Video Article
Direct Observation of Phagocytosis and NET-formation by Neutrophils in Infected Lungs using 2-photon Microscopy

Mike Hasenberg¹, Anja Köhler¹, Susanne Bonifatius¹, Andreas Jeron², Matthias Gunzer¹

¹Institute for Molecular and Clinical Immunology, Otto-von-Guericke University Magdeburg
²Department of Immunoregulation, Helmholtz Center for Infection Research

Correspondence to: Matthias Gunzer at matthias.gunzer@med.ovgu.de

URL: http://www.jove.com/details.php?id=2659
DOI: 10.3791/2659

Citation: Hasenberg M., Köhler A., Bonifatius S., Jeron A., Gunzer M. (2011). Direct Observation of Phagocytosis and NET-formation by Neutrophils in Infected Lungs using 2-photon Microscopy. JoVE. 52. http://www.jove.com/details.php?id=2659, doi: 10.3791/2659

Abstract
After the gastrointestinal tract, the lung is the second largest surface for interaction between the vertebrate body and the environment. Here, an effective gas exchange must be maintained, while at the same time avoiding infection by the multiple pathogens that are inhaled during normal breathing. To achieve this, a superb set of defense strategies combining humoral and cellular immune mechanisms exists. One of the most effective measures for acute defense of the lung is the recruitment of neutrophils, which either phagocyte the inhaled pathogens or kill them by releasing cytotoxic chemicals. A recent addition to the arsenal of neutrophils is their explosive release of extracellular DNA-NETs by which bacteria or fungi can be caught or inactivated even after the NET releasing cells have died. We present here a method that allows one to directly observe neutrophils, migrating within a recently infected lung, phagocyting fungal pathogens as well as visualize the extensive NETs that they have produced throughout the infected tissue. The method describes the preparation of thick viable lung slices 7 hours after intratracheal infection of mice with conidia of the mold Aspergillus fumigatus and their examination by multicolor time-lapse 2-photon microscopy. This approach allows one to directly investigate antifungal defense in native lung tissue and thus opens a new avenue for the detailed investigation of pulmonary immunity.

Protocol

1. Infection

1. Swollen conidia of the mold Aspergillus fumigatus (strain ATCC 46645) are generated by pre-incubation in RPMI 1640 (Biochrom AG, Berlin, Germany) supplemented with 5% FCS (v/v). 2x10⁷ resting conidia are resuspended in 5 ml medium and incubated in a 6-well tissue culture plate (TPP AG; Trasadingen, Switzerland, catalog # 92006) for 7h at 37°C.

2. Following the swelling period, the spores are fluorescently labeled by adding 10 μl of calcofluor white stock solution (Sigma Aldrich, Deisenhofen, Germany, catalog # F3543; 25 mg/ml in DMSO) to each 1 ml resting conidia determined by counting with a Neubauer chamber. Finally the spore suspension is spun down at 900xg at 5 min at room temperature, the supernatant is carefully removed, and the pellet resuspended to the final concentration of 1x10⁷ spores per 100 μl PBS.

3. For infection, 100 μl of spore solution is applied intratracheally into female mice (C57/BL6, 8–10 weeks old, Harlan, Germany). If neutrophils are to be observed as well, the use of Lys-EGFP mice, carrying EGFP under the lysozyme promotor is recommended. Begin by anesthetizing the animal with a single i.p. injection of 150 μl Ketamin/Rompun solution (Inresa Arzneimittel GmbH, Freiburg, Germany (Ketamin) and Bayer Vital GmbH, Leverkusen, Germany (Rompun), 1 ml Ketamin [50 mg/ml] + 0.5 ml Rompun [2 %] + 3.5 ml sterile NaCl [0.9 %]). 5 minutes later the anesthetized mouse can be fixed with an elastic band by its teeth to the ramp (i.e. a sloped surface) to facilitate the intubation (supplemental figure 1). Using forceps, the tongue is pulled to one side and a 22G indwelling venous catheter (B. Braun AG, Melsungen, Germany; Vasofix Braunüle) can be gently inserted into the trachea under permanent illumination with a goose neck lamp. The successful insertion of the catheter is verified by observing regular movement of the animal’s thorax with the frequency of the mechanical ventilator. When the successful intubation has been confirmed, 100 μl spore suspension is applied using a 100 μl micropipette. This volume should be inhaled by the animal without any additional help in 1-2 s. An enhanced distribution of fungal particles inside the lung is achieved by mechanically ventilating the infected animal for 2 minutes with a small animal respirator (MiniVent, Hugo Sachs, March-Hugestetten, Germany) at rate of 250 breaths per minute and an inhalation volume of 300 μl per breath (supplemental figure 2). After the infection animals are returned to their cage and observed every 5 minutes until ambulatory again. Mice do not show any evidence of pain or distress during the following incubation period.

