced surface expression of CD14 and CD16 and resulted in increased reactive oxygen formation (Figure 4E). These neutrophil activating effects were further enhanced by the presence of platelets (Figure 4D and 4E), indicating that platelets further accelerate neutrophil activation in response to HCMV.

Platelets Enhanced Leukocyte Adhesion and Transmigration in Response to HCMV

To determine whether HCMV and platelets influenced leukocyte adherence and transmigration, neutrophils, with or without platelets, were perfused at a shear rate of 50 s⁻¹ across a tumor necrosis factor-α–treated human umbilical vein endothelial cells (HUVEC) monolayer, in the presence or absence of HCMV. As shown in Figure 5A, treatment of leukocytes with HCMV tended to enhance adhesion to an HUVEC monolayer, whereas in the presence of platelets, HCMV significantly enhanced leukocyte adhesion (Figure 5A). In response to stimulation with HCMV, leukocyte transmigration was also significantly enhanced (Figure 5B), and this effect was further boosted in the presence of platelets. From these data, we conclude that HCMV induces leukocyte activation, adhesion, and transmigration and that platelets further amplify these processes.

HCMV Stimulated Platelets Released Proinflammatory and Proangiogenic Chemokines

Next, we analyzed surface expression and release of proinflammatory CD40L and interleukin-1β (IL-1β) and proangiogenic vascular endothelial–derived growth factor (VEGF) release on
stimulation with either HCMV or ADP. VEGF was measured after 15 minutes because it is immediately released on platelet degranulation, whereas CD40L is first expressed on the platelet surface followed by shedding, a process which takes minutes to hours. IL-1β can be freshly synthesized and released via microvesicles. Therefore, soluble CD40L (sCD40L) and IL-1β were measured after 2 hours. HCMV and ADP induced surface expression of CD40L after 15 minutes and release of sCD40L and IL-1β after 2 hours, but the effects were more pronounced in ADP-stimulated platelets compared with HCMV stimulation (Figure 6A–6C). VEGF rapidly increased in response to HCMV stimulation to a higher extent compared with ADP (Figure 6D). As shown in Figure 6E, conditioned supernatant from HCMV-stimulated and ADP-stimulated platelets promoted tube formation, whereas the conditioned supernatant from unstimulated platelets, or HCMV in buffer, had no proangiogenic effect. From these data, we conclude that HCMV triggers the release of proangiogenic and proinflammatory cytokines from platelets.

**Cytomegalovirus Induced Platelet Activation and Neutrophil Extravasation In Vivo**

To determine whether CMV activated platelets in vivo, we infected mice with MCMV and examined platelet activation in the
Platelets have been reported previously to express TLR2 either TLR2+/+ or TLR2−/− platelets, which were ex vivo stimulated to determine whether platelet TLR2 was involved in these effects. We platelet depleted and transfused wild-type compared with TLR2−/− mice (Figure 7D) significantly increased. In a thioglycollate-elicited sterile peritonitis model, we can show that MCMV shows a tendency to enhance neutrophil extravasation with effects more pronounced in TLR2−/− platelets. In line with this observation, also neutrophil recruitment was enhanced with acute and chronic allograft rejection and vasculopathy,23 confirming by studying murine platelets lacking TLR2, which did not respond to CMV stimulation, whereas the supernatant of CMV-treated wild-type platelets resulted in platelet degranulation in vivo. As shown in Figure 7H, we further show that ADP and ATP are involved in this secondary platelet activation because apyrase and ADP receptor antagonists could counteract this secondary activation. ADP release is a crucial cofactor for platelet activation in response to many agonists, which made blocking of the ADP receptor a widely used clinical target to prevent platelet activation. Apyrase has been shown previously to be a potent inhibitor of secondary platelet activation in response to TLR2 activation because it reduced P2X1-evoked calcium signals.22

