

## Real-Time Imaging of Bone Marrow Granulocyte Mobilization in Response to TLR Agonists Demonstrates a Necessity for Stromal TLR4

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Septicemia resulting from overwhelming bacterial infections is one of the leading causes of death around the world, and is therefore of great clinical importance and interest. Local infections are usually efficiently handled by the innate immune response; chief among the first responders are the neutrophils. However, septicemia occurs when the bacteria gain access the blood with systemic dissemination to sites such as the bone marrow. One of the hallmarks of host immune response during sepsis is an acute rise in the number of circulating neutrophils (i.e. neutrophilia) as a consequence of maturing neutrophil release from the bone marrow. This initial neutrophilia is later followed by a profound loss in circulating neutrophils (i.e. neutropenia). Recent studies have shown that the Toll-like Receptor 4 (TLR4), which recognizes the lipopolysaccharide (LPS) antigen of gram-negative bacteria, plays a significant role in the bone marrow's ability to produce more neutrophils in response to sepsis [1]. Furthermore, it has also been shown that the neutrophil response to LPS is not only dependant on TLR4 on the neutrophils but can also be influenced by TLR4 expression on endothelial cells [2]. To date scant reports are available to characterize the dynamic neutrophil response to LPS challenge within the bone marrow itself. This is of particular scientific interest as increased understanding of this process might shed further light on host's bone marrow response to sepsis, thus potentially aiding development of therapeutic approaches to enhance host recovery from life-threatening septicemia.

2-photon imaging of bone marrow was performed as previously described [3] in the calvarium of transgenic mice in which GFP is expressed under the control of the promoter to lysozyme M protein (LysM-GFP). Lysozyme M is highly expressed in neutrophils, with weak expression in monocytes. In untreated mice, LysM-GFP cells in the bone marrow can be observed throughout the bone marrow cavity, and, for the most part, appear to be in a quiescent state with only a few mobile cells exhibiting an average instantaneous speed of  $\sim 3 \mu\text{m}/\text{min}$ . (Fig. 1A). However, when these mice are challenged with LPS *i.v.* (100ng / mouse), we observed a massive mobilization of GFP<sup>+</sup> granulocytes (a process we refer to as “swarming”; Fig. 2A) beginning at  $\sim 30$  minutes following LPS injection. The average instantaneous speed under these conditions more than doubles to  $> 7 \mu\text{m}/\text{min}$ . (Fig. 1B). Interestingly, this phenomenon was not observed when ligands to other TLRs are used.

To determine if the observed “swarming” behavior is due to binding of LPS by the TLR4 on the granulocytes themselves or the non-hematopoietic stromal elements, we created bone marrow chimeras in which lethally irradiated TLR4<sup>-/-</sup> recipient mice were rescued with bone marrow cells from LysM-GFP donor mice. Interestingly, we did not observe any increase in swarming behavior at 30 minutes following injection of LPS in these chimera mice (Fig. 1C). This result suggests that functional response of TLR4 signaling of the bone marrow stromal elements, not of the bone marrow-resident neutrophils themselves, is responsible for the “swarming” phenomenon.

Sequential imaging of the LPS-challenged mice at later time points revealed that the bone marrow of wild type mice challenged with LPS were almost completely filled with the remnants of dead granulocyte debris, which gradually worsened over two weeks, at which time, few living LysM-GFP cells can be seen in the

marrow (Fig. 2B). This observation confirms the typical clinical scenario in which post-sepsis patients are at higher risk of secondary infection due to inability of the bone marrow to sustain continued granulocyte production. Our sequential intravital 2-photon technique provides a valuable model for imaging studies of the bone marrow response to sepsis and other marrow-related cellular response such as hematopoiesis and tumor metastasis.

