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Tracking of Inhaled Near-Infrared Fluorescent Nanoparticles in Lungs of SKH-1 Mice with Allergic Airway Inflammation

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ABSTRACT Molecular imaging of inflammatory lung diseases such as asthma has been limited to date. The recruitment of innate immune cells to the airways is central to the inflammation process. This study exploits these cells for imaging purposes within the lung, using inhaled polystyrene nanoparticles loaded with the near infrared fluorescence (NIRF) dye Itrybe (Itrybe-NPs). By means of *in vivo* and *ex vivo* fluorescence reflectance imaging (FRI) of an ovalbumin (OVA)-based allergic airway inflammation (AAI) model in hairless SKH-1 mice, we show that subsequent to intranasal application of Itrybe-NPs, AAI lungs display significantly higher fluorescence intensities than lungs of control mice for at least 24 h. *Ex vivo* immunofluorescence analysis of lung tissue demonstrates the uptake of Itrybe-NPs predominantly by CD68⁺CD11c⁺ECF-L⁺MHCII^{low} cells, identifying them as alveolar M2 macrophages (AMs) in the peribronchial and alveolar areas. The *in vivo* results were validated by confocal microscopy, overlapping tile analysis and flow cytometry, showing a significantly larger amount of Itrybe-NP-containing macrophages in lungs of AAI mice than in controls. A small percentage of NP-containing cells were identified as dendritic cells (DCs). Flow cytometry of tracheobronchial lymph nodes showed that Itrybe-NPs were negligible in lung draining lymph nodes 24 h after inhalation. This imaging approach may advance preclinical monitoring of AAI *in vivo* over time and aid the investigation of the role that macrophages play during lung inflammation. Furthermore, it allows for tracking of inhaled nanoparticles and can hence be utilized for studies of the fate of potential new nanotherapeutics.

KEYWORDS: Itrybe nanoparticles, *in vivo* near infrared fluorescence (NIRF) imaging, allergic airway inflammation, cell tracking, alveolar macrophages.

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3 Nanoparticles (NPs) are of increasing interest in preclinical and clinical research and
4 diagnosis. Because they are capable of conjunction with different cell types they provide a tool
5 for cell tracking and have been particularly explored in the field of stem cell therapy and cancer
6 to understand cell migratory pathways and the fate of grafted cells. Furthermore, the rapid
7 development of nanotechnology has generated a plethora of nanomaterials as candidates for
8 novel delivery systems for target-specific therapeutic drugs as well as for the diagnosis of
9 diseases and assessment of treatment efficacy. The chemical properties and the preparation of
10 NPs are well established and optimized with respect to resulting particle sizes, small size
11 distributions and various surface chemistries and also with respect to the preparation of
12 fluorophore-encoded particles.¹⁻⁴ However, there is still a need for their biological profiling and
13 examination *in vivo*. Several recent studies of the interaction of such nanoparticles with
14 biological systems have demonstrated the large influence of the particle surface functionalization
15 (chemical nature and number of surface groups) and charge on such interactions including the
16 formation of a protein corona and cellular uptake. However, no general statements can be made
17 at present because previous research has used a range of different model systems and sometimes
18 poorly analytically characterized particles and has applied different methods to assess possible
19 toxicity due to the lack of standardized measurements.⁵⁻¹⁰ Hence, it is often necessary to study
20 the influence of the respective tailor made particle systems on the specific biological system of
21 interest.

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24 Moreover, with the advent of nanotherapeutics in clinical practice,¹¹ and the increasing
25 manufacture and use of products based on nanotechnology,¹² there is an ever increasing need to
26 investigate the fate of NPs in the body and to determine in which cell types they accumulate. In
27 addition, the stability of NPs in the cellular microenvironment, as well as their trafficking and
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retention within different cells and organs require examination.^{13,14} Furthermore, established international regulatory authority governance of use of foreign substances in diagnostics and therapeutics dictates the safety thresholds for incorporation of foreign substances into healthcare. NPs are foreign substances and if administered for diagnostic or therapeutic purposes will need to undergo rigorous toxicological examination before incorporation into healthcare.

Tissue macrophages derive from monocytes and greatly increase in numbers in inflammation that commonly occurs in diseased tissue. Hence, this cell type is clearly an attractive target for new nanotherapeutics as well as for diagnostic purposes. The uptake of NPs by macrophages has been repeatedly shown in different biological systems and during disease. In particular, imaging of tumor-associated macrophages has been explored for the purpose of prognostic information, definition of tumor margins and measuring therapeutic response.^{15–17}

Along with magnetic NPs¹⁸, fluorescent NPs have been abundantly used for imaging macrophages, in both *in vitro* and *in vivo* assays.^{19,20} Above all, targeted, optically active contrast agents have improved the diagnosis and therapeutic assessment in oncology.²¹ We successfully applied polystyrene NPs (PSNPs) loaded with the bright NIR-emissive asymmetric cyanine Itrybe for tumor detection *in vitro* and in a mouse tumor model.²² PSNPs are especially attractive for *in vivo* imaging as polystyrene is generally considered as inert and non-toxic.^{23,24} PSNP preparation via miniemulsion polymerization is well established and these particles are commercially available in a large range of different sizes with a narrow size distribution and various surface chemistries.^{25,26} Moreover, control experiments performed with accordingly prepared dye-doped particles in a dispersion of 5% BSA/PBS buffer revealed the absence of dye leakage.²⁷

Here, we decided to use Itrybe-NPs to track macrophages in a mouse model of acute allergic airway inflammation (AAI) because the number of alveolar macrophages (AM) is significantly increased in AAI.²⁸ AAI is most commonly associated with asthma, which is a chronic inflammatory disease of the lungs characterized by a variable degree of reversible bronchoconstriction, airway hyperresponsiveness (AHR) and increased mucus production, features that have been consistently reproduced for decades in mice by sensitization with different allergens such as ovalbumin (OVA).²⁹ Allergen challenge induces a strong immune response that leads to profound immune cell infiltration of the lung airways, activation of AMs and thickening of the airway epithelium with a marked goblet cell hyperplasia, all of which are found in both asthmatic humans and airway immunized mouse models.³⁰ AMs are part of the innate immune system and one of the first cell types that allergens encounter. In a healthy lung, they form 95% of the cell load in bronchoalveolar lavage (BAL), the remainder being mainly lymphocytes. Particles inhaled into the lower airways are rapidly phagocytized by these cells and their number increases significantly during AAI.³¹ Controversy exists about the role AMs play during the development or progress of AAI. Recent evidence suggests that AMs are associated with both anti-inflammatory and pro-inflammatory functions.²⁸ Moreover, it is still unclear whether AMs play a part in inducing immune tolerance by antigen-presenting capabilities or if this function is largely attributed to dendritic cells.^{32,33}

To date, non-invasive preclinical animal studies of lung disease have mainly facilitated the anatomical or structural assessment of the diseased lung and/or require the use of radioactive agents or X-rays (PET, SPECT, CT).³⁴ Optical imaging has emerged as an important alternative, as it represents a rapid, inexpensive and relatively simple technique that enables the detection of specific targets and functional events in a live animal over time³⁵, in particular when combined

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with near infrared fluorescent (NIRF) probes. Such probes have revealed several advantages over other fluorescent dyes as NIR light can penetrate deeper into the tissue and measurements in this spectral region benefit from comparably lower tissue autofluorescence.³⁶ Moreover, they lack radioactivity and do not create toxic waste. Hence, NIRF imaging is considered as a real future alternative to nuclear imaging, the gold standard of functional imaging.

In this study we show that fluorescence reflectance imaging (FRI) in combination with bright NIR-emissive non-toxic Itrybe-NPs is an ideal technique to specifically monitor the presence of AAI in SKH-1 mice. We demonstrate that these NPs are predominantly taken up by macrophages in the lung and show, non-invasively, that AAI mice display significantly higher fluorescence intensities over the lung than controls. In addition, we find that such NPs provide a tool to track lung macrophages and examine their phagocytic capabilities during disease. This imaging approach could be applied to pursue the fate of inhaled fluorescent NPs in the future, thereby assessing their suitability in theranostic nanomedicine.

Results

SKH-1 mice are susceptible to AAI induction

Because the fur of mouse strains used in AAI research to date, such as BALB/c or C57BL/6, interferes with fluorescence imaging by absorbing and scattering of light and requires removal of the hair before imaging, we have considered the hairless but immunocompetent SKH-1 mouse strain a potential alternative.³⁷ To the best of our knowledge, SKH-1 mice have not previously been used in AAI or asthma research. Induction of AAI in SKH-1 mice using a conventional OVA model protocol³⁸ (Figure 1A) resulted in characteristic features of allergic inflammation, comparable to that in BALB/c mice, as demonstrated by lung histology, BAL cell count and cytokine expression. H&E staining of lung paraffin sections revealed typical infiltration of immune cells around the bronchi and blood vessels of the lung (Figure 1B). Periodic acid - Schiffs (PAS) staining of lung paraffin sections showed goblet cell hyperplasia and excess mucus production (Figure 1C). Eosinophil numbers in BALs of AAI mice was found to be on average 60 ± 1.6 % of the total cell count in OVA-induced immunized mice compared to 0.4 ± 0.38 % in control mice (Figure 1D). OVA-specific IgE and IgG1 were significantly elevated in sera of AAI mice (Figure 1E), as were mRNA expression levels of cytokines IL-4, IL-5, IL-10 and IL-13 in AAI lung tissues (Figure 1F). These results demonstrate that OVA-sensitization and challenge lead to a robust eosinophilic inflammation and Th2 response in SKH-1 mice. Macrophage count in BALs of AAI mice was well correlated with eosinophil numbers (Supplementary Figure 1) with a Pearson Correlation Coefficient r of 0.58.

