Tick-host-pathogen interactions in Lyme borreliosis
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The urokinase receptor (uPAR) facilitates clearance of *Borrelia burgdorferi*

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Abstract

The causative agent of Lyme borreliosis, *Borrelia burgdorferi*, has been shown to induce expression of the urokinase receptor (uPAR); however the role of uPAR in the immune response against *Borrelia* has never been investigated. uPAR not only acts as a proteinase receptor, but can also, dependently or independently of ligation to uPA, directly affect leukocyte function. We here demonstrate that uPAR is upregulated on murine and human leukocytes upon exposure to *B. burgdorferi* both in vitro as well as in vivo. Notably, *B. burgdorferi*-inoculated C57BL/6 uPAR knock-out mice harbored significantly higher *Borrelia* numbers compared to WT controls. This was associated with impaired phagocytic capacity of *B. burgdorferi* by uPAR knock-out leukocytes in vitro. *B. burgdorferi* numbers in vivo, and phagocytic capacity in vitro, were unaltered in uPA, tPA (low fibrinolytic activity) and PAI-1 (high fibrinolytic activity) knock-out mice compared to WT controls. Strikingly, in uPAR knock-out mice partially backcrossed to a more *B. burgdorferi* susceptible C3H/HeN background, higher *B. burgdorferi* numbers were associated with more severe carditis and increased local TLR2 and IL-1β mRNA expression. In conclusion, in *B. burgdorferi* infection, uPAR is required for phagocytosis and adequate eradication of the spirochete from the heart by a mechanism that is independent of binding of uPAR to uPA or its role in the fibrinolytic system. A better understanding of the role of uPAR in the course of Lyme borreliosis is of interest since various uPA/uPAR agonists/antagonists have been tested in oncological trials and disseminated Lyme borreliosis can be difficult to treat. Therefore, compounds modulating host immune responses may prove to be a valuable asset in the treatment of Lyme borreliosis in adjunction to antibiotics.

Authors Summary

Lyme borreliosis is caused by *Borrelia burgdorferi* and is transmitted through ticks. Since its discovery approximately 30 years ago it has become the most important vector-borne disease in the Western world. The pathogenesis is still not entirely understood. We here demonstrate that the urokinase receptor (uPAR) is upregulated in mice and humans upon exposure to *B. burgdorferi* in vitro and in vivo. Importantly, we describe the function of uPAR in the immune response against the spirochete; using uPAR knock-out mice we show that uPAR plays an important role in phagocytosis of *B. burgdorferi* by leukocytes both in vitro as well as in vivo. In addition, we show that the mechanism by which uPAR is involved in the phagocytosis of *B. burgdorferi* is independent of ligation to its natural ligand uPA or uPAR’s role in fibrinolysis. Understanding the role of uPAR in the course of Lyme borreliosis might be of clinical interest since various uPA/uPAR agonists/antagonists have been tested in oncological trials and disseminated Lyme borreliosis can be difficult to treat. Therefore, compounds modulating host immune responses may prove to be a valuable asset in the treatment of Lyme borreliosis in adjunction to antibiotics.
Introduction

Lyme borreliosis, an emerging tick-borne disease in both the New and Old world, is caused by spirochetes belonging to the *Borrelia burgdorferi* sensu lato group and is predominantly transmitted by *Ixodes* ticks [1]. In the United States *Borrelia burgdorferi* sensu stricto, from here on referred to as *B. burgdorferi*, is the only prevalent *Borrelia* species, whereas in Europe three *Borrelia* species - *B. burgdorferi*, *Borrelia garinii* and *Borrelia afzelii* – are able to cause Lyme borreliosis [2,3]. In humans, all three species frequently cause an erythematous cutaneous lesion, *erythema migrans*. In later stages of infection spirochetes can disseminate and cause disease that affects the joints, cardiac conduction system, central nervous system and the skin [4].

*Borrelia* has been shown to differentially express specific genes to inhibit, modulate or to bypass the host immune system [5] and to bind to host molecules in order to establish a persisting infection. In addition, *B. burgdorferi* can interact with the host fibrinolytic system [6]. *B. burgdorferi* abuses host plasminogen activators to activate plasminogen within the tick gut to facilitate migration through the arthropod vector [7]. However, plasminogen is not critical for transmission and infection, since plasminogen deficient mice do develop an infection after intradermal inoculation with *B. burgdorferi* [7]. In in vitro studies, the spirochete causes upregulation of the urokinase Plasminogen Activator (uPA) [8,9], the Plasminogen Activator Inhibitors (PAI)-1 and 2 [10,11], and the uPA Receptor (uPAR; CD87; PLAUR) [12,13]. uPAR is a multi-ligand receptor with a high affinity for uPA, but also vitronectin, many integrins and G-protein-coupled receptors, and is expressed by many different cell types, including leukocytes [14]. Binding of uPA to uPAR results in formation of plasmin at the leading edge of cells facilitating leukocyte migration by pericellular proteolysis of extracellular matrix proteins [14]. Besides functioning as a proteinase receptor, uPAR also affects leukocyte migration and adhesion [15–20], as well as phagocytosis [19,21], through intracellular signaling. This occurs, in part, independently of ligation of uPA by uPAR [22,23].

Importantly, uPAR has been shown to contribute to activation and mobilization of leukocytes in bacterial infections [14,15,19–24]. To elucidate the role and function of uPAR in the development of Lyme borreliosis in vivo we infected wildtype (WT) and uPAR knock-out C57BL/6 mice with *B. burgdorferi* sensu stricto and monitored *B. burgdorferi* numbers in multiple organs, histopathological changes of tibiotarsi and heart, and host immune responses. In addition, to investigate whether the observed phenotype in uPAR knock-out C57BL/6 mice was dependent on uPAR’s role in the fibrinolytic system or dependent on the interaction with uPA we also investigated the course of Lyme borreliosis in tPA, PAI-1 and uPA knock-out C57BL/6 mice. Moreover, we investigated the course of *Borrelia* infection in uPAR knock-out mice partially backcrossed to a C3H/HeN genetic background to assess the role of uPAR in mice more susceptible for infection with *B. burgdorferi*. 
Results

*Borrelia burgdorferi* upregulates uPAR expression in mice and humans

Previous reports have shown that uPAR is upregulated on both a monocytic cell line and primary monocytes upon activation with *B. burgdorferi* [12,13]. We here show that in vitro stimulation with different concentrations of viable *B. burgdorferi* resulted in significantly increased uPAR expression on both murine peritoneal macrophages and ex vivo generated – peripheral blood mononuclear cells-derived - human macrophages (Fig 1A and Supplemental fig 1A). In addition, using murine and human whole blood we observed similar results for granulocytes and monocytes (Fig 1B and supplemental B). By contrast, non-phagocytotic cells, i.e. T lymphocytes, did not upregulate uPAR upon ex vivo exposure to *B. burgdorferi* (Supplemental fig 1D). Other *Borrelia* species, such as *B. garinii* strain PBi and *B. afzelii* strain pKo - both able to cause Lyme borreliosis - also induced enhanced uPAR expression on leukocytes (data not shown). To determine whether uPAR is upregulated in humans upon *B. burgdorferi* infection, we quantified uPAR expression in transcutaneous skin biopsies from *B. burgdorferi* PCR and culture confirmed positive erythema migrans patients and healthy controls. We could not detect uPAR expression in control patients, where as we could easily detect uPAR expression in the diseased group (Fig 1C). Lastly, in WT C57BL/6 mice inoculated intraperitoneally with viable *B. burgdorferi* for 1 hour we observed a significant upregulation of uPAR on the surface of (F4/80 positive) macrophages (Supplemental fig 1C).