HCMV is released into the blood during active infection, allowing HCMV–platelet interactions. Here, for the first time, we show that HCMV predominately bound to a TLR2-positive platelet subpopulation, leading to the induction of platelet activation through a TLR2-dependent pathway. Platelet activation by HCMV could be completely abrogated on blocking TLR2 receptor or inhibition of phosphoinositide 3-kinase signaling, which is a key mediator in TLR2-induced platelet activation.19 Platelets have been reported previously to express TLR2 on the surface of 4% to 16% platelets.14–16 However, in our study, these levels were lower with 1.3% to 3% TLR2-positive platelets, which strongly correlated with the percentage of HCMV-bound platelets. Despite the low percentage of TLR2-positive platelets, ≥20% of platelets became P-selectin positive in response to HCMV, indicating that activation of the TLR2-positive subpopulation triggered further platelet activation in a TLR2-independent fashion. This observation was confirmed by studying murine platelets lacking TLR2, which did not respond to CMV stimulation, whereas the supernatant of CMV-treated wild-type platelets resulted in platelet degranulation even in the absence of a functional TLR2 receptor. We further show that ADP and ATP are involved in this secondary platelet activation because apyrase and ADP receptor antagonists could counteract this secondary activation. ADP release is a crucial cofactor for platelet activation in response to many agonists, which made blocking of the ADP receptor a widely used clinical target to prevent platelet activation. Apyrase has been shown previously to be a potent inhibitor of secondary platelet activation in response to TLR2 activation because it reduced P2X1-evoked calcium signals.22

Concentrations of 1 million HCMV particles per mL, which corresponds to a HCMV:platelet ratio of 1:300, were sufficient to trigger a significant increase in platelet degranulation. In organ transplant patients, where HCMV is associated with acute and chronic allograft rejection and vasculopathy,23...
circulating plasma levels of HCMV range between 0 and 30,000 virus copies/mL or ≤8 virus particles per 100 leukocytes, with higher levels at infection sites, making platelet activation in response to HCMV a likely in vivo scenario. However, HCMV-induced platelet activation did not result in platelet adhesion and aggregation of HCMV-treated platelets under high shear stress to a collagen-coated surface. Nor did HCMV induce surface expression of procoagulant platelet phosphatidylserine or increased fibrinogen binding of platelets.

We show that HCMV-stimulated platelets rapidly formed heterotypic aggregates with leukocytes, through platelet TLR2 signaling and subsequent platelet P-selectin binding to P-selectin glycoprotein ligand-1. Analysis of leukocyte subsets revealed that platelets predominantly interacted with monocytes, neutrophils, as well as with dendritic cells. In the lymphocyte population, only an increase in B-cell–platelet aggregates but not T-cell–platelet aggregates was observed in response to HCMV. Elevated levels of circulating platelet-leukocyte aggregates represent a risk factor for cardiovascular disease and are a predictor of acute myocardial infarction because the formation of platelet-leukocyte aggregates is known to promote leukocyte activation, formation of reactive oxygen species, and the exposure of leukocyte tissue factor. Indeed, we show that HCMV led to a rapid activation of neutrophils, resulting in degranulation and reactive oxygen formation, which was more pronounced when platelets were present. On stimulation with HCMV, leukocyte adhesion and transmigration through an endothelial monolayer were elevated and also these processes were further amplified in the presence of platelets. Platelet activation and platelet-leukocyte interaction are a known risk factors for various pathologies; however, the in vivo significances of platelet activation and leukocyte activation in response to HCMV need to be addressed in further studies.

HCMV infection modulates the type and quantity of bioactive proteins released from infected cells, and many of these factors play important roles in the pathogenesis of vascular diseases. However, it is currently unknown which factors platelets release in response to HCMV stimulation. Here, we show that platelet–HCMV interaction induced the release of proangiogenic cytokines such as VEGF, which are involved in endothelial migration, proliferation, and increased vascular permeability and can lead to leukocyte recruitment to the atherosclerotic plaque. In line with these results, we show that HCMV-stimulated platelet supernatants were able to induce endothelial tube formation. Moreover, we show that HCMV-activated platelets expressed and released CD40L. Platelet-derived CD40L is known to mediate the development of atherosclerosis by affecting platelet–platelet, platelet–leukocyte, and leukocyte–endothelium interactions. Furthermore, CD40L induces inflammatory responses in the endothelium, initiates the formation of reactive oxygen species, inhibits nitric oxide production, causes the release of chemokines, and upregulates adhesion receptors and tissue factor. Moreover, sCD40L is involved in
neointima formation and impairs angiogenic peripheral blood outgrowth cells. 33 We also show that HCMV-activated platelets secreted IL-1α that is a key mediator of the inflammatory host response that also exacerbates tissue damage. 34 These observations imply a role for HCMV–platelet interactions in the promotion of both inflammation and angiogenesis.