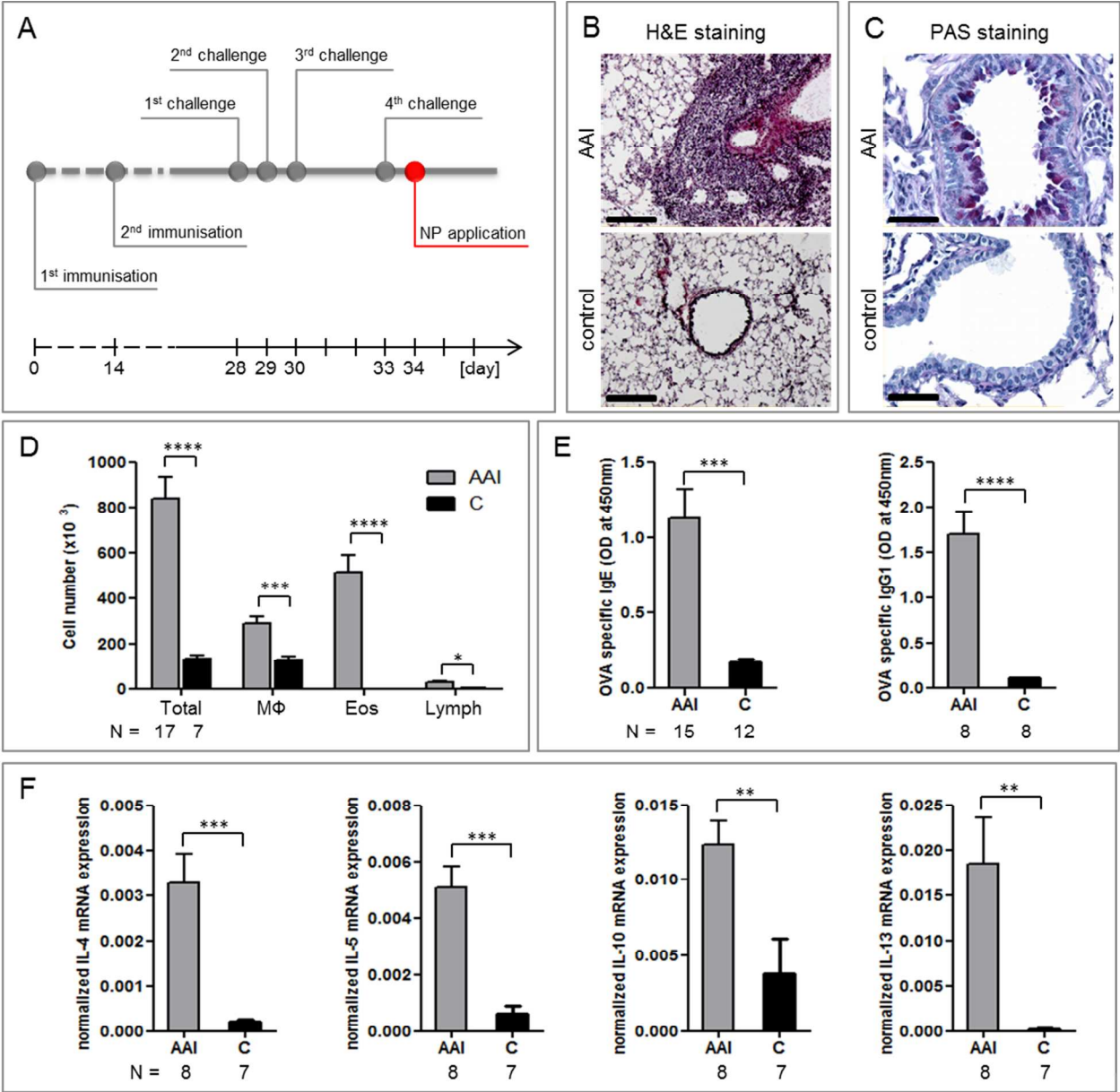
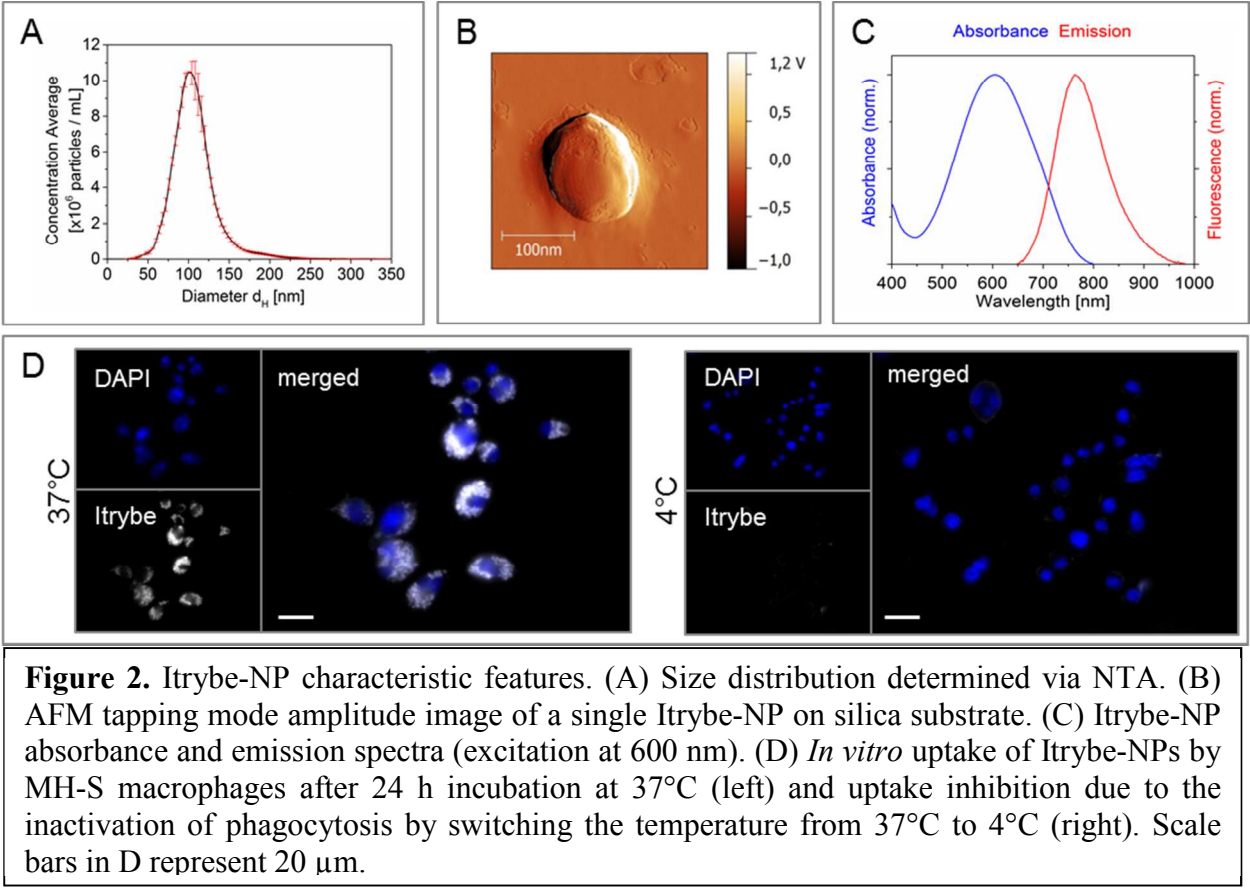


Figure 1. SKH-1 mice are susceptible to AAI induction. (A) General immunization and challenge scheme for the allergic inflammation model used in all investigations. (B) H&E and (C) PAS staining of lung paraffin sections of AAI (upper panel) and control mice (lower panel). (B) AAI mice exhibit typical cell infiltration around blood vessels and bronchi, as well as (C) goblet cells filled with secretory granules (magenta) and increased goblet cell hyperplasia (upper panel). (D) Cell composition in BALs shows increased total cells, macrophages (MΦ), eosinophils (Eos) and lymphocytes (Lymph) in AAI, (E) OVA-specific IgE and IgG1 are significantly increased in AAI. Serum was diluted 1:10 for IgE and 1:625 for IgG1, (F) Expression of IL-4, IL-5, IL-10 and IL-13 Cytokines expression in lung tissue is significantly elevated in AAI. Scale bars represent 200 μm (B) and 50 μm (C). Data were analyzed by t-test and * represents P<0.05

Characterization of Itrybe-NPs

Itrybe-NPs are 100 nm-sized carboxy-modified polystyrene nanoparticles loaded with the NIRF dye Itrybe, an asymmetric cyanine, via an established staining procedure.³⁹ In this procedure, a dye-containing tetrahydrofuran (THF) solution (0.5 mM) was added to an aqueous particle suspension (0.5 w%). The addition of THF leads to swelling of the polymer matrix. The hydrophobic fluorophore molecules can then diffuse into the matrix and adsorb on the apolar polymer chains. Thus, no functional groups or chemical reaction is necessary for dye labeling. Subsequently, the NPs are thoroughly washed with ethanol to ensure complete removal of all unincorporated fluorophores from the particle surface and the swelling solvent is exchanged for water to induce the contraction of the swollen polymer particles. This leads to the physical and sterical entrapment of the dye molecules inside the NPs.

The material and spectroscopic properties as well as the *in vitro* characteristics of Itrybe-NPs are summarized in Figure 2. Characterizing of the size distribution of colloiddally stable aqueous dispersions of Itrybe-NPs via nanoparticle tracking analysis (NTA) revealed a mean hydrodynamic diameter d_H of 108 +/- 8 nm and high monodispersity (Figure 2A). Atomic force microscopy (AFM) showed the spherical shape and non-porous surface of the NPs (Figure 2B). Itrybe-NPs show broad, unstructured and almost environment-insensitive absorption (500 nm – 700 nm) and emission (700 nm – 900 nm) bands with an absorption maximum at 605 nm and an emission maximum at 760 nm, respectively. The broad absorption band provides a great versatility in the choice of the excitation wavelength, rarely found in NIRF dyes, and the large Stokes Shift of about 3400 cm^{-1} is ideal for the separation of scattered excitation light from fluorescence (Figure 2C).



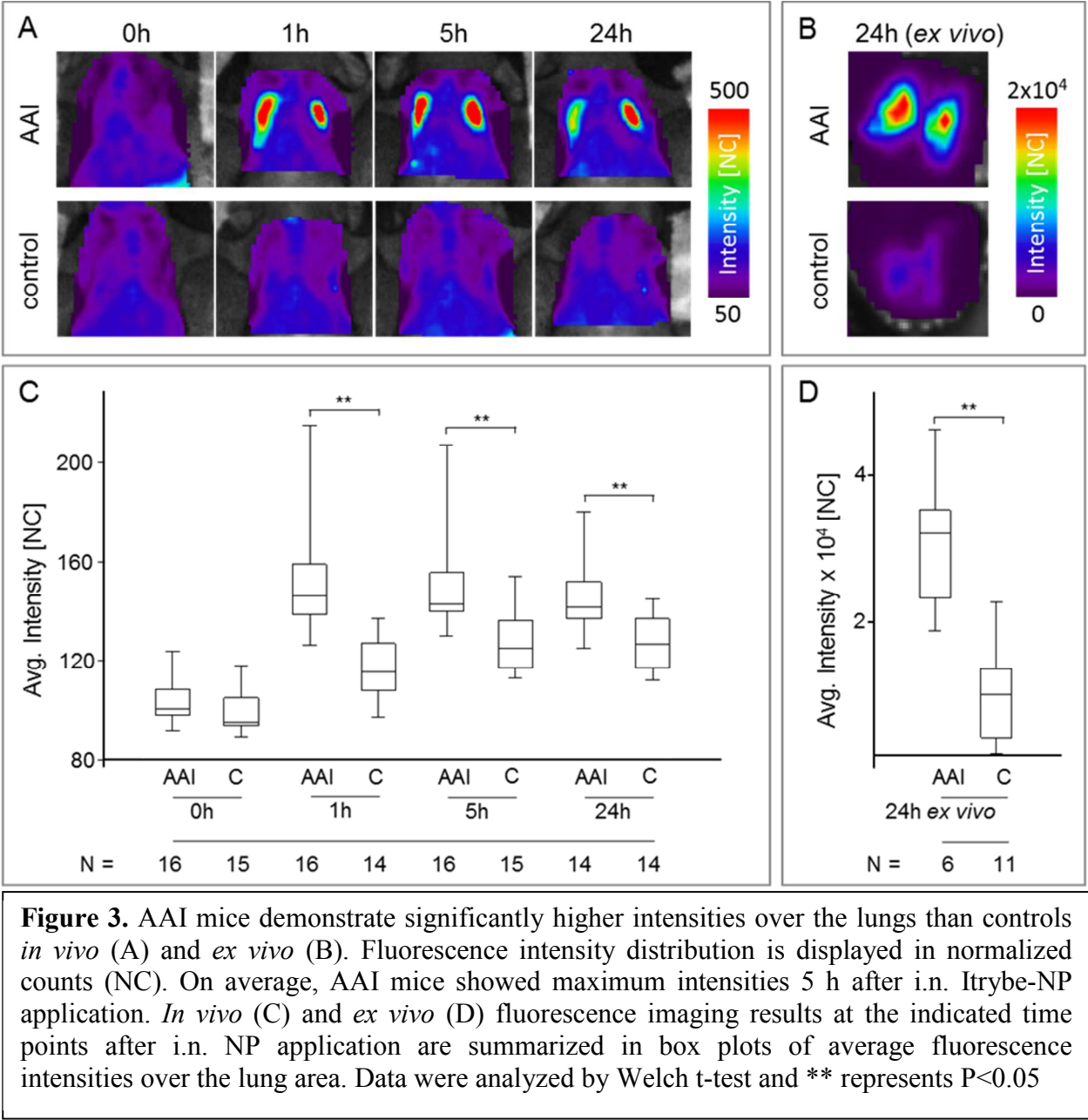
To test if Itrybe-NPs are taken up by AMs *in vitro*, we incubated 100 ng/ml NPs with 5×10^5 MH-S cells, an immortalized and adherent mouse alveolar macrophage cell line, over 5 h (data not shown) and 24 h (Figure 2D and E). The MH-S cells readily take up Itrybe-NPs within 5 h of incubation. As can be seen in the representative image in Figure 2D, a strong active uptake of the particles is observed after 24 h of incubation at 37°C, which was completely inhibited, when the cells were maintained at 4°C.

In vivo monitoring of AAI by means of Itrybe-NPs

To assess the suitability of Itrybe-NPs for monitoring the presence of AAI, we intranasally (i.n.) applied 100 nm NPs in OVA-induced AAI or control mice. Mice were scanned *in vivo* by FRI 1 h, 5 h and 24 h after NP application to investigate the distribution of Itrybe-NPs in whole bodies

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3 and in the lung over time. Full body scans revealed that fluorescence signals above background
4 are detectable over the lung area as early as 1 h after NP application. Some animals (AAI mice
5 and controls) also showed measurable fluorescence intensities in the stomach and intestine as
6 well as in the bladder (Supplementary Figure 2). To analyze the fluorescence intensities derived
7 from the lungs in more detail, we performed *in vivo* scans by choosing a region of interest (ROI)
8 outlining the lungs of the animals. These were performed with an increased raster resolution (1
9 mm) as well as a higher integration time (1 s). To verify that the measured fluorescence
10 intensities originated from the injected Itrybe-NP, we performed a lifetime (LT) analysis and
11 compared the results with the LT of the Itrybe-NPs *in vitro* in solution (Supplementary Figure 3).
12 The fluorescence LT measured *in vivo* over the lung region was 0.8 ns as measured *in vitro*,
13 which is substantially longer than the LT of the autofluorescence background measured in the
14 prescan in the same region (0.4 - 0.5 ns). This indicates that the fluorescence intensities
15 measured over the lung are specific for Itrybe.
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35 Figure 3A shows representative images of lung scans of OVA-induced AAI mice and healthy
36 control mice before and at 1 h, 5 h, and 24 h post i.n. NP application. We found a significantly
37 higher accumulation of Itrybe-NPs in lungs of OVA-immunized mice than in control mice at
38 every time point (Figure 3C). Peak average intensities over the lung were nearly identical at 1 h
39 and 5 h (153.8 and 154.2) post NP application. Some animals showed higher fluorescence
40 intensities in the lung at 1 h or 24 h after i.n. NP application, indicative of an inter-individual
41 variability in the progress of acute onset of AAI with a varying accumulation of immune cells in
42 the lungs of mice.
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AAI mice exhibited 2.9, 1.7 and 1.4 times higher NP-derived signals (average intensity over the lung minus average intensity of the prescan) in the lungs at 1 h, 5 h and 24 h respectively, when compared to control animals.