2. Lung preparation

1. 7 h after infection with the spores, the animal is euthanized by an overdose of isoflurane (Baxter GmbH, Unterschleißheim, Germany) until movements and breathing have ceased for more than 30 seconds. This is then followed by thoracotomy as a secondary method to assure clinical death and allow for visualization and access of the trachea and lungs. Then the chest is carefully opened to expose the lung and the upper respiratory tract. Then another 22G indwelling venous catheter is inserted into the trachea starting from the exposed epiglottis. Through this tubule the lungs are filled with 1 ml pre-warmed low melting agarose (2 % w/v, Promega, Mannheim, Germany) using a 1 ml Omnimix syringe (B.Braun). As soon as the lungs are filled the trachea is tied off with a short piece of sewing thread and the whole animal is put into a refrigerator at 4 °C.

2. 15 min later, when the agarose has solidified, the whole lung is excised beginning from the trachea. Using a pair of sharp pointed scissors, the right lung lobe is subsequently removed, the bottom surface is briefly dried on a sheet of tissue paper and then the whole lobe is glued to
3. 2-photon laser microscopy

1. The Petri dish containing the lung sample is then installed under the microscope objective so that the buffer can be warmed to 37°C by a temperature sensor-controlled heater (supplemental figure 4). 2-photon microscopy is then carried out over the whole cut surface using a Zeiss LSM 710 NLO microscope on an upright Axio Examiner stage equipped with a 20×NA1.0 water dipping lens (Zeiss, Jena, Germany). For imaging, different areas along the section are scanned down to 400 μm depth using an illumination wavelength of 800 nm detecting green (530 nm) and red (580 nm) fluorescence, as well as the second harmonic generation (SHG)-signal and the blue calcofluor fluorescence (at 400–470 nm emission) with external non descanned detectors (NDD). Besides 2-D images and time lapse movies (1 image every 5-10 seconds), single or repetitive 3-dimensional picture stacks can be subsequently rendered as voxel renderings or single extended focus images or 4-D data (3D over time) using different software packages. This microscopic setup allows the observation of cellular behavior in an almost intact environment that, as it constitutes the entry port for a variety of airborne pathogens, is of enormous immunological relevance. Cell motility, phagocytosis and NET production by endogenous neutrophils following infection with the mold Aspergillus fumigatus can be easily investigated under these in situ conditions.

4. Representative Results

If done properly the imaging will generate 2- or 3-color slides or movies. Although coloring is done in a post processing procedure and is thus freely adaptable, we generally select a coloring scheme that reflects the natural color of the dye/signal, which is detected in the relative channel. Thus, the Sytox dyes are depicted in red, the fungus as well as the SHG signal is shown in blue and, if present, EGFP-labeled cells are stained in green. In the first example (Fig. 1) the blue SHG signal depicts the tissue fibers of a non-infected lung and the red Sytox signal is produced by nuclei of lung-resident cells cut open by the vibratome. In the second example (Fig. 2) an infected lung is shown, where both, alveolar structures as well as fungal masses, appear in blue, while nuclei and NETs are stained red. The third example (Fig. 3) is from a Lys-EGFP transgenic animal where in addition to the blue and red structures the green neutrophils can also be seen. The migration of neutrophils and their phagocytosis of individual fungal elements in time lapse sequences is shown in the supplemental movie.

**Figure 1. The appearance of a non-infected lung slice in 2-color 2-photon microscopy.** A lung slice was prepared from a non-infected C57/Bl6 mouse and imaged as described in the protocol. Presented here are the SHG signal of the alveolar tissue structure (A), the Sytox signal of cell nuclei cut open during the preparation of the lung slice (B), and an overlay of the two channels (C). The boxed area in (C) is seen enlarged in (D). Please note the fibrous tissue at the bottom of the lung slice as compared to the clearly alveolar organization within the breathing-active areas of the lung above.
**Figure 2.** The appearance of a lung slice of an *Aspergillus* infected wild type animal in 2-color 2-photon microscopy. A lung slice from a C57/BL6 mouse infected 7 h before with *A. fumigatus* was prepared and imaged as described in the protocol. Shown is the combined fungal structure and SHG of the alveolar tissue (A), the Sytox signal of DNA NETs as well as cell nuclei cut open during the preparation of the lung slice (B), and an overlay of the two channels (C). The boxed area in (C) is seen enlarged in (D). Please note, the clearly distinct areas of fungal masses, alveoli, and NET-structures are marked with the letters F, A and N, respectively.