Animal models have provided solid in vivo evidence linking CMV to the acceleration of vascular disease processes. CMV infection of apolipoprotein E knockout mice leads to aggravated lesion size through local and systemic immune activation. 35 Interestingly, the effect of CMV on plaque development was independent of virus replication and also observed with ultraviolet radiation-inactivated virus, 35 indicating that surface receptor binding is sufficient for these processes. Because platelets are crucially involved in inflammatory and atherogenic processes and able to bind CMV, CMV–platelet interactions might be of high in vivo relevance.

Several lines of evidence point toward a role of TLRs in CMV-induced acceleration of atherogenesis. 36 To date, the extent to which endogenous and exogenous TLR2 agonists contribute to direct platelet activation and proinflammatory and proatherogenic consequences in atherosclerosis is unclear. It has previously been shown that exogenously stimulated bone marrow–derived TLR2 is involved in atherogenesis, 37 but the role of platelet TLR2 has never been investigated in this context. We were able to show in this study that murine cytomegalovirus increased CD40L expression, VEGF release, and platelet–leukocyte aggregate formation in vivo. In a sterile inflamed peritonitis model, we further show that CMV stimulation slightly enhanced neutrophil extravasation. To address the role of platelet-derived TLR2 in CMV infection, we ex vivo stimulated wild-type and TLR2 knockout platelets with CMV and transfused them into platelet-depleted mice. Ex vivo platelet stimulation resulted in in vivo platelet aggregate formation and a tendency to increase neutrophil extravasation in a TLR2-dependent fashion.

Although our study points toward a role for platelets in neutrophil activation and migration, further studies are warranted to elucidate the role of platelets in CMV-induced acceleration of atherogenesis and to determine the contribution of platelet TLR2 to atherosclerotic disease progression.

Taken together, we show that HCMV directly interacts with, and activates, a specific subset of platelets. Although this HCMV–platelet interaction does not induce immediate thrombotic events, it enhances the formation of platelet–leukocyte aggregates, promotes neutrophil activation and extravasation, as well as angiogenesis. These processes are critical for immune defense and wound repair, but they can also exacerbate tissue damage and enhance inflammation and atherogenesis. Therefore, the presence of HCMV in the circulation, from either primary infection or periodic viral reactivation, and the subsequent HCMV–platelet interactions could potentially contribute to the effects of HCMV in accelerating atherosclerosis.

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Disclosures

None.

References

In this study, we provide first evidence that human cytomegalovirus (CMV), a well-adapted herpesvirus, activates a subset of platelets via a Toll-like receptor 2–dependent mechanism. Platelet–CMV interaction did not result in platelet aggregation but triggered platelet degranulation, resulting in the release of proinflammatory and proangiogenic responses. On stimulation with CMV, platelets rapidly interacted with leukocytes and enhanced neutrophil activation and transmigration. Supernatants of CMV-stimulated platelets contained high levels of vascular endothelial–derived growth factor and accelerated angiogenic sprouting. In a mouse model, transfusion of ex vivo stimulation of platelets with CMV was sufficient to trigger platelet–leukocyte interactions in vivo, indicating that platelet could contribute leukocyte activation in vivo. These proinflammatory and proangiogenic responses of CMV-stimulated platelets might exacerbate tissue damage and contribute to CMV-associated acceleration of atherosclerosis.
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Material and Methods

Virus propagation and isolation
HCMV strain VR1814 (provided by G. Gerna, University of Pavia, Pavia, Italy) was prepared from cell-free supernatants of in vitro infected human umbilical vein endothelial cells (HUVECs), grown in EBM-2 medium with EGM-2 SingleQuots supplement (Clonetics, Cambrex), frozen, and stored at -85°C until use. VR1814 was then UV inactivated (UVStratalinker 1800; Stratagene), purified by gradient centrifugation as described and analysed and counted using a NanoSight LM10 instrument and Nanoparticle Tracking Analysis Software (NanoSight, Amesbury, UK).

Blood collection and isolation of human platelets and neutrophils
Venous blood was collected with a 24-G needle from healthy volunteers that were free of any anti-platelet medication for at least 2 weeks. The study was approved by the ethics committees of the Karolinska Institute (01-420). All human participants gave written informed consent.