To confirm that the intensities measured *in vivo* originated from the lung, we performed FRI scans of the excised lungs from animals sacrificed 24 h after i.n. NP application. *Ex vivo* scans

confirmed our *in vivo* data by displaying about 3.5 fold higher fluorescence intensities in AAI lungs than in control lungs (Figure 3B and D).

Itrybe-NPs are phagocytized by bronchial and alveolar macrophages

To analyze the location of the Itrybe-NP derived signals on a cellular level and thereby identify the cells that phagocytize the NPs *in vivo*, cytopins from BALs as well as frozen lung tissue sections were immunofluorescently stained with a fluorophore-labeled antibody against the widely used pan-macrophage marker CD68. Cytopins of BALs from lungs explanted 24 h after NP application demonstrated that Itrybe-NPs were taken up by CD68-positive (CD68⁺) cells in both AAI and control mice (Figure 4A) but not by any other cells, such as eosinophils, which are easily distinguished by their fragmented or donut shaped nucleus (Figure 4A, left panel).

Immunofluorescence microscopy of cryosections from entire lungs explanted 24 h after NP instillation also showed Itrybe-NPs in CD68⁺ immune cells in both AAI and control tissue (Figure 4B). As shown in Figure 4B, Itrybe-NP loaded CD68⁺ cells were predominantly found in alveolar spaces and in the bronchial lumen. In lungs from AAI mice, we never found NPs in CD68⁺ cells located within inducible bronchus-associated lymphoid tissue (iBALT) - an ectopic lymphoid tissue formed in the lung after pulmonary infection or inflammation. However, Itrybe NP-filled CD68⁺ cells often clustered around iBALT (Figure 4B), suggesting that these cells are not able to migrate into, but may be recruited by iBALT.

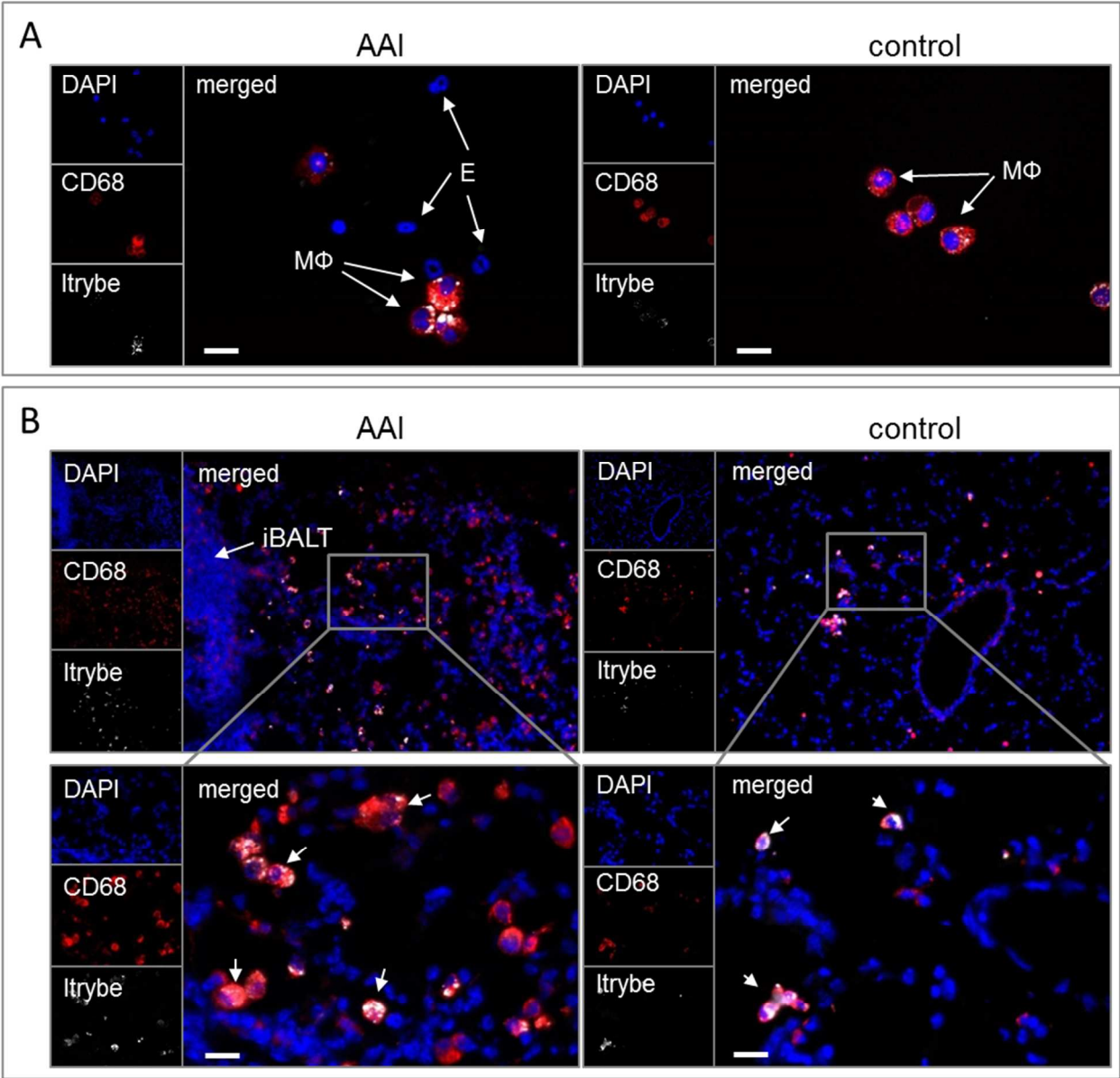


Figure 4. Itrybe-NPs are taken up by CD68⁺ cells. Anti-CD68 immunostaining of cytopspins from BAL 24 h after i.n. Itrybe-NP instillation demonstrates uptake of NPs by CD68⁺ cells in both AAI (A, left panel) and control (A, right panel) mice. Anti-CD68 immunostaining of cryosections from lungs of AAI (B, upper panel) and control mice (B, lower panel) shows a higher amount of Itrybe-NP containing CD68⁺ cells (arrows) in AAI mice compared to controls as well as clustering of Itrybe-NP containing CD68⁺ around iBALT in AAI. Note, that Itrybe-positive cells are never found in iBALT. Lower panels in B show magnifications of the selected areas in the upper panels. CD68 staining is shown in red, Itrybe-NPs in white. DAPI (blue) was used to stain cell nuclei in all images. MΦ: macrophages; E: eosinophils.

The total number of CD68- and Itrybe-NP-positive cells was obtained from merged, overlapping z-stacks, acquired with a confocal microscope and covering a representative area of $5.0 \times 2.8 \times 0.032 \text{ mm}^3$ (Figure 5A). Specificity of the detected Itrybe-NP signals was insured by measuring the emission spectrum in several Itrybe-NP containing ROIs and comparing it with the emission spectra of ROIs devoid of Itrybe-NPs and with the emission spectrum of the pure Itrybe-NP solution (Supplementary Figures 4 and 5). The emission maximum of Itrybe-NPs on tissue slices (located at about 710 nm) measured with the confocal microscope appears to be blue shifted compared to that measured *in vitro* (760 nm) with a calibrated spectrofluorometer (Figure 2C), which has been corrected for the wavelength-dependent spectral responsivity of the instrument.⁴⁰ This apparent spectral deviation is ascribed to the wavelength-dependent spectral responsivity of the confocal microscope originating from the detector and the transmission of other optical components in the detection channel, which distorts the measured spectrum on tissue slices in an instrument-specific manner and accounts for the apparent blue shift in emission.

One tiled image of each 6 AAI and 6 control lung cryosections was analyzed. In AAI lungs, we detected 4 times as many Itrybe-NP-containing CD68⁺ cells than in controls (Figure 5B). This difference clearly accounts for the increased fluorescence intensities detected by *in vivo* imaging in AAI lungs. As seen in Figure 5C, the number of Itrybe-NPs - containing cells form 2 clusters that correspond to AAI (red) and control (blue) mice, based on the fact that controls contain a lower total number of CD68⁺ cells when compared to AAI mice and thus have a lower amount of Itrybe-NP containing CD68⁺ cells. Stitched images of lung sections also revealed that in AAI lungs Itrybe-NPs accumulate more often in clusters around bronchi and blood vessels when compared to control lungs, where they were more evenly spread (Figure 5A).

Although macrophages in lung cryosections from AAI mice often appeared larger and consequently filled with more NPs when compared to controls, we could not find any significant difference in NP-load by quantification of mean fluorescence intensities in CD68/NP-colocalized cells (data not shown).

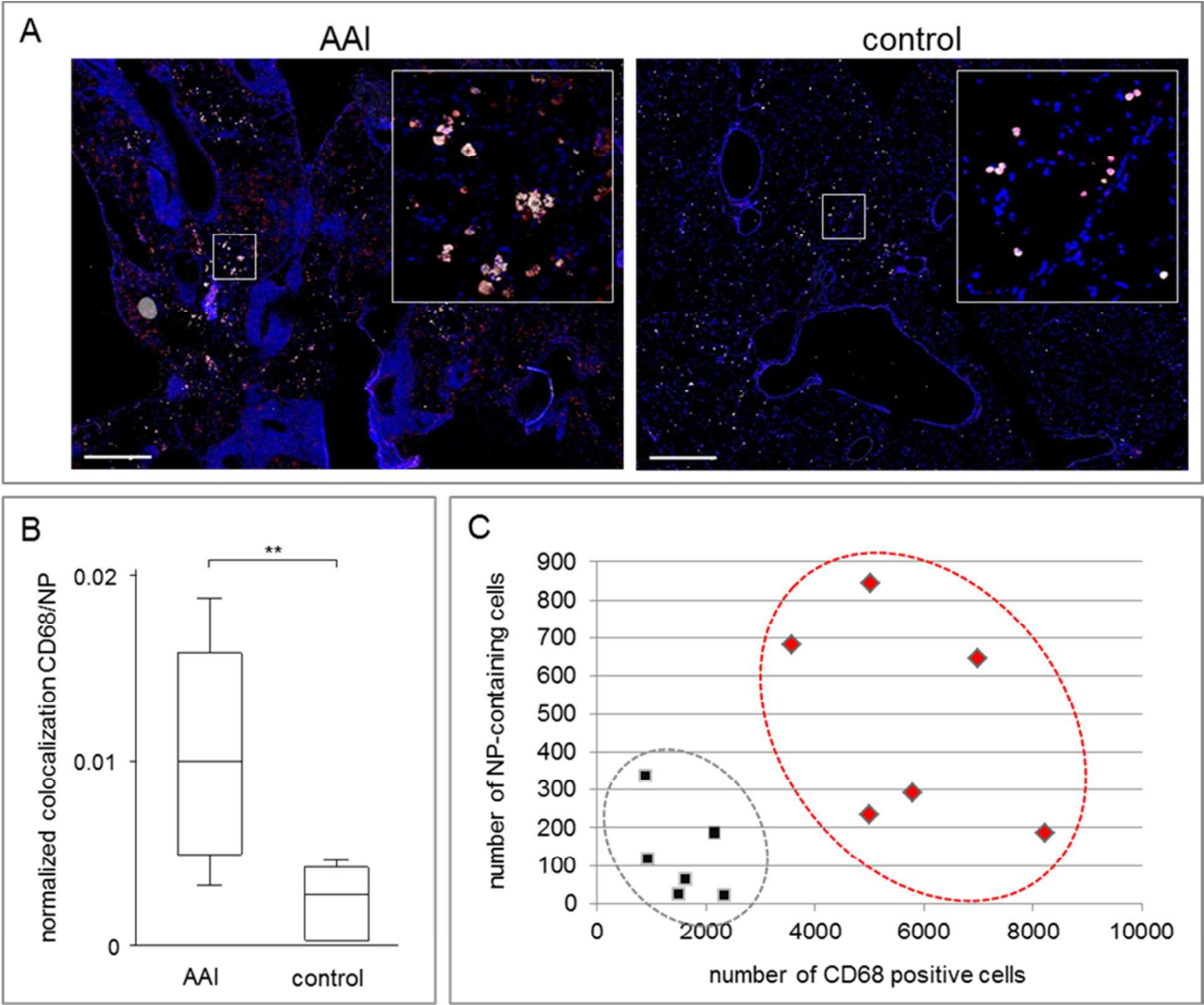


Figure 5. Distribution of Itrybe-NP-loaded CD68⁺ cells. (A) Representative stitched image of 12x9 tiles (456x456x32 μm each) selected in the center of an AAI and a control lung cryosection stained with anti-CD68 antibody showing an overview of Itrybe-NP distribution within the lung. (B) AAI lungs show a 4 times higher number of NP⁺/CD68⁺ cells than controls. Shown are colocalized CD68⁺/NP⁺ cells normalized to total nuclei. (C) The number of Itrybe-NP containing cells cluster for AAI (red) and controls (blue), as seen in the scatter plot of the total number of cells with colocalization of NP/CD68 from one tiled image each of 6 AAI and 6 control cryosections. DAPI was used to stain nuclei in all images. Scale bars in A represent 500 μm . Data were analyzed by Welch t-test and ** in B represents $p < 0.05$

Because reactivity of anti-CD68 antibodies has been reported with fibroblasts, activated endothelial cells, as well as dendritic cells (DCs)^{41,42}, we double-stained lung cryosections with anti-CD68 and anti-CD11c antibodies, as well as anti-MHCII antibodies, which are both highly expressed on DCs. As seen in Figure 6A and 6B, Itrybe-NPs were only found in CD68⁺CD11c⁺MHCII^{low} cells, suggesting that mainly macrophages and not DCs take up NPs. A low expression of MHCII on Itrybe-NPs positive cells also suggests that Itrybe-NPs are preferentially taken up by alternatively activated (M2) macrophages rather than by classically activated macrophages (M1), which tend to express higher MHCII. This was further confirmed by double-staining of lung cryosections with anti-CD68 and an antibody against ECF-L, a lectin primarily expressed on M2 macrophages. Figure 6C representatively shows that all Itrybe-NPs visualized by fluorescence microscopy were found in CD68⁺ECF-L⁺ cells, suggesting that NPs are predominantly taken up by alternatively activated M2 macrophages. In summary, the immunofluorescence staining suggests that Itrybe-NPs were predominantly phagocytized by CD68⁺CD11c⁺MHCII^{low}ECF-L⁺ cells, most likely representing bronchial and alveolar M2 macrophages.