C57BL/6 uPAR knock-out mice exhibit increased *B. burgdorferi* numbers in vivo and impaired phagocytosis of *B. burgdorferi* in vitro

To assess the role of uPAR in the immune response against *B. burgdorferi* vivo, we infected C57BL/6 WT and uPAR knock-out mice with *B. burgdorferi* and sacrificed mice two and four weeks post infection. By quantitative PCR we assessed *B. burgdorferi* numbers in skin, bladder and tibiotarsi post mortem. C57BL/6 uPAR deficient mice harbored higher *B. burgdorferi* numbers compared to WT animals in all tissues examined. This was most pronounced, and statistically significant, four weeks post infection (Fig 2A). These data were underscored by the fact that two weeks post infection only 3/8 bladder tissue cultures were positive in WT mice versus 7/7 in uPAR knock-out mice (Chi-square p= 0,026). We did not determine *B. burgdorferi* numbers in cardiac tissue in these experiments since we used the heart in toto for histopathology. In line with higher systemic *B. burgdorferi* numbers in uPAR deficient mice a significant increase in total IgG against *B. burgdorferi* over time (Fig 2B), and significantly higher IgG1 antibody levels four week post infection, were observed (Fig 2C). We detected no differences in IgM and IgG2b subclass-levels four weeks post infection (data not shown). To obtain a first insight into the mechanism by which uPAR deficiency could impact pathogen burden after infection with *B. burgdorferi* we stimulated leukocytes with viable spirochetes in vitro. We harvested peritoneal macrophages from C57BL/6 WT and uPAR knock-out mice, which we stimulated with viable *B. burgdorferi* (Cell:Borrelia = 1:50) for 16 hours. We demonstrate that *Borrelia* induced similar cytokine levels in WT and
uPAR facilitates clearance of B. burgdorferi

uPAR deficient macrophages (Fig 2D). We obtained comparable results when we stimulated whole blood in a similar fashion (data not shown). Next, because uPAR has been shown to play a crucial role in phagocytosis of Escherichia coli by neutrophils [19,21,22], we investigated whether WT and uPAR knock-out neutrophils and macrophages differed in their capacity to phagocytose B. burgdorferi. In these assays extracellular bacteria were quenched by addition of a quenching dye.
uPAR facilitates clearance of B. burgdorferi.

Data from the uPAR knock-out model support the hypothesis that uPAR is involved in the clearance of B. burgdorferi. Knock-out mice displayed higher systemic B. burgdorferi numbers compared to WT mice. DNA was extracted from tissues and subjected to quantitative PCR for B. burgdorferi and mouse β-actin. SHAM inoculated mice did not contain B. burgdorferi DNA. In addition, the IgG response was more rigorous in uPAR knock-out mice 2 and 4 weeks post infection. Macrophages isolated from uPAR knock-out mice produced similar levels of pro-inflammatory cytokines compared to WT mice. Importantly, the phenotype in uPAR knock-out mice was not explained by impaired influx of immune cells at early time points.

Figure 2. The urokinase receptor (uPAR) is involved in clearance of B. burgdorferi.
A. Urokinase receptor knock-out C57BL/6 mice display higher systemic B. burgdorferi numbers. WT and uPAR -/- mice were inoculated with B. burgdorferi and sacrificed two and four weeks post infection. DNA was extracted from the indicated tissues and subjected to quantitative Borrelia flab and mouse β-actin PCR. Data are representative of three independent experiments.

B and C. Urokinase receptor knock-out C57BL/6 mice develop more rigorous IgG responses. Sera from C57BL/6 WT and uPAR knock-out mice, 2 and 4 weeks post B. burgdorferi (B burg) or SHAM inoculation (SHAM) was used for whole cell ELISA. Thus, we determined total IgG directed against B. burgdorferi (B) and IgG subclasses, of which only IgG1 (C) is shown.

D. WT and uPAR -/- macrophages produce similar levels of pro-inflammatory cytokines when exposed to viable B. burgdorferi in vitro. Peritoneal macrophages were stimulated with control medium (medium) or B. burgdorferi (B burg) for 16 hours. The supernatant was analyzed for cytokine production using a mouse inflammation cytometric bead array.

E and F. Urokinase receptor deficient granulocytes and macrophages are incapable of adequately phagocytosing B. burgdorferi. Whole blood or peritoneal macrophages were incubated with CFSE labeled viable or heat-killed FITC-labeled B. burgdorferi at 37 °C or at 4 °C as a control. Phagocytosis was stopped by transferring the tubes to ice and extracellular bacteria were quenched by addition of a quenching dye containing Trypan blue. When whole blood was used erythrocytes were lysed before cells were stained and subjected to fluorescent microscopy (E) or stained for Gr-1 (granulocytes) and subjected to FACS analysis (F; left panel). Peritoneal macrophages were directly subjected to FACS analysis (F; right panel). Phagocytosis was depicted as the phagocytosis index [63,64]: mean fluorescence intensity (MFI) \times \text{percentage} \text{positive cells at 37°C minus (MFI} \times \% \text{positive cells at 4°C). Six to eight mice per group were used, graphs represent the mean \pm SEM and are representative of three independent experiments.

G. B. burgdorferi binds equally well to WT and uPAR -/- macrophages. A similar experiment as described in F was performed, albeit at 4 °C and without the addition of quenching dye to determine binding of B. burgdorferi to peritoneal macrophages. Binding is expressed as the binding index: \% CFSE positive cells x MFI. Four to six mice per group were used and bars represent the mean \pm SEM. The experiment was repeated twice. A P value < 0.05 was considered statistically significant. * indicating P < 0.05; ** P < 0.01 and *** P < 0.001. For color figure see page 261.

containing Trypan blue. We demonstrate that both uPAR knock-out neutrophils (in whole blood) and uPAR knock-out peritoneal macrophages were significantly less capable of phagocytosing B. burgdorferi, using either heat-killed FITC-labeled or viable CFSE-labeled B. burgdorferi (Fig 2E and F and Supplemental fig 2). Confocal microscopy confirmed labeled bacteria were localized intracellularly (Supplemental fig 3A and B). To distinguish between binding and phagocytosis we performed similar experiments, but at 4 °C and without the addition of quenching solution. These experiments showed no difference in the capacity of WT and uPAR deficient leukocytes to bind B. burgdorferi (Fig 2G). In addition, binding experiments with recombinant human uPAR and viable B. burgdorferi failed to show direct binding of the spirochete to uPAR (data not shown). Since uPAR has been shown to be of importance in the migration of leukocytes, we also investigated whether there was impaired migration of leukocytes in B. burgdorferi-infected uPAR knock-out mice. We intradermally inoculated C57BL/6 WT and uPAR knock-out mice with B. burgdorferi or controls and harvested skin at 0, 6 or 32 hours post infection. We did not observe influx of immune cells at t=0 (data not shown). By H&E, Ly6G and F4/80 stainings on sagittal skin sections we did observe an evident influx of immune cells and inflammation at t=6 hours, however there were no differences between WT and uPAR knock-out mice (Fig 3). As has been shown by others [25], the predominant cells at this early time point were granulocytes (Fig 3). Importantly, these data show that the phenotype in uPAR knock-out mice is not explained by impaired influx of immune cells at
the site of inoculation allowing for more dissemination of the spirochete. By contrast, later in the course of infection, at t=32 hours, we observed a more pronounced influx of macrophages in uPAR knock-out mice compared to WT controls, which probably is explained by the increased *Borrelia* burden in uPAR knock-out mice (Fig 3). In conclusion, higher *B. burgdorferi* numbers in C57BL/6 uPAR knock-out mice compared to WT mice could be explained by a decreased phagocytotic capacity of uPAR deficient leukocytes observed in vitro, and not by impaired migration of uPAR deficient leukocytes.