**Figure 3.** The appearance of a lung slice of an Aspergillus infected Lys-EGFP animal in 3-color 2-photon microscopy. A lung slice of a Lys-EGFP mouse infected 7 h before with *A. fumigatus* was prepared and imaged as described in the protocol. Shown is the combined fungal structure and SHG of the alveolar tissue (blue), the Sytox signal of cell nuclei and NETs (red), as well as numerous neutrophils (green).
Supplemental figure 1. Mouse fixation for intubation. Under Ketamin/Rompun anesthesia the animal is fixed with an elastic band at its teeth in order to facilitate the intubation with a 22G indwelling venous catheter.

Supplemental figure 2. Mechanical mouse ventilation. The intubated mouse is infected by an i.t. application of $1 \times 10^7$ spores resuspended in 100 μl PBS. An enhanced distribution of fungal particles inside the lung is achieved by mechanically ventilating the infected mouse with a small animal respirator.
Supplemental figure 3. Lung lobe fixation. After preparation of the right lung lobe the organ is fixed in a Petri dish by use of a laboratory-made flat washer that is covered by a set of parallel nylon threads.

Supplemental figure 4. 2-photon imaging setup. The Petri dish containing the right lung lobe is installed on a heating mat under the 2-photon microscope after addition of the DNA dye Sytox Orange.

Supplemental movie: Neutrophils migrating and phagocytosing fungal elements in Aspergillus infected lungs as seen by time lapse 2-photon microscopy of a living lung slice 7 h after infection with the fungus. Neutrophils are green, fungal elements and SHG are blue, and cell nuclei as well as NET-structures are depicted in red. The real time of the experiment is shown on the lower right. The scale bar depicts 50 μm. Click here to watch video.

Discussion

Real-time 2-photon microscopy in vivo or in intact organs has gained profound importance in studies dealing with the physiology of immune cells over the past 10 years. It was with this technique that important events like the dynamics of T-cell activation within the lymph nodes first became visible^{2-4}. More recently, researchers have also started to analyze specific cellular functions like the first steps in the generation of effector cells in lymphatic tissues using this approach^{5}.

However, although a number of new biological concepts have been revealed using this method, there are still challenging and important questions for which no intravital visualization studies have been published thus far. Notably this applies to the mammalian lung. The interesting aspect of this organ, as entry port for a variety of airborne pathogens, makes it one of the most crucial surfaces at which immunological processes take place in the mammalian body. With every breath over the entire life span, unwanted particles are inhaled some of which have the potential to induce life threatening infections^{6}. It is self-explanatory that at such a sensitive and endangered site a tight network of defense mechanisms needs to be present exhibiting the whole repertoire of immune responses. On the other hand it is very important that the induced immunological fight against potential pathogens at such a “dirty” place is tightly controlled. Exaggerated reactions of the immune system bear a high risk of harming the own body by massively injuring organ tissue upon stimulation of unspecific immune cell actions^{7,8}.
The protocol presented in this study for the observation of immune cells within the murine lung is still not an in vivo application, but rather a close approximation to the situation in a functionally intact lung. Ex vivo approaches for imaging lymphocytes, e.g. in explanted lymph nodes, have been shown to yield results equivalent to true in vivo observations and thus are highly relevant. The in situ observations in lung slices, which are only possible with our approach, take place in an infected lung shortly after excision. The 3D integrity during the cutting process is ensured by the agarose matrix, an essential step to allow a precisely controlled cutting process of the lung. Although it is necessary to cool the explanted lung for a short period to allow a solidification of the agarose matrix, it is possible to return to near physiological conditions for cells after cutting and rewarming of the tissue. This is clearly shown by our data, which demonstrate that under these conditions neutrophils are highly active and exhibit their full potential as agile phagocytes, which are necessary for the effective clearance of an Aspergillus fumigatus infection. They patrol the lung tissue passing through epithelial barriers in order to reach the inner parts of alveoli and furthermore they actively take up fungal spores 11. A key finding of this work was the appearance of structures resembling Neutrophil extracellular traps (NETs) in the Aspergillus infected organ. NETs are a very recent finding of a novel defense mechanism in neutrophils. However, since its initial description in 2004, the number of physiological or pathological conditions in animal models or humans where this phenomenon has been observed or is lacking has been explosively increasing. Interestingly, although so much work has been spent on these structures by so many different groups, most reports are still on a very descriptive level and not much is known about the mechanism of NET release and its regulation. With our protocol we were able for the first time to show NET fibers in an infected lung. Furthermore, we could demonstrate the importance of freshly recruited neutrophils as well as molecular fungal structures for their occurrence or inhibition. This clearly shows the potential of our method to investigate the single steps of NET formation in more detail. One could think for example to use neutrophils from suitable knock-out mice to observe their ability of NET formation in adoptive transfer experiments.