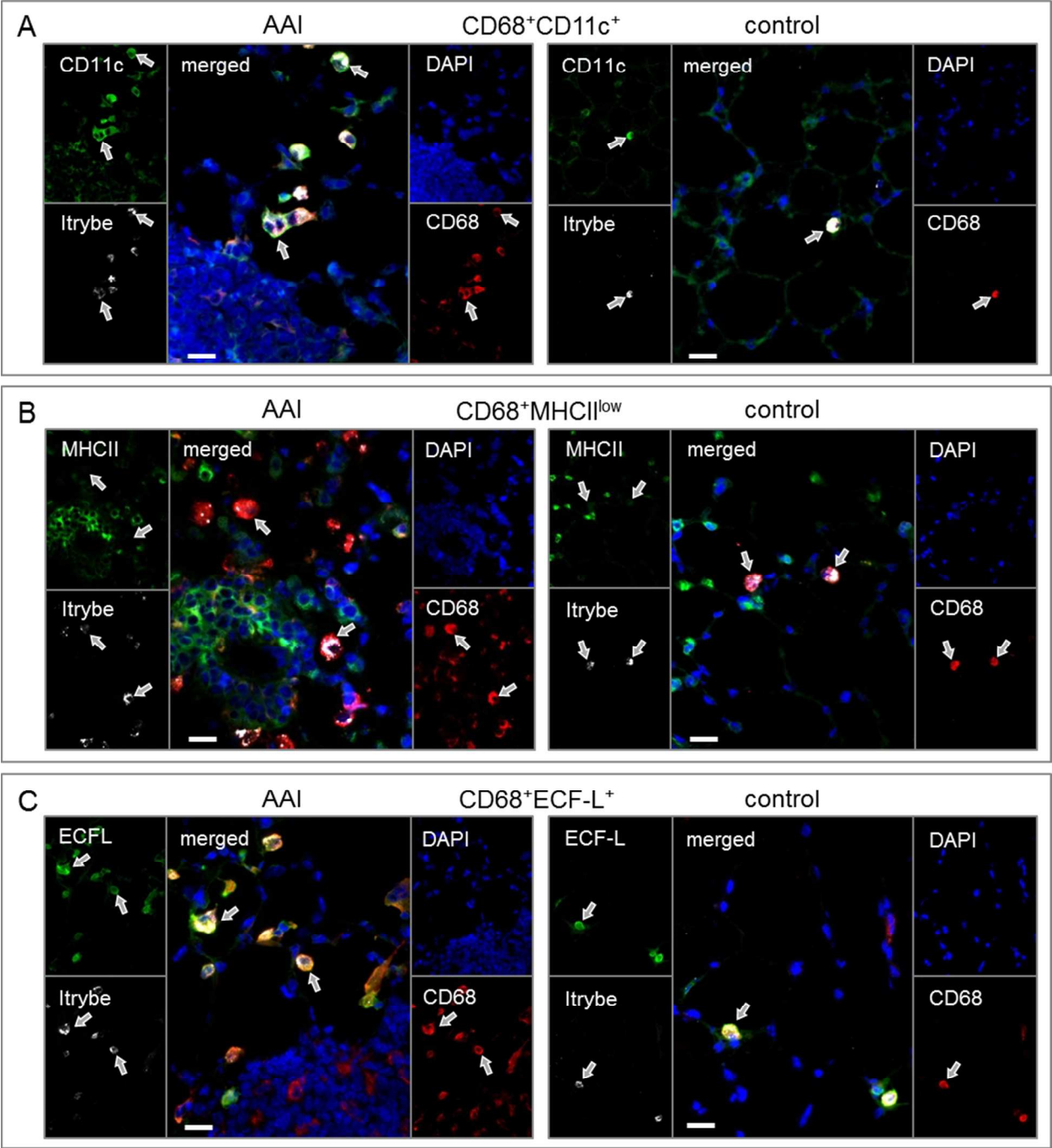


Figure 6. Itrybe-NPs are phagocytized by CD68⁺CD11c⁺ECF-L⁺MHCII^{low} M2 macrophages. Immunofluorescence microscopy of co-staining of AAI lung cryosections, 24 h after Itrybe-NP application with antibodies directed against CD68 and CD11c (A), MHCII (B) and ECF-L (C). Itrybe-NPs are shown in white; expression of CD68 is shown in red. Expression of CD11c, MHCII and ECF-L are shown in green. DAPI (blue) was used to stain nuclei in all images. Scale bars represent 20 μm.

The possible uptake of Itrybe-NPs by lung epithelial cells was also examined by immunofluorescence studies with antibodies directed against the transmembrane protein podoplanin, a heavily O-glycosylated protein, present on alveolar epithelial type I cells (AT1) as well as prosurfactant protein C (proSP-C), a marker for alveolar type II cells (AT2).⁴³ While we never found any Itrybe-NPs in proSP-C⁺ AT2 cells, we observed the location of some Itrybe-NPs in the cytoplasm of podoplanin⁺ AT1 cells (Figure 7).

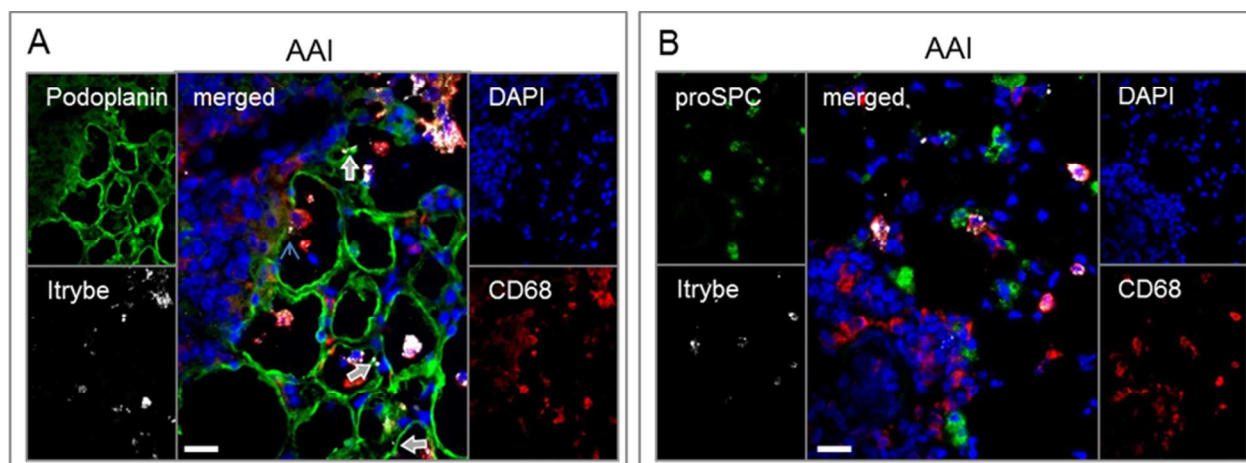


Figure 7. Occasional uptake of Itrybe-NPs by epithelial type I pulmonary cells. Co-staining of AAI lung cryosections with antibodies directed against CD68 and podoplanin (A) or CD68 and proSPC (B) 24 h after i.n. Itrybe-NP application. Few Itrybe-NPs were found in some podoplanin⁺ AT1 cells (A, arrows), but never in proSPC⁺ AT2 cells. CD68 expression is shown in red; podoplanin and proSPC expression are in green and Itrybe-NPs are shown in white. DAPI (blue) was used to stain nuclei in all images. Scale bars represent 20 μm.

To assess if the pattern of Itrybe-NP uptake is different at an earlier or later time point, we performed anti-CD68 immunostaining with cryosections of lungs explanted 5 h, 48 h or 72 h after i.n. Itrybe-NP instillation. Similar to the 24 h time point, we found Itrybe-NPs in CD68⁺ cells, in both AAI and control lungs. In contrast to findings obtained 24 h after Itrybe-NP instillation, at 5 h many free Itrybe-NPs were still seen in alveoli and bronchioles, representing NPs which were not or not yet phagocytized (Figure 8). At 48 h and 72 h after Itrybe-NP

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application, the pattern of NP-distribution was comparable to that obtained after 24 h (Figure 8), with most Itrybe-NPs phagocytized by CD68⁺CD11c⁺MHCII^{low}ECF-L⁺ alveolar M2 macrophages.

We also tested by immunofluorescence whether NP-containing cells can be found in lung draining lymph nodes 24 h, 48 h or 72 h post NP application. However, we could not detect any Itrybe-specific signals at any time point analyzed in either AAI or control lymph node cryosections (data not shown).

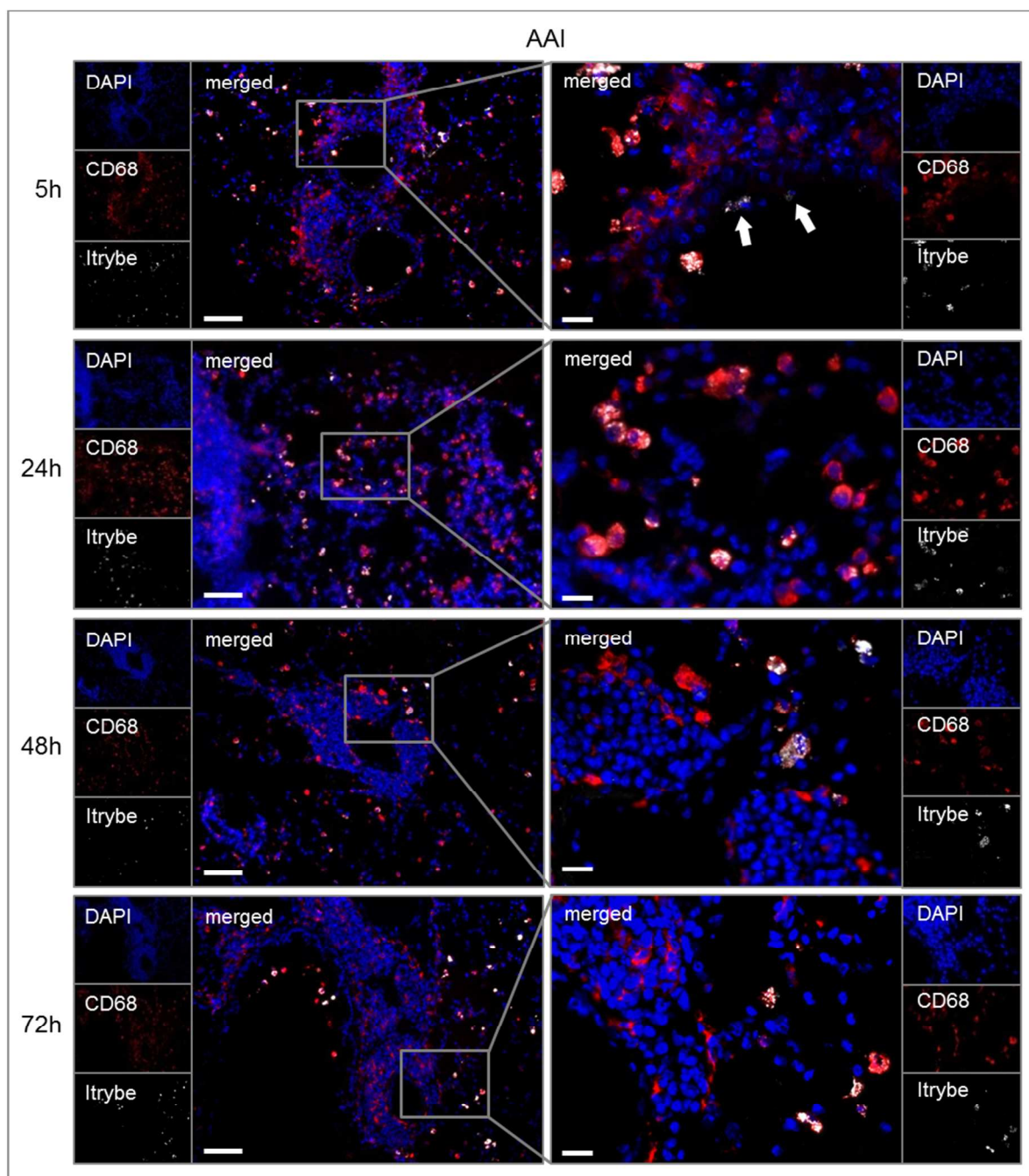


Figure 8. Itrybe-NP uptake by CD68⁺ cells in dependence of time. Immunostaining of AAI cryosections with anti-CD68 antibody. Five h after i.n. Itrybe-NP application, many NPs are within alveoli and bronchioles, where they have not yet been phagocytosed by cells (inset, white arrows). Forty eight h and 72 h after i.n. NP application show a similar pattern of Itrybe-NP localization as 24 h with all NPs taken up by AMs. CD68 expression is shown in red; Itrybe-NPs are in white. DAPI (blue) was used to stain nuclei. Scale bars represent 100 μm (left panel) or 20 μm (right panel).