**Higher *B. burgdorferi* numbers and impaired phagocytotic capacity in C57BL/6 uPAR knock-out mice are independent of ligation of uPA to uPAR**

Since uPAR has been suggested to affect function of leukocytes in both an uPA-dependent as well as an uPA-independent fashion we also assessed the course of *B. burgdorferi* infection in C57BL/6 uPA knock-out mice. Both 2 and 4 weeks post *B. burgdorferi* infection, C57BL/6 WT and uPA deficient mice displayed similar *Borrelia* numbers in all tissues examined as detected by quantitative PCR (Fig 4A). In addition, compared to WT controls, uPA deficient neutrophils and peritoneal macrophages were equally capable of phagocytosing *B. burgdorferi* (Fig 4B). These data suggest that the phenotype observed in C57BL/6 uPAR knock-out mice was independent of ligation of uPA to uPAR.
uPAR facilitates clearance of *B. burgdorferi*

Next, since uPAR has been shown to affect function of leukocytes through its role in the fibrinolytic system [14], we infected mice in which the activity of the fibrinolytic system was either impaired, i.e. C57BL/6 tPA deficient mice, or enhanced, i.e. C57BL/6 PAI-1 knock-out mice. First we demonstrated that *B. burgdorferi* infection did not influence fibrinolytic activity in citrate plasma in either mouse strain, or WT controls, as measured by amidolytic plasminogen activator activity assays (Table 1). Next, we showed that, compared to C57BL/6 WT mice, both C57BL/6 tPA and as PAI-1 knock-out mice display normal *Borrelia* numbers in various tissues two week post infection, as detected by quantitative PCR (Table 1). In line with these data, phagocytotic capacity of C57BL/6 tPA and PAI-1 deficient neutrophils was comparable to that of WT mice (Table 1). Importantly, uPAR knock-out mice, regardless whether they were infected with *B. burgdorferi*, have comparable fibrinolytic activity to WT mice (data not shown). Together these data indicate that the impaired phagocytotic capacity of uPAR deficient mice, resulting in higher spirochete numbers upon *B. burgdorferi* infection in vivo, is not dependent on the role of uPAR in fibrinolysis.

**Higher *B. burgdorferi* numbers and impaired phagocytotic capacity in C57BL/6 uPAR knock-out mice are independent of uPAR's role in the fibrinolytic system**

![Figure 4. The urokinase activator (uPA) is not involved in clearance of the spirochete. A. Urokinase activator knock-out C57BL/6 mice display similar systemic *B. burgdorferi* numbers compared to WT controls. WT and uPA -/- mice were inoculated with *B. burgdorferi* and sacrificed two and four weeks post infection. DNA was extracted from the indicated tissues and subjected to quantitative *Borrelia flab* and mouse β-actin PCR. Six to eight mice per group were used and *B. burgdorferi* numbers are depicted as described in figure 2A. B. Urokinase activator deficient granulocytes and macrophages (solid lines) are just as capable as WT controls (dotted lines) of phagocytosing *B. burgdorferi*. Phagocytosis assays were performed as described in figure 2 F. Six to eight mice per group were used, error bars represent SEM and the graphs are representative of two independent experiments. A P value < 0.05 was considered statistically significant.](image)
Chapter 9

The effect of uPAR deficiency on the development of Lyme carditis

We assessed carditis severity in *B. burgdorferi* inoculated C57BL/6 uPAR knock-out and WT mice two and four weeks post infection. Two weeks post infection, in hematoxylin and eosin (H&E) stained sagittal sections of mouse hearts, we found comparable carditis severity scores in C57BL/6 WT and uPAR knock-out mice (Supplemental fig 4A and B). The localization and severity of carditis in our experiments using C57BL/6 mice appeared to be similar to the localization and carditis severities reported by ourselves and others using the same, relatively resistant, mouse strain [26–29]. SHAM inoculated mice did not develop carditis (data not shown). We were unable to reliably score carditis four weeks post infection, since, as observed by others, at this stage, carditis was characterized by an organizing rather than ongoing inflammation (Supplemental fig 4A) [26].

Table 1. *B. burgdorferi* infection in WT, tPA -/- and PAI-1 -/- mice.

<table>
<thead>
<tr>
<th>PA activity (in %)*</th>
<th>SHAM</th>
<th><em>B. burgdorferi</em></th>
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<tbody>
<tr>
<td>WT</td>
<td>91.2 ± 1.4</td>
<td>89.6 ± 0.8</td>
</tr>
<tr>
<td>tPA -/-</td>
<td>10.7 ± 0.9*</td>
<td>9.9 ± 2.1*</td>
</tr>
<tr>
<td>PAI-1 -/-</td>
<td>132.7 ± 4.6*</td>
<td>141.7 ± 3.9*</td>
</tr>
</tbody>
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**Pathogen numbers** (per 1 x 10^6 β-actin)

<table>
<thead>
<tr>
<th></th>
<th>ankle</th>
<th>skin</th>
<th>bladder</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>–</td>
<td>1292 ± 473</td>
<td>872 ± 290</td>
</tr>
<tr>
<td>tPA -/-</td>
<td>–</td>
<td>636 ± 366</td>
<td>724 ± 301</td>
</tr>
<tr>
<td>PAI-1 -/-</td>
<td>–</td>
<td>560 ± 149</td>
<td>1463 ± 488</td>
</tr>
</tbody>
</table>

Phagocytosis index$ (% pos * MFI)

<table>
<thead>
<tr>
<th></th>
<th>ND</th>
<th>41015 ± 5826</th>
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<tbody>
<tr>
<td>WT</td>
<td>ND</td>
<td>39828 ± 3350</td>
</tr>
<tr>
<td>tPA -/-</td>
<td>ND</td>
<td>49928 ± 2752</td>
</tr>
<tr>
<td>PAI-1 -/-</td>
<td>ND</td>
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Note. C57BL/6 WT, tPA and PAI-1 knock-out mice (6-8 per group) were inoculated with *B. burgdorferi* strain B31 or SHAM and sacrificed two weeks later. * Plasminogen activity (PA) activity was measured in citrate plasma using amidolytic assays and expressed as a percentage.

$ B. burgdorferi$ numbers were determined by quantitative PCR and expressed as described in figures 2 and 3.

$ In addition, an in vitro phagocytosis assay was performed using naive mice (n = 6-8 per group) as described in figure 2. Whole blood was incubated with viable CFSE-labeled *B. burgdorferi* for 60 minutes at 37 or 4 °C as a control and phagocytosis was depicted as the phagocytosis index as described in figure 2.

$ PA activity was significantly lower in tPA knock-out mice compared to WT controls (P < 0.0001), regardless whether mice were inoculated with *B. burgdorferi* or SHAM.

$ PA activity was significantly higher in PAI-1 knock-out mice compared to WT controls (P < 0.0001), regardless whether mice were inoculated with *B. burgdorferi* or SHAM.

Results represent the mean ± SEM. Non-parametric statistical tests were used to analyze the differences between the groups. A *P* value < 0.05 was considered statistically significant.
However, in 4/8 uPAR deficient mice and 0/8 WT mice a mild active carditis, characterized by the presence of small cellular infiltrates at the aortic root, could still be observed 4 weeks post inoculation (Chi-square p = 0.021) (data not shown). By contrast, in 5/8 of WT mice and only in 2/8 uPAR deficient mice we observed organized inflammatory infiltrates, characterized by sharply delineated foci (Supplemental fig 4A) of mononuclear leukocytes situated in the atrial wall (Chi-square p = 0.0721). Together these findings suggest a difference with respect to the kinetics of the organization of carditis in C57BL/6 uPAR knock-out and WT mice. These data are in line with the previously described increased influx of macrophages in uPAR knock-out mice 32 hours after intradermal injection with*B. burgdorferi*(Fig 3), which is likely to be caused by the increased pathogen burden in these mice. Although we observed ankle swelling in both WT and uPAR knock-out mice during the course of infection, histological examination of H&E stained section of tibiotarsi did not reveal any signs of arthritis (data not shown). Finally, in line with the observed normal*Borrelia* numbers, in uPA, tPA and PAI-1 knock-out mice severity of carditis was comparable to that in WT mice (Supplemental fig 4C and D). Together these data demonstrate that, despite higher*B. burgdorferi* numbers, C57BL/6 uPAR knock-out mice develop carditis with a similar severity, albeit for a prolonged period of time, compared to WT controls.