Blood was anti-coagulated with 3.2% NaCitrate (BD Biosciences, NJ, USA) and platelet-rich plasma (PRP) generated by centrifugation of citrated blood at 125 g for 20 min. Platelets were isolated from PRP by centrifugation at 3000 g for 2 min in the presence of PGI2 (1 µM; Sigma-Aldrich, Stockholm, Sweden), followed by re-suspension in PBS containing 5% plasma from the same donor. Neutrophils were isolated by centrifugation of whole blood over a 2-step density gradient consisting of equal quantities of Histopaque 1077 and Histopaque 1119 (Sigma-Aldrich). The neutrophil layer was aspirated from the density interface and washed twice in PBS. Platelets were added to leukocytes at physiologically relevant ratios.

Flow cytometry
HCMV binding to blood cells was determined by positivity for gB-FITC (Virusys Cooperation, Taneytown, TA, USA). TLR2 and DC-SIGN positive cell subpopulations were determined with Alexa 647-conjugated anti-TLR2 and PerCP/Cy5.5-conjugated anti DC-SIGN (both antibodies from Biolegend, San Diego, CA, USA). Platelets were inhibited using PI3K inhibitor LY294002 (20µM; Cell Signaling Technology, Boston, MA, USA), apyrase grade VI (10U/ml; Sigma-Aldrich) or P2Y1 inhibitor MRS2179 and P2Y12 inhibitor MRS2159 (10µM; Sigma-Aldrich) or neutralising anti-TLR2 (25µg/ml; Invivogen, San Diego, CA, USA) 15 minutes prior to experiments. Platelet activation induced by ADP, HCMV or HCMV-stimulated platelet supernatant was determined by quantification of surface expression of activation markers using PE-conjugated anti-P-selectin (anti-CD62P), FITC-conjugated anti-CD40L or FITC-conjugated anti-CD63 (all antibodies from Biolegend)³. Surface expression of phosphatidylserine was analysed, based on FITC-labelled annexin V (Enzo Life Sciences, Lausen, Switzerland) binding³ and fibrinogen binding was analysed, based on the binding of Alexa 647-labelled fibrinogen (Molecular Probes, Invitrogen, Carlsbad, CA, USA). Formation of platelet-leukocyte aggregates was analysed by two-colour flow cytometry as described⁴ using Alexa 647-conjugated anti-CD61 and Pacific-Blue-conjugated anti-CD45 antibodies (BioLegend). Monocyte subset was analysed using an APC-Cy7 conjugated anti-CD14 (BD, Biosciences). Neutrophils were determined using Alexa488-conjugated anti-CD15 (BioLegend). Lymphocyte subsets were analysed using FITC-conjugated anti-CD3 and PE-conjugated anti-CD19 (BD, Biosciences). Neutrophil activation was determined using PE-conjugated CD16, APC-Cy7 conjugated anti-CD14 and APC-conjugated anti-CD11b (activated, clone CBRM1/5, BioLegend). Reactive oxygen species were determined by dihydrorhodamine 123 (DHR 123; Enzo Life Sciences, Lausen, Switzerland). Cytometric analyses were performed using a CyAN ADP flow cytometer and Summit V4.3 software (Beckman Coulter, Brea, USA).

Western blot
Platelets in the presence and absence of anti-TLR2 (25µg/ml) were stimulated with HCMV for 10 minutes and immediately lysed in Laemmli buffer. Lysates were separated by SDS-
PAGE, blotted to PVDF membrane and probed with rabbit primary Abs against phosphorylated AKT, ERK1/2 and p38 (Cell Signalling Technology, Boston, United States). For detection, a HRP-conjugated anti-rabbit IgG (Dako, Stockholm, Sweden) was used. After detection of phosphorylated proteins, the membranes were stripped (reblot plus strong solution, Milipore, Solna, Sweden), and total AKT, ERK1/2 and p38 (Cell Signalling Technology, Boston, United States) detected on the same blot. For each pair of proteins (phosphorylated and total), platelets from at least 3 donors were used. Quantification of images was done with ImageJ and relative ratios of the intensities of each protein pair were calculated.