Characterization of Itrybe-NP containing cells by flow cytometry

To further characterize the cells that take up Itrybe-NPs we performed *ex vivo* flow cytometry with BAL, lung and tracheobronchial lymph node cells from control (n=4) and AAI animals (n=6) which had received Itrybe-NPs i.n. 24h before. To assess the background fluorescence of Itrybe-NPs and define cells positive for Itrybe-NPs, samples of an untreated mouse were included in this assay. To exclude debris and identify single cells we used the FSC-A and SSC-A plots and SSC-W against SSC-A. Subsequently, we identified leukocytes as CD45⁺ cells. We noted the expected increase in CD45⁺ leukocytes among all cells in AAI lungs (33.3±4.0%) as compared to controls (23.4±7.7%, P=0.05, U test) and in AAI BAL (66.6±2.1%) compared to controls (29.3±11.1%, P=0.0061, t test).

For macrophage/monocyte detection we then gated F4/80⁺CD68⁺ cells and determined CD11c⁺ and CD11b⁺ expression (Supplementary Figure 7). As expected, nearly all leukocytes in BAL controls were F4/80⁺CD68⁺ macrophages (95%) but in BAL from AAI mice only 43% were macrophages (Figure 9A). The majority of these cells contained Itrybe-NPs (74% and 55% respectively) (Figure 9B). Among lung cells the percentage of macrophages was higher in AAI than in controls accounting for a higher total number of macrophages in AAI lungs. This result is in agreement with the higher fluorescence signal that we measured *in vivo*. In both control and AAI lung cells the majority of macrophages contained NPs (86% and 54%, respectively) (Figure 9B). However, there was a higher percentage of NP-containing macrophages in control lungs when compared to AAI and significantly higher mean fluorescence intensities (MFI) in control macrophages than in AAI macrophages (Figure 9C). Hence, either the number of Itrybe particles was limited and insufficient to “feed” all the AAI macrophages or AAI macrophages were impaired in their phagocytic capability.

We found that in control BAL and lung cells the majority of $CD45^+F4/80^+CD68^+$ cells were $CD11b^{low}$ and $CD11c^{high}$ (about 94% in BAL and 76% in lung cells) (Figure 9D), suggesting that these were AMs, and more than 70% of these cells contained Itrybe-NPs (Figure 9E). In AAI BAL and among lung cells the proportion of $CD45^+F4/80^+CD68^+CD11b^{high}CD11c^{low}$ macrophages increased (57% in BAL and 74% in lungs) (Figure 9G), which may represent mainly interstitial macrophages (IMs). The $CD45^+F4/80^+CD68^+CD11b^{high}CD11c^-$ cells may represent mainly monocytes. When we gated for $CD45^+F4/80^+CD68^+CD11b^{high}$ and $CD45^+F4/80^+CD68^+CD11c^{high}$ populations we found that both populations contained NPs (Figure 9E and H). The MFI of NPs was higher in control macrophages from both populations (Figure 9F and I). $CD45^+F4/80^+CD68^+CD11c^{high}$ cells had the highest MFI values for NPs (Figure 9F). Cells with this expression profile were identified as AMs earlier⁴² and were therefore the most efficient phagocytes or had the best access to the NPs.

In lymph nodes of both AAI and controls the percentage of NP-containing $CD45^+F4/80^+CD68^+CD11c^+CD11b^+$ cells was very low (0.5-2%) and of these only 3-4% contained NPs, which was in the range of background when compared to cells from mice not exposed to NPs. About 9% of $CD45^+F4/80^-CD68^-$ also showed Itrybe-NP fluorescence. However, the MFI of Itrybe-NPs in $CD45^+F4/80^-CD68^-$ cells was very low (data not shown), suggesting that the unspecific binding of Itrybe-NP to other leukocytes is very minor. Moreover, all lymph node cells showed very low MFIs for Itrybe-NPs, which was in the range of background (Figure 9 C, F, I).

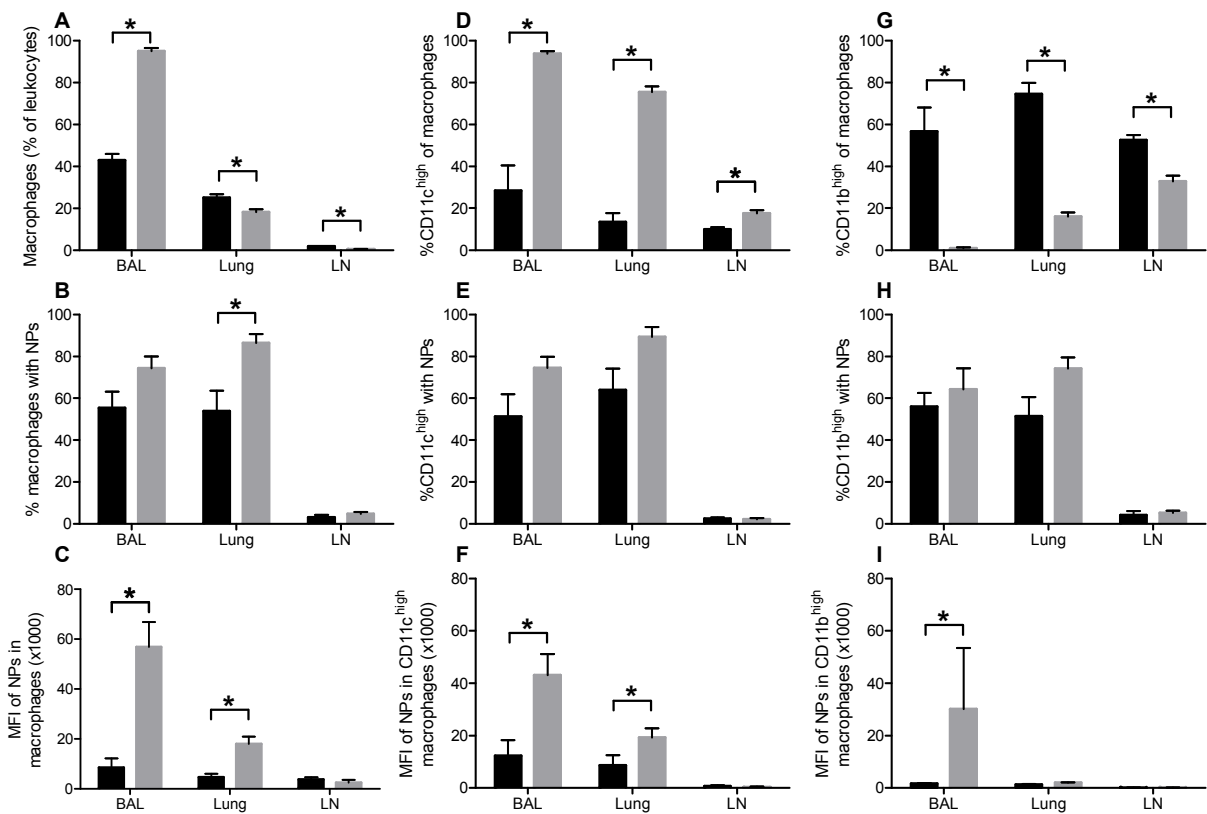


Figure 9. Itrybe-NP uptake by macrophages. Percentage of macrophages (A, D, G), percentage of their corresponding NP uptake (B, E, H) and MFI of NPs (C, F, I) in single cells from BAL, lung and lymph nodes (LN) as identified by flow cytometry. Leukocytes were defined as CD45⁺ cells. Macrophages were defined as CD45⁺F4/80⁺CD68⁺CD11b⁺CD11c⁺. Black bars = AAI, grey bars = controls. Data represent mean ± SEM of 6 mice for AAI and 4 mice for controls. Data were analyzed by U test and significant differences (P≤0.05) are indicated by *.

To determine the uptake of NPs by DCs, we gated for CD45⁺CD11c^{high}MHCII^{high}CD68⁺ cells (Supplementary Figure 7). As expected we found low numbers of DCs in BAL, and 65% of these contained NPs (Figure 10A and B). One to 3% of viable CD45⁺ cells from both control and AAI lungs were CD11c^{high}MHCII^{high}CD68⁺ DCs (Figure 10A), which to a large part were able to take up NPs (68% in AAI and 85% in controls) (Figure 10B). About 50-75% of NP-containing CD11c^{high}MHCII^{high}CD68⁺ DCs were also CD103⁺ (data not shown), further suggesting that at

least subsets of DCs can take up NPs, although they constitute overall a minor proportion of Itrybe-NP loaded cells. The higher MFI for NPs in BAL controls than in BAL AAI suggests that either control DCs take up more NPs or that less NPs are available per DC in AAI (Figure 10C). Among tracheobronchial lymph node cells less than 1% of $CD45^+$ cells were $CD11c^{high}MHCII^{high}CD68^+$ DCs in both AAI and controls and only 1-3% of these cells contained NPs (Figure 10B). However, these frequencies were similar to the rare unspecific events in our untreated control mouse (data not shown) and can therefore not certainly be considered as positive event. This result confirms our findings with immunofluorescence microscopy and suggests that Itrybe-NPs may not be transported to lung draining lymph nodes, at least within the first 24 hrs after NP application.

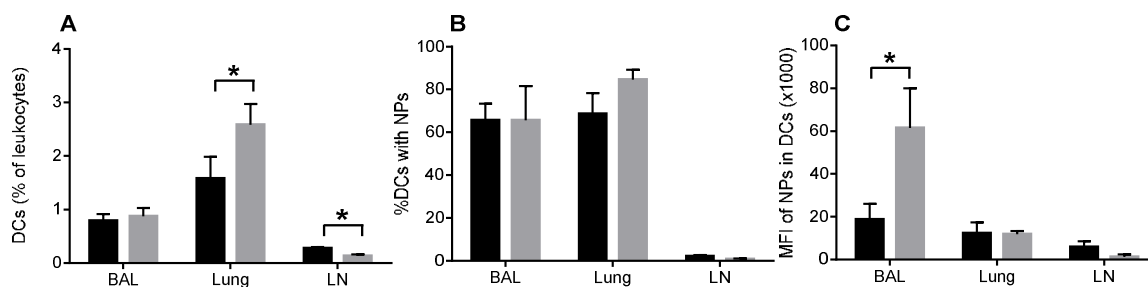


Figure 10. Itrybe-NP uptake by DCs. Percentage of DCs (A), percentage of their corresponding NP uptake (B) and MFI of NPs (C) in single cells from BAL, lung and lymph nodes (LN) as identified by flow cytometry. Leukocytes were defined as $CD45^+$ cells. DCs were defined as $CD45^+ CD11c^{high}MHCII^{high}CD68^+$. Black bars = AAI, grey bars = controls. Data represent mean $\pm$ SEM of 6 mice for AAI and 4 mice for controls. Data were analyzed by U test and significant differences ($P \leq 0.05$) are indicated by *.