**The course of*B. burgdorferi* infection in uPAR deficient mice on a*B. burgdorferi* susceptible genetic background**

To further investigate the effect of uPAR deficiency on the development of Lyme borreliosis symptoms we generated uPAR deficient mice on a more*Borrelia* susceptible genetic background. It is well-known that C57BL/6 mice are relatively resistant to*B. burgdorferi* and develop less severe symptoms after infection with the spirochete, and that C3H/HeN mice are more susceptible and develop more severe symptoms after infection with*B. burgdorferi*[30]. In addition, it has been described that F1 of WT C57BL/6 crossed with (x) C3H/HeN mice are intermediately sensitive to*B. burgdorferi* infection [30]. Therefore we investigated the course of Lyme borreliosis in F2 of C57BL/6 x C3H/HeN uPAR knock-out mice and WT littermate controls. We first showed that, similar to uPAR knock-out mice on a pure C57BL/6 background, these mice harbor higher*Borrelia* numbers in multiple tissues compared to WT littermate controls two weeks post infection (Fig 5A), indicating that the lack of uPAR in these mice also resulted in impaired phagocytosis and increased pathogen burden. Indeed, in in vitro phagocytosis assays, compared to WT littermate controls, C57BL/6 x C3H/HeN uPAR deficient neutrophils were significantly less capable of phagocytosing*B. burgdorferi*(Fig 5B). Strikingly, compared to WT littermate controls (Fig 5C), C57BL/6 x C3H/HeN uPAR knock-out mice developed significantly more severe carditis (Fig 5D), reflected by influx of greater numbers of leukocytes in more and larger parts of cardiac tissue two weeks post infection (Fig 5E). As has been shown by others the main cells involved in inflammation were macrophages, as determined by F4/80 immunostaining (Fig 5F and G). By multiplex ligation-dependent probe amplification (MLPA), we detected significantly increased levels of interleukin (IL)-1β, IL-1 Receptor Associated Kinase (IRAK)-3, and toll-like receptor (TLR)2 mRNA in hearts from uPAR knock-out mice compared to WT littermate controls two weeks post infection (Fig 5H),
Figure 5. The course of Lyme borreliosis in uPAR knock-out mice on a B. burgdorferi susceptible mixed C57BL/6 x C3H/HeN genetic background. 
A. Urokinase receptor deficient mice on the mixed genetic background also display higher B. burgdorferi numbers compared to WT littermate controls. C57BL/6 mice were backcrossed twice to a C3H/HeN background. We intercrossed F2 mice and used the homozygous and nullizygous offspring (F2 homozygous uPAR deficient C57BL/6 x C3H/HeN mice and WT littermate controls) for our experiments. Mice were inoculated with B. burgdorferi or SHAM and sacrificed two weeks post infection. DNA was extracted from the indicated tissues and samples were subjected to quantitative Borrelia flab and mouse β-actin PCR. B. burgdorferi numbers are depicted as described in figure 2. Six to eight mice per group were used.
B. Urokinase receptor deficient leukocytes from mice on the mixed genetic background are not as capable of phagocytosing B. burgdorferi as are granulocytes from WT littermate controls. Phagocytosis assays with whole blood were
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performed as described in figure 2. Six to eight mice per group were used. C, D and E. Peak carditis in these uPAR -/- mice (D) is more severe compared to carditis in WT littermate controls (C). Mice were inoculated with *B. burgdorferi* and sacrificed two weeks post infection. Pictures of hematoxyline and eosin stained sagittal sections depict representative sections. Carditis was scored as described in supplementary figure 4 within the same session (E). Six to eight mice per group were used. F and G. The main cell involved in murine Lyme carditis is the macrophage. Representative pictures of F4/80 stained sagittal sections of hearts from *B. burgdorferi* infected uPAR deficient mice (G) and WT littermate controls (F). H. More severe inflammation in *B. burgdorferi* infected uPAR deficient animals (*n* = 7) compared to WT littermate controls (*n* = 7) as measured by multiplex ligation-dependent probe amplification (MLPA). MLPA was performed on RNA obtained from half of sagitally dissected hearts from *B. burgdorferi* or SHAM inoculated mice. Depicted are mRNA expression of TNF-α, CCL3, TLR2, CD14, IL1-β, IRAK3, ICAM1 and TBP (housekeeping gene [69]). Other genes included in the assay were IL6, IL10, INF-γ, TFPI, F3, PROCR, SERPINE1P, PLAT, PLAUR, TLR4, TLR9, LY96, IRAK1, F2R, NFkB1a, NOS3, ITGA5, B2M, ITGAV, ITGAB3, TFRC, HIF1A, MMP2 and HP. Bars represent the mean ± SEM. A *P* value < 0.05 was considered statistically significant. * indicating *P* < 0.05; ** *P* < 0.01. For color figure see page 264.

consistent with the observed higher *B. burgdorferi* numbers and more severe cardiac inflammation in uPAR knock-out mice. Since, uPAR has also been shown to enhance migration of leukocytes towards the site of infection for some, but not all bacteria, in these mice we performed in vitro migration assays with WT and uPAR deficient macrophages (Supplemental fig 5A). We observed impaired migration of uPAR deficient macrophages to C5a (Supplemental fig 5B), however not to supernatant from a cardiomyoblastic rodent cell line (Supplemental fig 5C), compared to migration of WT macrophages, which is in line with our observations in the in vivo migration assays in WT C57BL/6 mice. In this in vitro setting, whether or not this cell line was stimulated with viable *B. burgdorferi* did not affect migration of WT and uPAR deficient macrophages. WT and uPAR deficient C57BL/6 x C3H/HeN mice developed comparable ankle swelling during the course of *B. burgdorferi* infection (Supplemental fig 5D), however despite the more susceptible phenotype of these mice compared to C57BL/6 mice, these mice did not develop any histological signs of arthritis, as determined by hematoxyline and eosin staining, but also Ly6G - a marker for granulocytes - immunostaining (data not shown). In line with these data, post mortem radiological examination of the hind limbs did not reveal any signs of arthritis (Supplemental fig 5E).

**Discussion**

Since its discovery approximately 30 years ago Lyme borreliosis has become the most important vector-borne disease in the Western world. We here demonstrate, to our knowledge for the first time, that uPAR plays an important role in the antibacterial innate immune response against *B. burgdorferi*. We show that uPAR expression is upregulated in response to *B. burgdorferi* on human and murine leukocytes both in vitro, as well in vivo. Importantly, we describe the role of uPAR in the immune response against *B. burgdorferi*. By using C57BL/6 WT and uPAR knock-out mice we show that uPAR plays an important role in phagocytosis of *B. burgdorferi* - a prerequisite for the eradication of the spirochete - by leukocytes. Moreover, experiments with C57BL/6 uPA, tPA and PAI-1 knock-out mice show that the mechanism by which uPAR is involved in the phagocytosis...
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of *B. burgdorferi* is independent of ligation to uPA or uPAR's role in fibrinolysis. Finally, we show that, in mice relatively susceptible to *Borrelia* infection - mice on a mixed C57BL/6 and C3H/HeN background - uPAR deficiency also impaired phagocytotic capacity in vitro, which was associated with higher *B. burgdorferi* numbers, more local inflammation and more severe carditis, compared to WT littermate control animals, further underscoring the in vivo relevance of our findings. Together these data demonstrate an important role for uPAR in the innate immune response against, and the clearance of, the causative agent of Lyme borreliosis.

Earlier studies documented that membrane bound uPAR and uPAR mRNA are upregulated in human peripheral blood-derived monocytes and the human monocyte-like cell line U937 upon exposure to viable and heat-killed *B. burgdorferi* [12,13]. We here show that viable *B. burgdorferi* induces upregulation uPAR (Fig 1 and Supplemental fig 1), not only on murine and human monocytes, but also on macrophages and granulocytes in vitro. Notably, uPAR expression in response to *B. burgdorferi* in vivo has never been investigated. We here show that in skin from Lyme borreliosis patients with erythema migrans uPAR mRNA expression is significantly increased and could be readily detected by quantitative RT-PCR (Fig 1). In addition, in a small pilot experiment, intradermal syringe inoculation of C57BL/6 WT mice with viable *B. burgdorferi* (or PBS as a control) also appeared to increase uPAR mRNA levels at the site of inoculation 6 hours post injection, but - due to small group numbers - these differences were not statistically significant (data not shown). Increased levels of uPAR are likely to be caused by influx of leukocytes to the site of the tick-bite. Indeed, in preliminary experiments in which we inoculated human skin ex vivo with viable *B. burgdorferi* - a model in which there is no influx of leukocytes [31]- we did not observe an increase in uPAR expression as determined by uPAR immunostaining on snapfrozen sagittal skin sections (data not shown). Erythema migrans lesions are characterized by perivascular infiltrates in the dermis composed primarily of lymphocytes and macrophages [32]. We do not know which infiltrating cell type is responsible for the elevated uPAR levels, but based on our in vitro data we speculate that the macrophage is the most likely candidate. Indeed, macrophages from intraperitoneally *B. burgdorferi*-inoculated WT C57BL/6 mice did upregulate uPAR expression, further indicating that *Borrelia*-phagocyte interaction in vivo results in induction of uPAR expression (Supplemental fig 1). Upregulation of uPAR appeared not to be specific for *B. burgdorferi* since, in our in vitro experiments, other bacteria, i.e. *Klebsiella pneumoniae* and *Burkholderia pseudomallei*, also induce upregulation of uPAR to a similar extent (data not shown).