Platelet adhesion under shear stress
Whole blood was stimulated with ADP (25µM) or HCMV (6x10^9 particles/ml) for 5 min, perfused over collagen IV-coated channel slides (iBiDi, Martinsried, Germany) for 4 min and monitored by real-time capture of microscopic phase contrast images (Olympus CKX41, Tokyo, Japan). The flow rate was set to 5 ml/min, to give a shear rate of 1000 sec^{-1}. After a 5-min washout with PBS, pictures of adherent platelets were taken at a 10x magnification and analysed using ImagePro (DataCell, Finchampstead, UK) and ImageJ (Wayne Rasband, NIH, Bethesda, USA).

Platelet aggregation
Platelet aggregation was performed in an optical 4-channel aggregometer (490-4D, Chronolog Corp., Havertown, PA, USA). Transmission was measured between 0% and 100% set with platelet suspension and buffer, respectively. Platelet suspensions were stirred for 1 minute in siliconised glass cuvettes in the aggregometer before adding the aggregating agents. ADP, HCMV or supernatant from HCMV-stimulated neutrophils (1x10^7 neutrophils stimulated for 20 min with 1x10^9 HCMV, following centrifugation at 1 000xg for 10 min) from the same blood donor and platelet aggregation monitored over 8 minutes.

Immunofluorescence
Formation of platelet-leukocyte aggregates in response to HCMV was confirmed by confocal microscopy. Leukocytes were then stained with murine anti-CD45 antibody (Dako, Glostrup, Denmark), followed by Alexa 594-conjugated goat anti-mouse antibody (Molecular Probes, Invitrogen), and platelets were stained with Alexa 647-conjugated anti-CD61 (BioLegend). Cells were analysed using a Leica TCS SP5 confocal microscope with 40-x objective (Leica, Wetzlar, Germany).

Measurement of secreted pro-angiogenic and pro-inflammatory proteins
VEGF, CD40L and interleukin-1β (IL-1β) in the supernatant from HCMV-stimulated platelets (following centrifugation at 1 000 x g for 90 sec in the presence of 1µM PGI_2) were determined by ELISA (R&D Systems, Abingdon, United Kingdom), according to the instructions of the manufacturer.

Leukocyte recruitment assay
Primary cultures of HUVECs were dissociated with trypsin-EDTA (Sigma-Aldrich) and passaged into channel slides (µ-Slide VI; iBiDi, Martinsried, Germany). Confluent HUVECs were TNF-α treated (50U/µl, for 4h) and channel slides mounted on a phase-contrast microscope stage. One end of a channel was attached to a Harvard withdrawal syringe pump (Harvard Apparatus, South Natick, MA), set to low shear rates (50 s^{-1}). The other end of the channel was attached to an electronic valve (Lee Products, Gerards Cross, United Kingdom), allowing smooth transition between reservoirs containing neutrophil suspension, platelet-neutrophil suspension or cell-free buffer. Residual TNF-α was first removed by perfusion with PBS with 1% BSA. Then a 4-minute bolus of either unstimulated or HCMV-treated neutrophils in the absence or presence of platelets was perfused through the microslide, followed by washout with PBS with 1% BSA. Video-microscopic recordings of leukocyte-HUVEC interactions were made for 10 sec in separate fields along the centerline of the channel.
Images were analysed off-line using a computerised image analysis system (ImagePro; DataCell, Finchampstead, United Kingdom). Leukocytes were classified as: (i) phase-bright cells rolling slowly over the HUVEC surface; (ii) phase-bright, activated, stationary cells; (iii) transmigrated phase-dark cells. Adherent cells were easily distinguished from non-adherent flowing cells, which were visible only as faint streaks. Adhesion data were calculated by counting all cells interacting with the monolayer and expressed as cells per mm²/per 10⁶. Untreated HUVEC did not support any neutrophil recruitment and TNF stimulated monolayers provided a positive control.

**Capillary tube assay**

To assess capillary tube formation primary cultures of HUVECs⁵ (2x10⁵ cells/ml) were seeded on growth factor reduced matrigel (BD, Biosciences) coated angiogenesis µ-slides (iBiDi, Martinsried, Germany) and stimulated with conditioned supernatant from HCMV-stimulated and unstimulated platelets for 6 hours. As a control HUVECs were also treated with HCMV in PBS and supernatant of ADP-stimulated platelets. Capillary tube formation was recorded (at 10×magnification) with phase-contrast microscopy (Olympus CKX41, Tokyo, Japan) and analysed using ImagePro Software (DataCell, Finchampstead, UK).