Discussion

To our knowledge this is the first application of NIRF-loaded NPs for the *in vivo* detection of inflammatory response by FRI in a conventional OVA-induced allergic airway inflammation

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3 mouse model (AAI). We could demonstrate that uncomplicated i.n. application of non-toxic
4 Itrybe-NPs was sufficient to distinguish between AAI and control mice, showing that the
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6 measured fluorescence intensities over the lung area were significantly higher than those from
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8 controls.
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13 Molecular imaging of lung diseases and in particular AAI using fluorescence imaging has been
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15 very limited up to now. Three *in vivo* approaches have been reported with NIRF labeled probes
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17 so far where higher fluorescence intensities were observed in AAI versus controls for OVA-
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19 induced allergic asthma models: one targeting eosinophilia by using a NIRF-labeled anti-SiglecF
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21 antibody ⁴⁴ and the others monitoring the increase of typical inflammation markers such as
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23 matrix metalloproteinases ⁴⁵ or P-Selectins.⁴⁶ All of these approaches used NIRF-labeled probes,
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25 as NIRF poses a number of advantages for FRI, such as high signal-to-background ratio and high
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27 tissue penetration. Our own approach also exploits these advantages of NIRF by utilizing NPs
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29 loaded with the NIRF dye Itrybe. In addition, we established AAI in hairless and
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31 immunocompetent SKH-1 mice and demonstrated that these mice respond equally well to the
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33 induction of allergic inflammation as commonly used mouse strains, such as BALB/c, by
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35 developing a pronounced eosinophilia, goblet cell hyperplasia, mucus production and iBALT in
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37 their lungs. These mice also displayed typical features of a Th2 allergic response such as
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39 elevated OVA-specific IgE and IgG1 serum levels as well as significantly higher IL-4, IL-5, IL-
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41 10 and IL-13 cytokine expression than controls. The use of hairless SKH-1 mice allows a further
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43 improvement of fluorescence detection owing to the reduction of light scattering and absorbance
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45 due to the lack of fur.
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55 In addition, our intranasal application of the NP probe, presents the most direct route to lung
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57 tissue. Intravenously applied NPs tend to be rapidly cleared by the liver or kidneys, depending on
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the size, or accumulate in the spleen and lymph nodes, as these organs contain a high number of macrophages.⁴⁷ While some animals displayed fluorescence signals in the stomach due to swallowing of the probe, we did not detect any NP-derived signals in the liver, kidney, spleen or lymph nodes. This underlines the high potential of intranasally applied Itrybe-NPs for non-invasively distinguishing inflamed from healthy lungs. Hence, this NP-based approach in general, and our Itrybe-NPs specifically, present a promising tool for the *in vivo* monitoring of macrophages in allergic lung pathologies. In our imaging strategy, we found that macrophages readily capture and retain our NIRF-probe in the lung, as they represent one of the innate immune cells which significantly increase in numbers upon airway inflammation. NPs have been used to image macrophages before, for example using iron oxide or gadolinium particles in combination with MRI. In this way, inflammatory processes and the related macrophage infiltration were detected in atherosclerosis, myocardial infarction, stroke, multiple sclerosis, rheumatoid arthritis and kidney transplantation in small animal models.⁴⁸ Nanomaterial-based contrast agents for computer tomography (CT) have also been used for tracking macrophages. However, X-rays require relatively high concentrations of NPs to delineate individual cells.⁴⁹ Even NP-loaded macrophages themselves have been used as contrast agents. For example Madsen *et al* (2013) used MRI to detect the migration of NP-loaded exogenous macrophages into the brain.⁵⁰ In a previous study we tracked intratracheally applied barium sulfate-loaded macrophages in the lung of mice with AAI by using synchrotron radiation inline free propagation phase contrast CT.⁵¹ Imaging of endogenous pulmonary macrophages has to date only been shown with gold-NPs⁵² and superparamagnetic iron oxide NPs (SPIOs).⁵³ To the best of our knowledge our study is the first to use fluorescently labeled NPs to track pulmonary macrophages. Because macrophages increase in number during the process of AAI and are the

main phagocytic cell in the lung, we explored the source of the increased *in vivo* signal which we detected in lungs of AAI mice by undertaking an *ex vivo* characterization of the uptake of Itrybe NPs. Our tiling experiments confirmed the *in vivo* results by demonstrating a higher number of Itrybe-NP-containing AMs in AAI than in controls. It also enabled a semi-quantitative calculation of the fluorescence intensity in AAI as compared to controls, which may be used as an indication of the percentage of NPs that can reach the different parts of the lung. Moreover, our results revealed that Itrybe-NP-containing macrophages cluster around bronchi and blood vessels in AAI, whereas there was a more homogenous distribution in controls. Future experiments may involve spectrally resolved, 3-dimensional, overlapping, confocal scans of the entire lung tissue sections, which would allow for absolute measurements of amount of NP present, as well as their localization within specific cell organelles or lung area.

As we demonstrate by immunofluorescence, Itrybe-NPs were readily taken up by AMs, but flow cytometry analysis suggested the uptake also by DCs and interstitial macrophages. We cannot exclude the possibility that some of the macrophages and DCs share common staining characteristics and/or phagocytic capacity. In accordance with the study by Geiser *et al*⁵², Itrybe NPs were taken up by macrophages of both diseased and healthy animals. The role of the two major types of AMs, M1 (classically activated) and M2 (alternatively activated) macrophages, in human allergic asthma as well as mouse AAI, are complex and controversial. It has been proposed that M1 are Th1-driven, while M2 cells are induced by and can drive Th2 responses, but increasing evidence suggests the parallel development and involvement of both these cell types in asthma.²⁸ It seems that while M1 macrophages may be beneficial to prevent allergic sensitization, in already established asthma they may promote the development of M2 macrophages.⁵⁴ M2 macrophages are associated with asthma, but it is still unclear if they

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3 actively contribute to asthma pathogenesis.⁵⁵ In our immunofluorescence experiments, we
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5 detected NPs in M2 cells, regardless of disease status. We therefore assume that the uptake of
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7 NPs per se by M2 cells is not related or dependent on the presence of AAI, in accordance with
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9 the fact that these cells are involved in phagocytosis of foreign pathogens and apoptotic cells.
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11 However, by flow cytometry we found higher mean fluorescence intensities of NPs in control
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13 macrophages than in those from AAI mice, suggesting that control macrophages may be better
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15 phagocytes than those of AAI mice or that less NPs were available per macrophage in AAI. A
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17 systematic analysis of the uptake and processing of Itrybe-NPs in relation to disease status and
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19 the effect NPs may have on the exacerbation of AAI is in preparation and will provide important
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21 information about the role AMs play in the relationship between AAI and inhaled NPs.
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28 We further found that NP-filled AMs often clustered around iBALT. This could indicate that
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30 cells accumulated in iBALT, in particular B-cells, positively influence the activation and
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32 recruitment of macrophages, a mechanism that has recently been suggested for B-cells in iBALT
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34 of cigarette smoke – induced COPD.⁵⁶ We also detected small amounts of Itrybe-NPs in AT1
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36 cells, the structural cells of the lung which are responsible for gas exchange. These findings
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38 confirm former *in vitro* and *in vivo* studies that epithelial cells are capable of taking up NPs.^{57,58}
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43 As NPs are increasingly considered as targeted drug delivery systems,⁵⁹ their characterization
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45 and rigorous toxicological examination is of growing importance for clinical application. This
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47 includes establishing their fate and within this, the kinetics of the uptake, distribution and
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49 dispersal. Transport via macrophages and DCs is clearly involved in the latter and uptake of NPs
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51 by macrophages has been recently exploited for novel nanotherapeutics.⁶⁰ Identification of
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53 location in tissues and cells then assists in the direction of toxicology focusing on the potential
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55 toxic effects on tissues and cell compartments of accumulation. Macrophages and DCs can
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traffic from the lungs to other organs and examination of their capacity to deliver NPs between sites is therefore of value in assessing NP kinetics. Nanoparticles may not be inert passengers within immune cells, but may have significant effects on their function and even influence their longevity. Interestingly, we detected only a negligible amount of Itrybe-NPs in lung draining lymph nodes and therefore found no evidence of NP-filled macrophage or DC trafficking to lymph nodes, at least within the first 24 h after application of NPs. A possible explanation for this may be that NPs impede migration of both macrophages, but at least DCs to lymph nodes. It is also possible that the less phagocytic DCs migrate to the lymph nodes and the more phagocytic DCs, which are more akin to macrophages stay behind in the lung.

Evidence of such influence would be important for the interpretation of findings obtained from research of biological systems in which NPs are used as research tools. In this regard, Itrybe-NPs in combination with *in vivo* optical imaging and high resolution fluorescence microscopy represent a powerful tool to trace macrophages in the entire lung and provide new information of the fate of inhaled NPs. In addition, the biological stability which is provided by the encapsulation of the Itrybe dye grants an important precondition for the tracking of nanomaterial within living systems.

Conclusion

Our results show that 100 nm Itrybe-NPs are a highly suitable tool for distinguishing between healthy mice and mice with allergic lung inflammation *in vivo* and, subsequently, localizing NP-loaded immune cells by *ex-vivo* NIRF microscopy. Our approach may facilitate a broader

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3 understanding of the cellular and molecular events in lung disease and may be used as a model to
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5 assess nanotherapeutics.
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10 11 **Materials and Methods**

12 13 14 *Materials and cells*

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18 100 nm carboxy-modified polystyrene particles were purchased from Kisker Biotech GmbH and
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20 stained with Itrybe using a previously described staining procedure.³⁹ All particles were
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22 ultrasonically treated prior to use. Itrybe was obtained from Otava Ltd. and used as received.
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26 For *in vitro* experiments, 5×10^5 cells of the immortalized and adherent mouse alveolar cell line
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28 MH-S (CRL-2019, ATCC)⁶¹ were grown on 25 mm Poly-L-Lysin coated coverslips in complete
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30 RPMI medium, supplemented with 10% FCS and 0.05 mM 2-mercaptoethanol. MH-S cells were
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32 incubated for 24 h with 100 ng/ml of Itrybe-NPs either at 37°C or at 4°C, washed twice with
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34 phosphate-buffered saline (PBS) and then fixed for 10 min at RT with 4% paraformaldehyde
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36 (PFA) before counterstaining the nuclei with 4 µg/ml DAPI and visualization using an Axiovert
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38 200 M inverted microscope (Carl Zeiss Microscopy GmbH).
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43 44 *Nanoparticle characterization*

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47 The size distribution was analyzed by NTA using a NanoSight LM14 equipped with a 405 nm
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49 laser diode and scientific CMOS camera from Malvern Instruments GmbH. The aqueous
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51 suspensions containing about 5×10^8 particles/ml were measured 5 times for 60 s and analyzed
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53 with the NanoSight NTA 2.3 Analytical Software Suite.
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For the characterization of the morphology, Itrybe-NPs were dried on a silica substrate and imaged with an Agilent 5500 atomic force microscope (AFM) in the tapping mode using soft tapping cantilevers with a spring constant of nominally 5 N/m and a tip radius of <10 nm (Tap150Al-G from BudgetSensors).

Absorption measurements of 0.1 w% particle suspensions were carried out with a Cary 5000 spectrophotometer from Varian Inc. Fluorescence measurements were performed with a calibrated Spectronics Instruments 8100 spectrofluorometer (Aminco Bowmann) equipped with Glan Thompson polarizers. The excitation polarizer was set to 0° and the emission polarizer to 54.7°. The resulting emission spectra were corrected for the spectral responsivity of the fluorometer's emission channel.

Animals

Pathogen free female immunocompetent and hairless SKH-1mice (CrI: SKH1-*Hr^{hr}*), 6-8 weeks of age, were purchased from Charles River Laboratories Inc. All animals were housed in a controlled environment at a regular 12-hour dark:light cycle, at 22°C and were fed laboratory chow and tap water ad libitum. Seven days prior to imaging experiments the standard food was replaced by chlorophyll free chow (Scientific Animal Food & Engineering) to reduce autofluorescence from the stomach and gut of the animals. All animal experiments were performed in accordance with German animal ethics regulations and were approved by the local ethics office (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, LAVES, ethics approval no. 33.12-42502-04-12/0834).

Induction of AAI

On days 0 and 14 mice were immunized via intraperitoneal (i.p.) injection with 50 μg OVA (Hyglos) and 0.5% aluminium hydroxide gel adjuvant (Invivogen) in a volume of 0.2 ml PBS, as well as intranasally (i.n.) with 50 μg OVA in 25 μl of PBS. On days 28, 29, 30 and 33 post-immunization, mice were challenged i.n. with 250 μg OVA in 25 μl PBS to induce an acute reaction. Control mice received PBS instead of OVA for all described steps except for the last two challenges on days 30 and 33, where they received 250 μg OVA in 25 μl PBS. Itrybe-NPs were given i.n. one day post challenges and mice were scanned over a given period of time by optical imaging. All i.n. applications were performed under short 2% isoflurane, 2 l/min oxygen anesthesia. Mice were sacrificed by CO_2 overdose after the last scan.