To investigate the role of uPAR in the immune response against *B. burgdorferi* and the course of murine Lyme borreliosis we inoculated C57BL/6 WT and uPAR knock-out mice with *B. burgdorferi*. We demonstrate by quantitative PCR and culture that mice lacking uPAR display significantly increased *B. burgdorferi* numbers in all tissue examined, indicative of a more disseminated infection (Fig 2), although also in these mice there appeared to be clearance of *B. burgdorferi*, as suggested by lower numbers 4 weeks compared to 2 weeks post infection. The increased *B. burgdorferi* burden in uPAR deficient mice was underscored by a more abundant, putatively reactive, IgG response (Fig 2). The role of uPAR in leukocyte adhesion and migration, leading to recruitment of these cells to
the site of infection, has been the topic of investigations for many years. Several in vivo studies show that migration of uPAR deficient leukocytes is impaired in response to, for example, *Pseudomonas aeruginosum* [22] and *Streptococcus pneumoniae* [23]. In other studies, e.g. in *E. coli*-induced peritonitis [20] and pyelonephritis [21] uPAR deficiency did not affect leukocyte recruitment, indicating that the role of uPAR in migration of leukocytes is dependent on the pathogen, the site of infection and the disease model. Interestingly, in the mouse model for Lyme borreliosis uPAR is not crucially involved in migration of leukocytes to *B. burgdorferi* infected tissues, as can be deducted from our in vivo migration experiments (Fig 3). Strikingly, the fact that we observed more macrophages 32 hours after injection with *B. burgdorferi* in uPAR knock-out skin compared to WT controls, but no differences in H&E staining, suggests that the quality of the inflammatory infiltrate is affected rather than the quantity; presumably due to higher *B. burgdorferi* numbers in the uPAR knock-out mice. Interestingly, recently it was shown that uPAR also facilitates phagocytosis of the gram-negative bacterium *E. coli* by neutrophils [19,21]. We here show, by fluorescent microscopic assays, and FACS-based phagocytosis assays, that both uPAR deficient granulocytes and macrophages are significantly less capable of phagocytosing viable spirochetes (Fig 2 and Supplemental fig 2 and 3). Importantly, uPAR deficiency did not affect binding of the spirochete to the surface of leukocytes (Fig 2). In addition, in an in vitro killing assay uPAR appeared not to be involved in killing of the spirochete following phagocytosis (data not shown), indicating that uPAR is involved strictly in the process of internalization of *B. burgdorferi* by leukocytes. Others have previously shown that phagocytosis of spirochetes by immune cells can be crucial for adequate cytokine induction and leukocyte activation [33–35]. We did not observe defects in pro-inflammatory cytokine production in uPAR deficient leukocytes when stimulated in vitro with *B. burgdorferi*. In contrast to the studies described above our results describe more subtle differences in phagocytotic capacity between WT and uPAR deficient leukocytes; we demonstrate diminished, but not absent, phagocytosis in uPAR deficient macrophages compared to WT controls. Thus, there could still be activation of intracellular signaling pathways and/or recognition by intracellular/internalized TLRs that could contribute to the production of normal pro-inflammatory cytokines in uPAR deficient leukocytes. Secondly, our phagocytosis assays clearly show that early phagocytosis is not affected by uPAR deficiency. Most likely early events are critical for the rapid induction of innate pro-inflammatory cytokines.

The role of uPAR in phagocytosis of *B. burgdorferi* appeared to be independent of uPA and uPAR's role in the fibrinolytic system, since in our phagocytosis assays uPA, tPA and PAI-1 knock-out mice all displayed normal phagocytotic capacity of the spirochete compared to WT mice (Fig 3 and table 1). In addition, in vivo experiments clearly show that when these mice were inoculated with *B. burgdorferi* and sacrificed two weeks post infection, normal *B. burgdorferi* numbers were detected (Fig 3 and table 1). There are numerous in vitro studies reporting that *B. burgdorferi* interacts with the fibrinolytic system (reviewed in [6]). Extrapolating these data to the in vivo situation, this interaction, mainly through binding to host derived plasminogen, was thought to enable the spirochete to penetrate tissues, the blood-brain barrier and migrate through the extracellular matrix [8,36–38]. Indeed, for the spirochetal causative agent of relapsing fever, using plasminogen
knock-out mice, it has been clearly shown that plasminogen is required for dissemination of the spirochete to the heart and brain in vivo [39]. To our knowledge, for *B. burgdorferi* however, there is only one previously published study that describes the effect of diminished fibrinolytic activity on the course of *B. burgdorferi* infection in vivo [7]. In this study, in which plasminogen deficient mice were used, plasminogen was shown to be important for dissemination of the spirochete within the feeding tick. Strikingly, despite a short-lived spirochetemia, all plasminogen deficient mice developed infection after intradermal inoculation with *B. burgdorferi*. Importantly, there were no differences in *B. burgdorferi* numbers in any of the tissues examined in plasminogen knock-out and WT mice at several time points post infection [7]. In line with these data, our results demonstrate that the fibrinolytic system per se does not affect the course of *B. burgdorferi* infection. Strikingly, we here show that one of the key players in the fibrinolytic system, uPAR, independently of ligation to uPA or its presumptive role in fibrinolysis, is importantly involved in the course of experimental murine Lyme borreliosis.

The fact that we show that the requirement of uPAR in phagocytosis of *B. burgdorferi* is independent of uPA or uPAR's role in the fibrinolytic system suggests that the requirement of uPAR in internalization of *Borrelia* is dependent on interaction of uPAR with other cell surface molecules. Indeed, uPAR has been shown to facilitate various leukocyte functions, among which adhesion, migration and phagocytosis through interaction with αβ-integrins and other cell surface molecules, but also vitronectin [14,15]. This implies a role for uPAR as a signaling receptor. However, because uPAR is a glycosyl-phosphatidylinositol linked receptor and lacks a cytosolic domain it needs to form functional transmembrane units with other molecules, such as multiple αβ-integrins, G-protein-coupled receptors, and caveolin in order to induce intracellular signaling events leading to cytoskeleton rearrangements and consequent cell movement [14,15]. Since both uPAR and *B. burgdorferi* share many molecules with which they can interact, for example αβ-integrins and vitronectin, it will be challenging to identify the surface molecule with which uPAR associates to facilitate phagocytosis of *B. burgdorferi*.