**Mice strains and murine platelet isolation**

TLR2⁻/⁻ mice on C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Blood was obtained by orbital sinus venopuncture and immediately anticoagulated (3.2% trisodium citrate). TLR2⁻/⁻ mice were bred as heterozygotes and only littermates were used for experimentation. Blood was diluted (1:4) in HEPES Tyrode buffer and centrifuged (50x g for 10 min) to obtain PRP. PRP was stimulated with murine CMV (MCMV) for 15 min and platelet activation determined by flow cytometric measurement of P-selectin expression on the platelet surface (anti-CD62P-FITC, Emfret Analytics GmbH, Germany). MCMV-stimulated platelet supernatant was prepared by centrifugation of MCMV-stimulated platelets at 3 000x g for 60 seconds.

**In vivo effects of MCMV on platelet activation**

C57BL/6 mice were intraperitoneally injected with either MCMV (2x10⁵ pfu) or PBS (100µl). After 3 hours, mice were anesthetised by isoflurane and blood was collected by cardiac puncture and immediately anticoagulated (3.2% NaCitrate, PPACK and PGI₂). Experiments were approved by the Stockholm North Committee for Experimental Animal Ethics (N110/11). Platelet-leukocyte interactions were determined by flow cytometric measurement of double-positivity for FITC-conjugated anti-CD45 and Alexa 647-conjugated anti-CD61 (Biolegend) cells. Surface expression of CD40L was determined by PE-conjugated anti-CD40L (Biolegend). Fibrinogen binding was analyzed by platelet binding to Alexa 647-conjugated fibrinogen (Molecular Probes, Invitrogen). Plasma levels of VEGF were determined by ELISA (R&D Systems) according to the instructions of the manufacturer.

**Platelet depletion and transfusion**

For platelet depletion and transfusion mice were short-term anesthetised with isoflurane (Forane, Abbot GesmbH, Vienna, Austria). For platelet transfusion, blood from TLR2⁻/⁻ and respective littermate TLR2⁺/+ control mice was collected retro-orbitally into citrated tubes and pooled to a volume of 1.5mL donor blood per recipient mouse. Blood was centrifuged (50xg, 10 min) and PRP treated for 15 min with MCMV (1x10⁶) or left untreated. PRP was then centrifuged in the presence of 1/25 ACD and 0.5 U/mL apyrase (Sigma-Aldrich) and platelets resuspended in sterile PBS to a density of 7x10⁹/ml.

Recipient C57BL/6J mice were platelet depleted by intravenous retro-orbital injection of anti-CD42b (0.5µg/g body weight; Emfret Analytics GmbH) and after 1 hour 200µl of MCMV-treated or untreated washed platelets were transfused.

**In vivo neutrophil recruitment assay**
For the neutrophil recruitment assay, TLR2−/− and respective littermate TLR2+/− control mice or platelet depleted C57BL/6, treated with TLR2+/+ or TLR2−/− platelet transfusions, were intraperitoneally injected with 0.5 ml sterile 3% thioglycollate (Sigma-Aldrich). After 3 h, mice were anesthetised by injection of ketamine (100 mg/kg body weight; ketaminol, Intervet International GmbH, Germany) and xylazine (10 mg/kg body weight, Xylasol, Dr. E. Gräub AG, Switzerland). Cell composition of peritoneal lavage fluid (retrieved by injection of 8 ml PBS) and cell counts were determined by flow cytometry using anti-CD45-Alexa647 (BioLegend) and anti-Ly-6G/6C-PE (BioLegend).

Statistical analysis
Results are presented as mean value ± standard deviation (SD). Effects of multiple treatments were tested using analysis of variance (ANOVA) followed by a Bonferroni post hoc testing. Effects of single treatments were tested by an unpaired t-test using GraphPad Prism 5.00 (GraphPad Software, San Diego, CA, USA). Graphics of the calculated data were drawn with SigmaPlot 10.0. (Systat Software, San Jose, CA, USA) * P values < 0.05; ** P values < 0.01 were considered significant.

References