## References

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- [2] G. Andonegui et al., *J. Clin. Inv.* 119 (2009) 1921.
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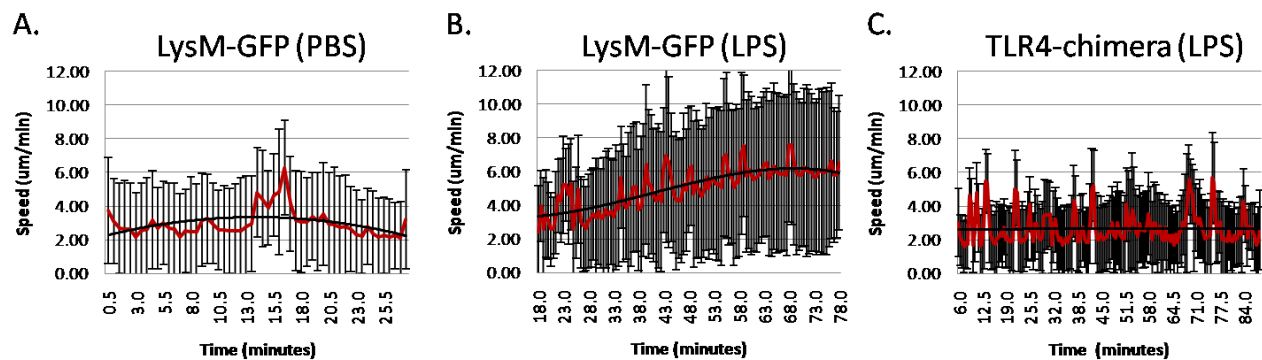


FIG. 1. Average Instantaneous speed of LysM-GFP cells in the bone marrow. A) PBS injected LysM-GFP control. B) LysM-GFP mouse injected with 100 ng LPS I.V. C) LysM-GFP  $\rightarrow$  TLR4<sup>-/-</sup> chimera injected with 100 ng LPS I.V. Time in B and C indicates minutes after LPS injection. Spikes in instantaneous speed correspond to movement of the entire field of view and are therefore artifacts of the intravital imaging.

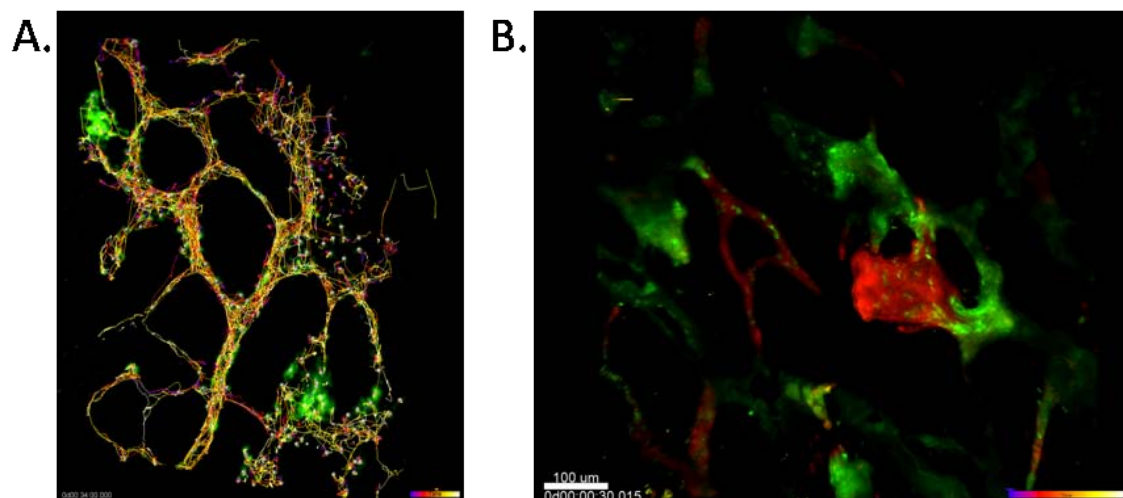


FIG. 2. Representative images of bone marrow containing LysM-GFP cells. A) Tracks of LysM-GFP cells after I.V. LPS injection (100 ng). B) Bone marrow of LysM-GFP mouse 2 weeks after LPS injection.