When we infected uPAR knock-out mice on a mixed C57BL/6 and C3H/HeN background with *B. burgdorferi* these mice exhibited higher *B. burgdorferi* numbers in cardiac tissue two weeks post infection compared to WT littermate controls, which was also associated with decreased phagocytosis of *B. burgdorferi*. Strikingly, in these mice we observed a significantly increased influx of leukocytes, predominantly macrophages, at the atrioventricular junction and at the aortic root compared to WT littermate controls (Fig 5). This further indicates that uPAR's role in migration of leukocytes towards *B. burgdorferi* infected and inflamed organs can be compensated for and is not of in vivo importance, since, otherwise we would have observed diminished influx of leukocytes in hearts from uPAR knock-out mice. In line with this hypothesis, in our vitro migration assays using cells from these mice, we observed impaired migration of uPAR deficient macrophages to C5a, but not to supernatant from a cardiomyoblastic rodent cell line (Supplemental Fig 5). The fact that cells did not migrate better to *B. burgdorferi* stimulated H9c2 cells could be due to production of both
stimulating and inhibitory chemotactic stimuli of these cardiomyoblastic cells upon exposure to *B. burgdorferi*, as has been recently shown for neutrophils [40]. Furthermore, our data indicate that, although the underlying mechanisms appear to be the same, the consequences of uPAR deficiency for the course of murine Lyme borreliosis are dependent on the genetic background of the host. Others have shown that C57BL/6 and C3H/HeN mice harbor similar *B. burgdorferi* numbers after infection, but the severity of symptoms was more pronounced in C3H/HeN mice [30], indicating that the extent of the immune response that is mounted against the spirochete is dependent on the genetic background of the host. Indeed, we have demonstrated that uPAR deficiency in *Borrelia* resistant C57BL/6 mice leads to higher *B. burgdorferi* loads, but to comparable, albeit longer-lived active carditis compared to WT controls. By contrast, uPAR deficient mice on a more susceptible mixed C57BL/6 x C3H/HeN background also exhibited higher *B. burgdorferi* numbers, but more pronounced influx of leukocytes and more severe (peak) carditis. Local cytokines and chemokines induced by *B. burgdorferi* are thought to mediate Lyme carditis. A cytokine that has been implicated to be of paramount importance for local inflammation and migration of leukocytes is IL-1β [41–43]. Also in *B. burgdorferi* infected mice and patients IL-1β has been shown to be upregulated in heart or joints [44–46]. Interestingly, by MLPA we found significantly higher levels of mRNA coding for IL-1β, IL-1 receptor associated kinase (IRAK)-3 (predominantly expressed in macrophages) and TLR2 (the TLR preferentially recognizing *B. burgdorferi* lipoproteins) in hearts from *B. burgdorferi* infected uPAR knock-out mice on the mixed genetic background compared to WT littermate controls (Fig 5). Interestingly, in previous studies we showed that (human) peripheral blood-derived dendritic cells stimulated with the TLR2 ligand lipoteichoic acid (LTA) or viable *B. burgdorferi* produced high levels of IL-1β [47]. By contrast, when the same cells were stimulated with the TLR4 ligand LPS IL-1β levels were undetectable. Macrophages have also been shown to be able to produce IL-1β upon *B. burgdorferi* stimulation [48]. We postulate that, in the in vivo infection experiments, decreased uptake of *B. burgdorferi* resulted in higher local and systemic *B. burgdorferi* numbers. These higher *Borrelia* numbers might have led to an increased influx of leukocytes, as we demonstrated by pathology in mouse hearts and mouse skin, in turn leading to an increased induction of pro-inflammatory molecules. One could argue against the use of F2 mice in our studies, however the fact that F1 WT C57BL/6 x WT C3H/HeN mice are already intermediate susceptible to *Borrelia* infection [30], encouraged us to perform our experiments with F2 mice. In these mice we also obtained surface ECGs during the course of *B. burgdorferi* infection. Using signal averaged ECG analysis with the digital acquisition and analysis Power Lab/4SP system (AD instruments, Oxfordshire, UK) we did not find statistical differences between *B. burgdorferi* infected uPAR deficient and WT animals, although we did observe tachycardia in both groups following *B. burgdorferi* infection (data not shown). On histological examination we did not observe arthritis in *B. burgdorferi* infected WT and uPAR knock-out C57BL/6 mice, not even when mice were sacrificed 6 weeks post infection (data not shown). By backcrossing uPAR deficient mice to the more *Borrelia* susceptible genetic C3H/HeN background, we speculated, based on finding by others [30], we would be able to observe the effect of uPAR deficiency on arthritis. We measured swelling of the hindpaws during the course of infection, however we did not find
significant differences in ankle swelling between the groups (Supplemental fig 5). Furthermore, two and three weeks post infection we could not detect histopathological changes suggestive of arthritis in the hindlimbs of C57BL/6 x C3H/HeN uPAR knock-out and WT littermate controls. The development of edema without histopathological changes has been described previously in response to *Borrelia* infection [29]. Alternatively, we might have sacrificed the mice too early in the course of infection, which could be underscored by the fact that the ankles had not reached maximal swelling yet (Supplemental fig 5). In line with the absence of histopathological changes, no signs of arthritis were observed in post mortem radiological examination of hindpaws three weeks post infection (Supplemental fig 5).

In conclusion, we here show that uPAR is importantly involved in the host defense against *B. burgdorferi* by facilitating leukocyte phagocytosis of *B. burgdorferi*. A better understanding of the role of uPAR in the course of Lyme borreliosis might be of clinical interest since several uPA/uPAR agonists and antagonists have already been tested in oncological trials in humans [49,50] and it has been well-established that late in the course of Lyme borreliosis the infection tends to be more difficult to treat [51]. Therefore compounds modulating the host immune response, in adjunction to antibiotics, may prove to be a valuable asset in both the treatment of early Lyme borreliosis, when the bacterium needs to be cleared, as well as the treatment of late Lyme borreliosis, when ongoing inflammation might be causative of persisting symptoms and when the immune response might need to be dampened.

**Material and methods**

**Mice, spirochetes and infection.** Specific pathogen-free wildtype C57BL/6 mice were purchased from Harlan Sprague Dawley Inc. (Horst, The Netherlands) and uPAR knock-out C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) [52]. In addition C57BL/6 uPAR knock-out mice were backcrossed twice to a C3H/HeN - purchased from Jackson Laboratories – background, generating F2 C57BL/6 x C3H/HeN heterozygous uPAR deficient mice. F2 mice were crossed among each other to generate homozygous C3H/HeN x C57BL/6 uPAR knock-out mice and WT littermate controls. uPA, tPA and PAI-1 knock-out mice were also purchased from Jackson Laboratories. All mice were bred in the animal facility of the Academic Medical Center (Amsterdam, The Netherlands). Age-and sex-matched animals were used in each experiment and the Animal Care and Use Committee of the University of Amsterdam approved all experiments. Six to eight-week old mice were infected by intradermal syringe inoculation with $1 \times 10^6$ *B. burgdorferi* sensu stricto strain B31 clone 5A11 [53], that had previously been recovered from an experimentally infected mouse [29]. Spirochetes were cultured in BSK-II medium, enumerated and inoculated in the midline of the back or with BSK-II medium as a control (SHAM), as described previously [29,54]. Mice were sacrificed by bleeding from the inferior vena cava at the indicated time points, i.e. 2, 4 (or 6 weeks) post infection. Heparin or citrate plasma was stored at -20 °C for future
use. Skin (inoculation site), urinary bladder, heart and tibiotarsi were saved for histopathological examination, culture or quantitative Polymerase Chain Reaction (q-PCR).

**Q-PCR.** DNA from murine tissues was obtained with the DNeasy KIT (Qiagen, Venlo, The Netherlands) as previously described [29]. Quantitative PCR detecting *Borrelia flaB* and mouse β-actin was performed, as described previously [29]. Standards consisted of dilutions of genomic DNA from *B. burgdorferi* or mouse β-actin (252 bp) cloned into the PCR2.1-TOPO vector (Invitrogen, Breda, The Netherlands), as described previously [29,54].

**Arthritis, paw swelling and radiological examination.** Histopathological changes in tibiotarsi were assessed as previously described [29,55]. We monitored ankle swelling of both tibiotarsal joints using a Mitutoyo pressure controlled microcaliper (Mitutoyo, Kanagawa, Japan). Measurements were performed several times throughout the course of the infection by the same observer blinded to the experimental design. Lastly, we performed post mortem radiological examination of formalin fixed right hind paws, as described previously [56].

**Carditis.** Five μm-thick paraffin embedded sections of sagittally dissected hearts were processed and H&E stained by routine histological techniques. Carditis was scored on a scale from 0 to 3 by a pathologist blinded to the experimental design, essentially as previously described [27,28,54], with 0: no carditis; 1: mild carditis; 2: moderate carditis and 3: severe carditis. As described previously [26], 2 weeks post infection, carditis was characterized by disperse inflammation at the atrioventricular junction and aortic root, while as four weeks post infection, organizing inflammation was characterized by the presence of sharply delineated foci in the atrial walls. An F4/80 immunostaining (BMA Biomedicals, Augst, Switzerland) was performed to detect influx of macrophages [57].