Thus, although the direct observation of neutrophil immigration from the peripheral blood is not possible with this system due to the lack of blood supply after organ explantation, we still believe that our protocol is a valuable and relatively easy to handle approach that allows the imaging of early or late steps in the immune defense against lung infections. This is, therefore, an important step towards investigating this phenomenon within the breathing lung of live animals.

Disclosures
We have nothing to disclose.

Acknowledgements
The authors would like to thank Dr. Lars Philipsen for help with optimizing the intravital movies, Dr. Jonathan Lindquist for carefully reading the manuscript, and all members of the Gunzer laboratory for helpful discussions and comments during the development of the method. This work was funded by grants from the Deutsche Forschungsgemeinschaft (DFG, SFB 854) to M.G.

References

1. Faust, N., et al., "Insertion of enhanced green fluorescent protein into the lysozyme gene creates mice with green fluorescent granulocytes and macrophages," Blood 96, 719 (2000).
2. Gunzer, M., et al., "A spectrum of biophysical interaction modes between T cells and different antigen presenting cells during priming in 3-D collagen in vivo," Blood 104, 2801 (2004).
3. Mempel, T. R., Henrickson, S. E., & von Andrian, U. H., "T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases," Nature 427, 154 (2004).
4. Bousso, P. & Robey, E., "Dynamics of CD8(+) T cell priming by dendritic cells in intact lymph nodes," Nat. Immunol. 4, 579 (2003).
5. Beunev, H., et al., "Visualizing the functional diversification of CD8(+) T cell responses in lymph nodes," Immunity 33, 412 (2010).
6. Amanianpa, V., et al., "Surface hydrophobin prevents immune recognition of airborne fungal spores," Nature 460, 1117 (2009).
7. Snelgrove, R. J., et al., "A Critical Role for LTA4H in Limiting Chronic Pulmonary Neutrophilic Inflammation," Science 330, 90 (2010).
8. Chou, R. C., et al., "Lipid-Cytokine-Chemokine Cascade Drives Neutrophil Recruitment in a Murine Model of Inflammatory Arthritis," Immunity 33, 266 (2010).
9. Nitschke, C., et al., "3-D and 4-D imaging of immune cells in vitro and in vivo," Histochem. Cell Biol. 130, 1053 (2008).
10. Niesers, R. A., Andresen, V., & Gunzer, M., "Intravital 2-photon microscopy – focus on speed and time resolved imaging modalities," Immuno Rev 221, 7 (2008).
11. Bruns, S., et al., "Production of Extracellular Traps against Aspergillus fumigatus in vitro and in Infected Lung Tissue is Dependent on Invading Neutrophils and Influenced by Hydrophobin RodA," PLoS Pathog. 6, e1000873 (2010).
12. Miller, M. J., et al., "Autonomous T cell trafficking examined in vivo with intravital two-photon microscopy," Proc. Natl. Acad. Sci. U.S.A. 100, 2604 (2003).
13. Brinkmann, V., et al., "Neutrophil Extracellular Traps Kill Bacteria," Science 303, 1532 (2004).
14. Marcos, V., et al., "CXCR2 mediates NADPH oxidase-independent neutrophil extracellular trap formation in cystic fibrosis airway inflammation," Nat Med. 16, 1018 (2010).
15. Clark, S. R., et al., "Platelet TL4 activates neutrophil extracellular traps to ensnare bacteria in septic blood," Nat Med. 13, 463 (2007).
16. Bianchi, M., et al., "Restoration of NET formation by gene therapy in CGD controls aspergillosis," Blood 114, 2619 (2009).