In vivo fluorescence reflectance imaging (FRI)

Two-dimensional *in vivo* FRI was performed with an Optix MX2 System (ART), which comprises an interface for inhalation anesthetics and 4 pulsed laser diodes (635, 670, 730 and 785 nm) as described before.⁶² The Optix MX2 system works in a raster mode, illuminating each raster point with a multitude of short laser pulses (<150 ps, repetition rate 8 MHz). For measurement of the emitted fluorescence light a single photon counting detector is used that, being triggered with each excitation pulse, records the amount of detected fluorescence photons and the time delay for the first detection event. From these values a time-of-flight histogram is created for each raster point (temporal point spread function, TPSF) which contains information about fluorescence intensity, concentration and depth of the fluorophore in the analyzed subject as well as the fluorescence lifetime. The lifetime is an additional parameter characterizing the fluorophore, which can be used to discriminate between probe specific signals and unwanted fluorescence derived from autofluorescence. Raster point separation is possible between 0.5 – 2 mm, and photon collection time per raster point can be modified (integration time). Whereas the

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3 first allows for a more detailed analysis, increasing the integration time improves the statistics in
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9 During *in vivo* scans, mice were anaesthetized by inhalation with 2% isoflurane, 2l/min oxygen
10 for 15 - 20 min per scan. OVA challenged and control mice were prescanned to assess
11 autofluorescence of the animals. One day post challenges the animals were given 160 µg of 100
12 nm Itrybe-NPs in 30 µl of PBS by i.n. application and were then scanned after 1 h, 5 h and 24 h
13 (n=16 for AAI; n=15 for controls). Itrybe fluorescence was measured using an excitation of 635
14 nm in combination with a 670 nm band-pass emission filter with 40 nm bandwidth. Scans were
15 performed with a 1.5 mm (whole body scans) or 1.0 mm (lung scans) raster, a photon collection
16 time (integration time) of 0.3–1 s per scan point and varying laser power. Intensity data and
17 fluorescence lifetime were analyzed with the OptiView-2-02-00 software (ART).
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31 Fluorescence intensity data are displayed in normalized counts (NC), where the measured
32 fluorescence intensities (counts) were normalized for varying laser power and integration times,
33 allowing for comparison of measurements with different settings. Data were quantified as
34 average fluorescence intensity over a chosen region of interest over the lung and subsequently
35 corrected for autofluorescence by subtracting the average fluorescence intensity from the same
36 region of interest in the respective prescans.
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46 Following imaging, mice were sacrificed and lungs were harvested for *ex vivo* scans and then
47 cryofrozen for later microscopy. Bronchoalveolar lavage (BAL) was performed with some mice
48 before harvesting of the lung.
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55 *Bronchoalveolar lavage*
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Five - 72 h after NP application, mice were sacrificed by CO₂ overdose. BAL was performed by washing the airways gently three times with 500 µl of 2% FCS/PBS after exposing and cannulating the trachea. Volumes were pooled and then washed once in the same buffer. Recovered cells were counted in a haemocytometer and 3×10^4 cells were used for obtaining cytopins with a Shandon Cytospin 4 (Thermo Scientific), followed by Giemsa staining (Sigma Aldrich) for differential cell counting. Where indicated, cytopins were fixed with 1:1 acetone/methanol solution, immunostained and counterstained with DAPI (4 µg/ml) for visualization of nuclei.

Isolation of lung and lymph node cells

To obtain single-cell suspensions lungs were lavaged and digested as described elsewhere⁶³. Briefly, the left lungs were washed with sterile PBS and minced in Petri dishes with RPMI 1640 medium (Gibco). The dissected lung tissue was digested in medium that contained Collagenase Type IV (Sigma-Aldrich; 0,5 mg/ml) and type IV bovine pancreatic DNase (Sigma-Aldrich; 25 U/ml) at 37°C for 45 min. Cells were dispersed with a 10-ml syringe (BD Biosciences) that was fitted with an 18-gauge needle and filtered with a 70 µm cell strainer (BD Falcon). Red blood cells were depleted with RBC lysis buffer (Biolegend) at 37°C for 1 min.

Tracheobronchial lymph nodes were carefully excised, washed in PBS and cells filtered through a 70 µm cell strainer using a syringe plunger.

Flow cytometry

Single cell suspensions were incubated with antibody dilutions according to manufacturer's instructions for 30 min at 4 °C in the dark. The panel of antibodies used, included: CD45-Pacific

Blue (clone 30-F11), CD11c-PE (clone N418), CD68-PerCP Cy5.5 (clone FA-11), F4/80-Brilliant Violet 510 and FITC (clone BM8), CD11b-Brilliant Violet 510 (clone M1/70), CD103-biotin (clone 2E7), all from Biolegend; MHC II (I-A)-FITC (clone NIMR-4, eBioscience). Streptavidin-BUV395 was from BD Biosciences. Isotype controls (rat IgG2a, rat IgG2b, armenian hamster IgG) were purchased from Biolegend. Flow cytometry was performed using a BD LSRII flow cytometer and data were analyzed with FlowJo software (TreeStar).

Immunofluorescence staining

Explanted lungs were cannulated and filled with 600 µl of Tissue Tek OCT compound (Sakura Finetek) and immediately frozen in liquid nitrogen at -80°C. Frozen lung sections of 5 µm or 20 µm from AAI and control mice were obtained by cutting the lungs with a Jung Frigocut 2800E cryostat microtome (Leica Microsystems).

For NIRF microscopy, all sections were stained with DAPI (4 µg/ml) for visualization of nuclei. For detection of macrophages 5 µm cryosections were fixed with acetone, stained with rat-anti-mouse CD68 antibody (clone FA-11, Abcam,1:300), followed by incubation with biotinylated goat-anti-rat antibody (BioLegend, 1:200) and Streptavidin-Alexa Fluor 555 (Life Technologies, 1:400). CD11c cell expression was assessed with Alexa Fluor 488 conjugated anti-mouse CD11c (clone N418, BioLegend, 1:200) and MHCII expression with Alexa Fluor 488 conjugated rat-anti-mouse MHCII antibody (clone M5/114.15.2, Biolegend, 1:200). ECF-L was stained with goat anti-mouse ECF-L antibody (R&D, 1:150), followed by donkey anti-goat-Alexa Fluor 488 secondary antibody (Abcam, 1:400). AT1 cells were detected with hamster-anti-mouse podoplanin (Santa Cruz Biotechnology, 1:200), followed by goat anti-hamster-Alexa Fluor 488 secondary antibody (Life Technologies, 1:400). AT2 cells were detected by rabbit anti-mouse

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3 proS-PC antibody (Millipore, 1:2000), followed by goat anti-rabbit Alexa Fluor 488 (Life
4 Technologies, 1:400). Images were acquired with an Axiovert 200 M inverted microscope (Carl
5 Zeiss Microscopy GmbH) equipped with a Xenon lamp and a high sensitivity ORCA-AG digital
6 camera (Hamamatsu). Image generation and processing were performed with the AxioVision
7 Rel.4.6 software (Carl Zeiss Microscopy GmbH) and FIJI (National Institutes of Health,
8 Bethesda, USA).⁶⁴
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12 For quantification of NP-positive macrophages in AAI and control lungs 20 μ m sections were
13 stained with anti-mouse CD68 antibody as described above. Representative 5.0 x 2.8 x 0.032 mm
14 areas of the lung containing nuclei, CD68- and Itrybe-NP-positive cells were acquired by
15 scanning and then merging 108 overlapping z-stacks with a Leica SP5 confocal laser scanning
16 microscope (CLSM, Leica Microsystems) equipped with a 20x immersion objective (HCX PL
17 APO CS, 0.70NA), a motorized stage, a tunable laser (470-670 nm) and hybrid detectors. DAPI,
18 Alexa Fluor 555 and Itrybe-NPs were excited with 405, 555 and 650 nm, respectively, and their
19 individual emissions collected at 420-500 nm, 560-620 nm and 665-795 nm. Imaris 7.7.1
20 (Bitplane) was then used to visualize the fluorescence signal and generate ellipsoid spots for all
21 nuclei, CD68- and Itrybe-positive cells based on the original CLSM multi-channel images.
22 Briefly, three-dimensional objects were generated for each nucleus, CD68 and Itrybe-positive
23 cell and counted. To improve accuracy, each of the signal sources were modeled after an
24 ellipsoid shape, whose major axis was parallel to the z-axis, so as to account for the lower z-axial
25 optical resolution. The size of the ellipsoid model shape varied proportionally with respect to the
26 size of the detected fluorescence cluster. Ellipsoids representing CD68 or Itrybe-NPs were
27 considered to colocalize, when the distance of their center coordinates was less than 0.5 μ m.
28 Further visualization was conducted with the FIJI image processing package, while data analysis
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was done with Microsoft Excel. To account for differences in the total number of cells between AAI and control lungs, CD68⁺/NP⁺ colocalization was normalized to the total cell number measured as DAPI stained nuclei.

Determination of OVA-specific antibodies by ELISA

Sera were obtained 48 h after the last challenge. Serum levels of OVA-specific IgE and IgG1 were measured by ELISA. Briefly, 96-well plates (Nunc) were coated with 100 µg/ml OVA. After addition of serum samples, OVA specific IgE was detected with a commercial kit (Biolegend) according to the manufacturer’s protocol and OVA specific IgG1 was detected with a biotinylated anti-IgG1 antibody (Southern Biotechnology) and amplified with Streptavidin HRP (Southern Biotechnology) as described before.⁶⁵ Optical densities of the samples were measured at 450 nm.

Determination of cytokine mRNA expression by quantitative reverse transcription PCR

Pieces of cryofrozen lung tissue were homogenized with a tissue homogenizer and total RNA extracted with an RNAeasy Universal Plus kit (Qiagen) according to the manufacturer’s protocol. RNA concentrations and purity were measured with a NanoDrop (Thermo Scientific). The SuperScript® First-Strand Synthesis System for RT-PCR (Life Technologies) was used to synthesize first-strand cDNA. Real-time PCR was performed using the QuantiTect SYBR Green PCR Kit (Qiagen) and a LightCycler 480II (Roche Diagnostics). Data were expressed as the calculated ratio of the values obtained for the specific cytokines vs the values of the housekeeping gene HPRT1. Forward and reverse primers for all genes are listed in Supplementary Table 1.

Histology

For demonstration of AAI induction in SKH-1mice, AAI and control lungs were explanted 2 days after the last challenge, fixed in 10% buffered formalin and embedded in paraffin. 3 μ m sections were cut and H&E staining was performed to evaluate the degree of lung infiltrating mononuclear cells. PAS staining was performed with a PAS staining kit according to the manufacturer's protocol (Merck) to show excess mucus production in paraffin sections from AAI mice.

Statistical analysis

Statistical analysis for significant difference of the mean values between two groups was performed with PAST⁶⁶ using a Welch t-test. The non-parametric U test was employed for the analysis of flow cytometry data, which were frequently not normally distributed. P-values ≤ 0.05 were considered significant. Cluster analysis was performed with PAST⁶⁶ and calculated clusters were manually encircled in the scatter plot created by Microsoft Excel. Pearson's Correlation Coefficient was calculated by Microsoft Excel.

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Supplementary Information Available

Supplementary Figure 1

Supplementary Figure 2

Supplementary Figure 3

Supplementary Figure 4

Supplementary Figure 5

Supplementary Figure 6

Supplementary Figure 7

Supplementary Table 1

This material is available free of charge via the Internet at <http://pubs.acs.org>.

References

(1) Landfester, K. Miniemulsions for Nanoparticle Synthesis. In *Colloid Chemistry II*; Antonietti, P. D. M., Ed.; Topics in Current Chemistry; Springer Berlin Heidelberg, **2003**; pp. 75–123.

(2) Sapsford, K. E.; Algar, W. R.; Berti, L.; Gemmill, K. B.; Casey, B. J.; Oh, E.; Stewart, M. H.; Medintz, I. L. Functionalizing Nanoparticles with Biological Molecules: Developing Chemistries That Facilitate Nanotechnology. *Chem. Rev.* **2013**, *113*, 1904–2074.

(3) Mu, Q.; Jiang, G.; Chen, L.; Zhou, H.; Fourches, D.; Tropsha, A.; Yan, B. Chemical Basis of Interactions Between Engineered Nanoparticles and Biological Systems. *Chem. Rev.* **2014**, *114*, 7740–7781.