**Multiplex ligation-dependent probe amplification.** MLPA was performed in essence as described before [58]. The genes that were analyzed are listed in the figure legend of Supplemental Figure 3. Equal amounts of mRNA were included per reaction and all samples were tested in a single experiment using the same batch of reagents. The levels of mRNA for each gene were expressed as a normalized ratio of the peak area of the fluorescent intensity (in arbitrary units) and divided by the cumulative peak area of all genes in the assay, resulting in the relative abundances of mRNAs of the genes of interest [59].

**Whole cell *B. burgdorferi* ELISA.** *Borrelia burgdorferi* sensu stricto strain B31 specific total immunoglobulin (Ig)G and IgG subclasses were measured in heparin plasma from infected animals and controls by ELISA as described previously [29]. All measurements were performed in duplicate.

**Amidolytic assays of PA activity.** Plasminogen activator was measured as a measure for the activity of the fibrinolytic system using an amidolytic assay as described earlier [23,60]. Briefly, citrate plasma was incubated with S-2251 (Chromogenix, Mölndal, Sweden), plasminogen and cyanogen bromide fragments of fibrinogen (Chromogenix, Milano, Italy). Conversion of plasminogen to
plasmin was assessed by subsequent conversion of the chromogenic substrate S-2251 and was detected with a spectrophotometer.

**Stimulation assays.** Whole blood and peritoneal macrophages from three naive uPAR knock-out or WT mice were harvested as described [61]. Briefly, 1 x 10⁷ adherent macrophages and heparinized whole blood were stimulated in duplo in 96-well microtiter plates (Greiner) with 1 x 10⁶ or 1 x 10⁷ viable *B. burgdorferi* suspended in Roswell Park Memorial Institute (RPMI) 1640 medium or medium as a negative control for 16h. Supernatants were collected and stored at -20°C until cytokine production was measured by CBA. For assessment of uPAR expression by fluorescence activated cell sorter (FACS), cells were harvested and stained with murine anti-CD87-Phycoerythrin (PE) (BD Pharmingen, Maasssen, The Netherlands). To assess uPAR expression on specific cells, cells were double-stained with anti-GR1-flourescin isothiocyanate (FITC) (BD Pharmingen) (granulocytes) or F4/80-allophycocyanin (APC) (BD Pharmingen) (monocytes and macrophages). In addition, in non-phagocytosing cells, i.e. CD4⁺ and CD8⁺ T cells - stained with anti-CD3-APC (BD Pharmingen) and anti-CD4-FITC or anti-CD8-PerCP respectively (BD Pharmingen) - we also assessed uPAR expression by FACS analysis. Similarly, uPAR expression on human cells derived from heparinized whole blood was analyzed with a human biotin-labeled antibody against uPAR (R&D Systems, Minneapolis, MN) in combination with streptavidin conjugated to PE; these cells were triple-stained with anti-CD15-APC (BD Pharmingen) (granulocytes) and anti-CD14-Cy-Chrome 5 (Cy5) (BD Pharmingen) (monocytes) (BD Pharmingen). Human macrophages were generated as described previously [62]. Briefly, human peripheral blood derived mononuclear cells were isolated from buffy coats by centrifugation over a Ficoll-Paque gradient. Subsequently, adherent monocytes were cultured in X-VIVO medium (BioWhittaker, Walkersville, MD) with 1% heat-inactivated autologous plasma to allow for differentiation to human monocyte-derived macrophages in 7 days. Antibodies were used in concentrations recommended by the manufacturer and FACS analysis was performed using the BD FACSscalibur (BD Biosciences, Breda, The Netherlands). Endotoxin concentration in the *B. burgdorferi* culture media was approximately 1 IU/ml, as determined by a Cambrex QCL LAL assay (Cambrex). We established that the maximal amount of LPS that could have possibly contaminated the final *Borrelia* preparation used for the in vitro stimulations - after extensive washing and resuspension in different cell culture media - was insufficient to influence uPAR expression (data not shown). In a separate experiment viable *B. burgdorferi* (1 x 10⁷) were injected into the peritoneal cavity of C57BL/6 WT or uPAR knock-out mice for one hour. Hereafter cells were harvested, stained for F4/80, and CD87 (uPAR) expression was measured by FACS analysis.

**Detection of uPAR mRNA expression in human samples.** Transcutaneous skin biopsies were collected from healthy volunteers, i.e. non-inflamed skin, or patients with active Lyme erythema migrans at the Academic Medical Center, Amsterdam, The Netherlands and New York Medical College, NY. IRB approval was obtained from both institutes. All Lyme patient skin samples were tested positive for *B. burgdorferi* spirochetes by in vitro culture and PCR. Skin samples were frozen-ground to fine powder using a china grinder and RNA was extracted using the TRIZOL reagent...
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from Invitrogen (Carlsbad, CA, U.S.A). RNA samples were treated with TURBO DNase (Applied Biosystems, Foster City, CA, U.S.A) to remove DNA contaminants. RNA was then converted to cDNA using an Affinity Script kit (Stratagene, La Jolla, CA, U.S.A). Quantification of uPAR was performed by Taqman PCR (Applied Biosystems) and normalized to β-actin (ACTB). The primers and probes used for uPAR were forward 5’AATCCTGGAGCTTTGAAAATCT 3’, reverse 5’CCACTTTTAGTACGCAGGAGA 3’, and probe 5’6FAM-ACTGCCGAGGCCCCCATGAATC 3’-TAMRA. Human β-actin primers and probe were inventoried products of Applied Biosystems.

**Phagocytosis assays.** Phagocytosis assays were performed in essence as described before [63–65]. Viable *B. burgdorferi* were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen) as described by others [66] or heat-inactivated (30 min at 56 °C) non-motile, but intact, *B. burgdorferi* were labeled with fluorescein isothiocyanate (FITC). Adhered peritoneal macrophages (derived from 6–8 mice per group) were incubated with CFSE-labeled *B. burgdorferi* (Cell:*Borrelia* = 1:50) in serum-free RPMI 1640 medium in 24-well microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands) for 0, 15 and 60 minutes at 37 °C. Phagocytosis was stopped by transferring the cells to 4 °C. Extracellular signal of *B. burgdorferi* was eliminated by addition of a quenching solution for one minute - containing Trypan blue that absorbs the fluorescence emission of both FITC and CFSE (Orpegen, Groningen, The Netherlands; [65]) - and three washes with ice-cold PBS. For each sample and each time point 4 °C controls were performed, however there was hardly any phagocytosis detectable under these conditions (data not shown). Cells were resuspended in FACS buffer (PBS supplemented with 0,5% bovine serum albumin (BSA), 0,01% NaN₃ and 0,35 mM EDTA) followed by FACS analysis. At 37 °C the majority of spirochetes was internalized as was determined by control experiments in which we did not add the quenching solution (data not shown). Similarly, to determine neutrophil phagocytosis capacity, 50 μl of whole blood was incubated with 2 x 10⁶ viable CFSE-labeled *B. burgdorferi* for the indicated time, after which quenching solution was added for one minute and samples were washed twice with ice-cold FACS buffer. Thereafter cells were incubated with BD Lyse/Fix solution (BD Biosciences) and neutrophils were labeled using anti-Gr-1-PE (BD Pharmingen). Live cells were electronically gated and phagocytosis was determined using FACS. The phagocytosis index of each sample was calculated as previously, described: (mean fluorescence intensity (MFI) x percentage (%) positive cells) at 37°C minus (MFI x % positive cells) at 4°C [63,64].

**Migration assays.** In vitro migration experiments with murine peritoneal macrophages from WT and uPAR knock-out mice were performed essentially as described[67,68]. Prior to experimentation cells were labeled with CellTracker Green (Molecular Probes, Eugene, Or) in serum-free Dulbecco’s modified Eagle’s medium (DMEM). The dye was fixed by 1 h incubation in DMEM plus 10% FCS. Thereafter cells were washed and resuspended in serum-free medium and transferred to 3 μM pore size HTS FluoroBlok Cell Culture Inserts (BD Falcon) which were inserted in fitting 24-well plates containing various attractants (*B. burgdorferi*, activated complement factor 5 (C5a)) also in DMEM serum-free medium. Fluorescence, representing the number of cells on the bottom side of the insert, was read.
every 2 min on a Series 4000 CytoFluor Multi-Well Plate Reader (Perseptive Biosystems, Framingham, MA). Raw fluorescence data were corrected for background fluorescence and no-attractants controls were subtracted at each measured time point to correct for random migration. Migration start points were set to zero. To mimic the in vivo situation more closely we also performed experiments with an embryonic rodent heart-derived cell line, H9c2 cells (CRL-1446, American Type Culture Collection, Queens Road, Teddington, UK). These cardiomyoblasts were maintained in DMEM with 10% foetal bovine serum (FBS). Prior to experimentation, cells were washed and resuspended in serum-free DMEM and incubated with viable *Borrelia* (Cell:*Borrelia* =1:50) or medium as a control for 16 h. The supernatants were centrifuged for 5 minutes at 1200 x g to remove cells and other particles, followed by centrifugation at 4000 x g for 15 minute to remove the spirochetes. Supernatants were used undiluted or diluted (data not shown) as chemoattractants in the indicated experiments. All experiments were performed in duplo or in triplo and repeated three times. In addition, we also assessed migration of leukocytes in skin from C57BL/6 WT and uPAR deficient mice in response to *B. burgdorferi* in vivo (n=5 per group). In these set of experiments we intradermally injected C57BL/6 WT mice with 1 x 10^6 *B. burgdorferi* in PBS in the midline of the neck and mice were sacrificed 0, 6 or 32 hours post inoculation. Control animals were injected with PBS. Skin was harvested, formalin fixed and imbedded in paraffin. Five μm-thick sagittal skin sections were processed and H&E, Ly6G and F4/80 stained by routine histological techniques [57]. The control animals did no display influx of leukocytes (data not shown). Slides were scored for influx of leukocytes by an independent pathologist who was blinded to the experimental design. Influx was semi-quantitatively scored on a scale from 0-3, with 0 being no, 1 mild, 2 moderate, and 3 being severe diffuse infiltration.

**Statistical analysis.** Differences between the groups were analyzed using the two-sided non-parametric Mann-Whitney U test (Graphpad Prism Software version 4.0, San Diego, CA). Where indicated a two-sided Chi-square indicated was applied. Data are presented as the mean ± standard errors of the mean (SEM). A *P* value of < 0.05 was considered significant, where * indicated *P* < 0.05, ** *P* < 0.01 and *** *P* < 0.001. For ECG data statistical analysis was performed using a multivariate repeated measurements model (SPSS statistics software 17.0).

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Supporting information

**Supplemental figure 1.** *Borrelia burgdorferi* induces upregulation of the urokinase receptor on leukocytes in vitro and in vivo.

A. Viable *B. burgdorferi* induces uPAR expression on ex vivo generated human macrophages. Cells were incubated with viable *B. burgdorferi* for 16 hours. Thereafter cells were stained with anti-CD87 (uPAR), electronically gated and analyzed by FACS analysis. Representative cytograms and histograms are shown.

B. Viable *B. burgdorferi* induces uPAR expression on murine granulocytes. Whole blood was incubated with viable *B. burgdorferi* for 16 hours. Erythrocytes were lysed, cells were co-stained with anti-GR-1 and anti-CD87 (uPAR), electronically gated and analyzed by FACS analysis. Representative cytograms and histograms are shown.

C. Viable *B. burgdorferi* (1 x 10^8) were injected into the peritoneal cavity of C57BL/6 WT (n=6) or uPAR knock-out (n=4) mice for one hour. Hereafter cells were harvested, stained for F4/80, and CD87 (uPAR) expression was measured by FACS analysis. A *P* value < 0.05 was considered statistically significant. * indicating *P* < 0.05; ** *P* < 0.01

D. In non-phagocytosing cells, i.e. CD4+ and CD8+ T cells - double stained with anti-CD3-APC (BD Pharmingen) and anti-CD4-FITC and anti-CD8-PerCP, respectively - we also assess CD87 (uPAR) expression by FACS analysis. Error bars represent the mean of triplicates within one experiment ± SEM.
Supplemental figure 2. Impaired phagocytosis of *B. burgdorferi* by uPAR deficient leukocytes.
After the assays whole blood was lysed and stained with anti-GR-1 (granulocytes), viable cells were gated (left panel A) and GR-1 positive cells (right panel A) were analyzed for intracellular *B. burgdorferi*-CFSE signal over time (B). Assays were performed as described in figure 2. Marker (M)1 encompasses positive cells.
Supplemental figure 3. Confocal microscopy of *B. burgdorferi* phagocytosis. A and B. Confocal microscopy confirmed that *B. burgdorferi* in in vitro phagocytosis assays were localized intracellularly. Cells incubated with CFSE-labeled *B. burgdorferi* were subjected to confocal microscopy. Nuclei of cells were stained with DAPI. In Panel A we depicted the widest transversal section of a segmented nucleus of a granulocyte stained with DAPI and a CFSE-labeled *B. burgdorferi* spirochete. Superimposing the brightfield image confirms the bacterium is localized intracellularly. Panel B shows another granulocyte and *B. burgdorferi* from different view points (left panel) and a picture from a stack movie (right panel) further verifying that we are assessing internalized bacteria in the in vitro phagocytosis assays. For color figure see page 266.
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Supplemental figure 4. Carditis in WT, uPAR, uPA, tPA and PAI-1 knock-out mice. A and B. Peak carditis in C57BL/6 uPAR -/- is of similar severity compared to WT controls, although active carditis persists longer in uPAR -/- mice. WT and uPAR -/- mice were inoculated with B. burgdorferi and sacrificed two or four week post infection. Sagittal sections of formalin fixed and paraffin embedded hearts were H&E stained. The severity two weeks post infection was scored by a pathologist blinded to the experimental design on a scale of 0-3, with 0: no carditis; 1: mild carditis; 2: moderate carditis and 3: severe carditis. SHAM inoculated mice did not develop...
carditis (data not shown). Pictures depict representative sections.

C and D. Peak carditis in C57BL/6 uPA, tPA and PAI-1 knock-out mice is comparable to peak carditis in WT C57BL/6 mice. Carditis was scored as described above. Six to eight mice per group were used and bars represent the mean ± SEM. A P value < 0.05 was considered statistically significant.

Supplemental figure 5. Migration and arthritis in WT and uPAR knock-out mice on a B. burgdorferi susceptible genetic background.

A, B and C. Urokinase receptor deficient macrophages from mice on the mixed genetic background can migrate to cardiogenic stimuli just as well as macrophages from WT littermate controls. Migration of CellTracker Green labeled WT or uPAR deficient macrophages towards several chemotactic stimuli was investigated in vitro (A). As chemotactic stimuli we used B. burgdorferi or activated complement factor 5 (C5a) (B) and supernatant from the cardiomyoblastic rodent cell line H9c2 stimulated with B. burgdorferi or control medium for 16 hours prior to experimentation (C). All experiments were performed in duplo in serum free DMEM medium without the addition of antibiotics and migration was corrected for the no-attractant control. Graphs represent the mean of three independent experiments ± SEM. The fluorescent signal in the lower chamber (indicative of migration) was measured in real time every two minutes (cycle).

D and E. Only edema, no arthritis in uPAR knock-out mice (n=7) and WT littermate controls (n=8). Ankle swelling was measured using a microcaliper during the course of infection (D). In this particular experiment mice were monitored for three weeks. Post mortem, but before decalcification, radiological examination of the right hindlimb was performed (E). No differences between SHAM inoculated and B. burgdorferi infected animals were observed. A P value < 0.05 was considered statistically significant. * indicating P < 0.05.

For color figure see page 268.
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