- (4) Liu, Q.-H.; Liu, J.; Guo, J.-C.; Yan, X.-L.; Wang, D.-H.; Chen, L.; Yan, F.-Y.; Chen, L.-G. Preparation of Polystyrene Fluorescent Microspheres Based on Some Fluorescent Labels. *J. Mater. Chem.* **2009**, *19*, 2018–2025.
- (5) Albanese, A.; Tang, P. S.; Chan, W. C. W. The Effect of Nanoparticle Size, Shape, and Surface Chemistry on Biological Systems. *Annu. Rev. Biomed. Eng.* **2012**, *14*, 1–16.
- (6) Clift, M. J. D.; Rothen-Rutishauser, B.; Brown, D. M.; Duffin, R.; Donaldson, K.; Proudfoot, L.; Guy, K.; Stone, V. The Impact of Different Nanoparticle Surface Chemistry and Size on Uptake and Toxicity in a Murine Macrophage Cell Line. *Toxicol. Appl. Pharmacol.* **2008**, *232*, 418–427.
- (7) Fröhlich, E.; Salar-Behzadi, S. Toxicological Assessment of Inhaled Nanoparticles: Role of *in vivo*, *Ex vivo*, *in Vitro*, and *in Silico* Studies. *Int. J. Mol. Sci.* **2014**, *15*, 4795–4822.
- (8) Fröhlich, E.; Meindl, C.; Roblegg, E.; Griesbacher, A.; Pieber, T. R. Cytotoxicity of Nanoparticles Is Influenced by Size, Proliferation and Embryonic Origin of the Cells Used for Testing. *Nanotoxicology* **2012**, *6*, 424–439.
- (9) Xia, T.; Kovochich, M.; Liong, M.; Zink, J. I.; Nel, A. E. Cationic Polystyrene Nanosphere Toxicity Depends on Cell-Specific Endocytic and Mitochondrial Injury Pathways. *ACS Nano* **2008**, *2*, 85–96.
- (10) Lewinski, N.; Colvin, V.; Drezek, R. Cytotoxicity of Nanoparticles. *Small Weinsh. Bergstr. Ger.* **2008**, *4*, 26–49.
- (11) Jain, R. K.; Stylianopoulos, T. Delivering Nanomedicine to Solid Tumors. *Nat. Rev. Clin. Oncol.* **2010**, *7*, 653–664.
- (12) Aitken, R. J.; Chaudhry, M. Q.; Boxall, A. B. A.; Hull, M. Manufacture and Use of Nanomaterials: Current Status in the UK and Global Trends. *Occup. Med. Oxf. Engl.* **2006**, *56*, 300–306.
- (13) Drescher, D.; Orts-Gil, G.; Laube, G.; Natte, K.; Veh, R. W.; Österle, W.; Kneipp, J. Toxicity of Amorphous Silica Nanoparticles on Eukaryotic Cell Model Is Determined by Particle Agglomeration and Serum Protein Adsorption Effects. *Anal. Bioanal. Chem.* **2011**, *400*, 1367–1373.
- (14) Schipper, M. L.; Iyer, G.; Koh, A. L.; Cheng, Z.; Ebenstein, Y.; Aharoni, A.; Keren, S.; Bentolila, L. A.; Li, J.; Rao, J.; *et al.* Particle Size, Surface Coating, and PEGylation Influence the Biodistribution of Quantum Dots in Living Mice. *Small Weinsh. Bergstr. Ger.* **2009**, *5*, 126–134.
- (15) Bertrand, N.; Wu, J.; Xu, X.; Kamaly, N.; Farokhzad, O. C. Cancer Nanotechnology: The Impact of Passive and Active Targeting in the Era of Modern Cancer Biology. *Adv. Drug Deliv. Rev.* **2014**, *66*, 2–25.
- (16) Daldrup-Link, H. E.; Golovko, D.; Ruffell, B.; Denardo, D. G.; Castaneda, R.; Ansari, C.; Rao, J.; Tikhomirov, G. A.; Wendland, M. F.; Corot, C.; *et al.* MRI of Tumor-Associated Macrophages with Clinically Applicable Iron Oxide Nanoparticles. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **2011**, *17*, 5695–5704.
- (17) Gadde, S.; Even-Or, O.; Kamaly, N.; Hasija, A.; Gagnon, P. G.; Adusumilli, K. H.; Erakovic, A.; Pal, A. K.; Zhang, X.-Q.; Kolishetti, N.; *et al.* Development of Therapeutic Polymeric Nanoparticles for the Resolution of Inflammation. *Adv. Healthc. Mater.* **2014**, *3*, 1448–1456.
- (18) Faraj, A. Al; Shaik, A. S.; Afzal, S.; Sayed, B. Al; Halwani, R. MR Imaging and Targeting of a Specific Alveolar Macrophage Subpopulation in LPS-Induced COPD Animal Model

- Using Antibody-Conjugated Magnetic Nanoparticles. *Int. J. Nanomedicine* **2014**, *9*, 1491–1503.
- (19) Hoffmann, K.; Behnke, T.; Drescher, D.; Kneipp, J.; Resch-Genger, U. Near-Infrared-Emitting Nanoparticles for Lifetime-Based Multiplexed Analysis and Imaging of Living Cells. *ACS Nano* **2013**, *7*, 6674–6684.
- (20) Key, J.; Leary, J. F. Nanoparticles for Multimodal *in vivo* Imaging in Nanomedicine. *Int. J. Nanomedicine* **2014**, *9*, 711–726.
- (21) Hellebust, A.; Richards-Kortum, R. Advances in Molecular Imaging: Targeted Optical Contrast Agents for Cancer Diagnostics. *Nanomed.* **2012**, *7*, 429–445.
- (22) Behnke, T.; Mathejczyk, J. E.; Brehm, R.; Würth, C.; Gomes, F. R.; Dullin, C.; Napp, J.; Alves, F.; Resch-Genger, U. Target-Specific Nanoparticles Containing a Broad Band Emissive NIR Dye for the Sensitive Detection and Characterization of Tumor Development. *Biomaterials* **2013**, *34*, 160–170.
- (23) Clift, M. J. D.; Rothen-Rutishauser, B.; Brown, D. M.; Duffin, R.; Donaldson, K.; Proudfoot, L.; Guy, K.; Stone, V. The Impact of Different Nanoparticle Surface Chemistry and Size on Uptake and Toxicity in a Murine Macrophage Cell Line. *Toxicol. Appl. Pharmacol.* **2008**, *232*, 418–427.
- (24) Olivier, V.; Duval, J. L.; Hindié, M.; Pouletaut, P.; Nagel, M. D. Comparative Particle-Induced Cytotoxicity toward Macrophages and Fibroblasts. *Cell Biol. Toxicol.* **2003**, *19*, 145–159.
- (25) Gessner, A.; Lieske, A.; Paulke, B.-R.; Müller, R. H. Functional Groups on Polystyrene Model Nanoparticles: Influence on Protein Adsorption. *J. Biomed. Mater. Res. A* **2003**, *65*, 319–326.
- (26) Sun, X.; Rossin, R.; Turner, J. L.; Becker, M. L.; Joralemon, M. J.; Welch, M. J.; Wooley, K. L. An Assessment of the Effects of Shell Cross-Linked Nanoparticle Size, Core Composition, and Surface PEGylation on *in vivo* Biodistribution. *Biomacromolecules* **2005**, *6*, 2541–2554.
- (27) Behnke, T.; Würth, C.; Hoffmann, K.; Hübner, M.; Panne, U.; Resch-Genger, U. Encapsulation of Hydrophobic Dyes in Polystyrene Micro- and Nanoparticles via Swelling Procedures. *J. Fluoresc.* **2011**, *21*, 937–944.
- (28) Balhara, J.; Gounni, A. S. The Alveolar Macrophages in Asthma: A Double-Edged Sword. *Mucosal Immunol.* **2012**, *5*, 605–609.
- (29) Bates, J. H. T.; Rincon, M.; Irvin, C. G. Animal Models of Asthma. *Am. J. Physiol. - Lung Cell. Mol. Physiol.* **2009**, *297*, L401–L410.
- (30) Blyth, D. I.; Pedrick, M. S.; Savage, T. J.; Hessel, E. M.; Fattah, D. Lung Inflammation and Epithelial Changes in a Murine Model of Atopic Asthma. *Am. J. Respir. Cell Mol. Biol.* **1996**, *14*, 425–438.
- (31) Draijer, C.; Robbe, P.; Boorsma, C. E.; Hylkema, M. N.; Melgert, B. N. Characterization of Macrophage Phenotypes in Three Murine Models of House-Dust-Mite-Induced Asthma. *Mediators Inflamm.* **2013**, *2013*, 632049.
- (32) Thepen, T.; Claassen, E.; Hoebe, K.; Brevé, J.; Kraal, G. Migration of Alveolar Macrophages from Alveolar Space to Paracortical T Cell Area of the Draining Lymph Node. *Adv. Exp. Med. Biol.* **1993**, *329*, 305–310.
- (33) Willart, M. A. M.; Hammad, H. Alarming Dendritic Cells for Allergic Sensitization. *Allergol. Int. Off. J. Jpn. Soc. Allergol.* **2010**, *59*, 95–103.

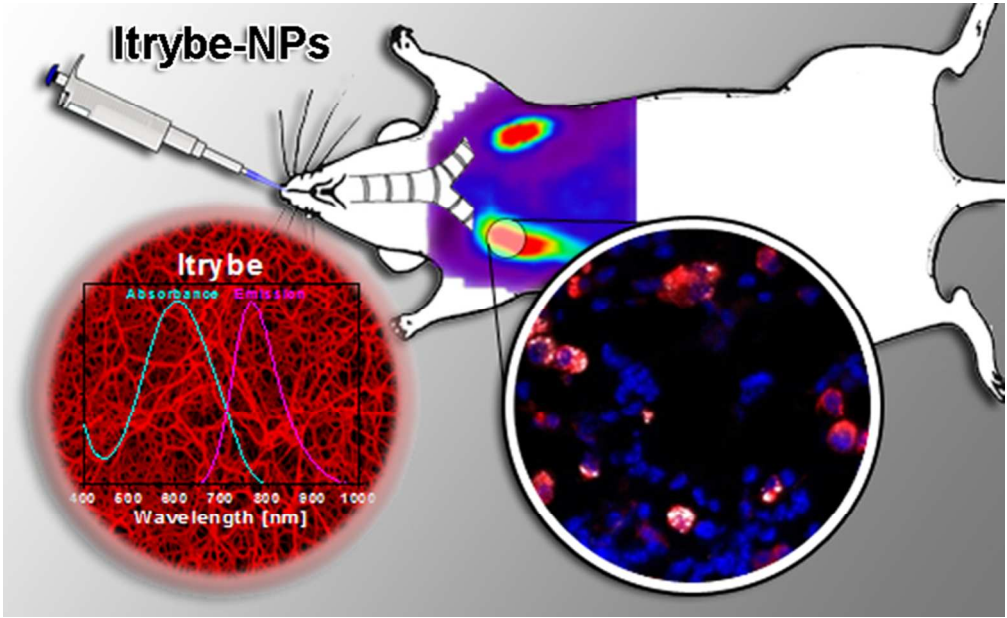
- (34) Gammon, S. T.; Foje, N.; Brewer, E. M.; Owers, E.; Downs, C. A.; Budde, M. D.; Leevy, W. M.; Helms, M. N. Preclinical Anatomical, Molecular, and Functional Imaging of the Lung with Multiple Modalities. *Am. J. Physiol. - Lung Cell. Mol. Physiol.* **2014**, *306*, L897–L914.
- (35) Napp, J.; Mathejczyk, J. E.; Alves, F. Optical Imaging *in vivo* with a Focus on Paediatric Disease: Technical Progress, Current Preclinical and Clinical Applications and Future Perspectives. *Pediatr. Radiol.* **2011**, *41*, 161–175.
- (36) Weissleder, R.; Ntziachristos, V. Shedding Light onto Live Molecular Targets. *Nat. Med.* **2003**, *9*, 123–128.
- (37) Schaffer, B. S.; Grayson, M. H.; Wortham, J. M.; Kubicek, C. B.; McCleish, A. T.; Prajapati, S. I.; Nelson, L. D.; Brady, M. M.; Jung, I.; Hosoyama, T.; *et al.* Immune Competency of a Hairless Mouse Strain for Improved Preclinical Studies in Genetically Engineered Mice. *Mol. Cancer Ther.* **2010**, *9*, 2354–2364.
- (38) Nabe, T.; Zindl, C. L.; Jung, Y. W.; Stephens, R.; Sakamoto, A.; Kohno, S.; Atkinson, T. P.; Chaplin, D. D. Induction of a Late Asthmatic Response Associated with Airway Inflammation in Mice. *Eur. J. Pharmacol.* **2005**, *521*, 144–155.
- (39) Behnke, T.; Würth, C.; Laux, E.-M.; Hoffmann, K.; Resch-Genger, U. Simple Strategies towards Bright Polymer Particles via One-Step Staining Procedures. *Dyes Pigments* **2012**, *94*, 247–257.
- (40) DeRose, P. C.; Resch-Genger, U. Recommendations for Fluorescence Instrument Qualification: The New ASTM Standard Guide. *Anal. Chem.* **2010**, *82*, 2129–2133.
- (41) Kunisch, E.; Fuhrmann, R.; Roth, A.; Winter, R.; Lungershausen, W.; Kinne, R. W. Macrophage Specificity of Three Anti-CD68 Monoclonal Antibodies (KP1, EBM11, and PGM1) Widely Used for Immunohistochemistry and Flow Cytometry. *Ann. Rheum. Dis.* **2004**, *63*, 774–784.
- (42) Zaynagetdinov, R.; Sherrill, T. P.; Kendall, P. L.; Segal, B. H.; Weller, K. P.; Tighe, R. M.; Blackwell, T. S. Identification of Myeloid Cell Subsets in Murine Lungs Using Flow Cytometry. *Am. J. Respir. Cell Mol. Biol.* **2013**, *49*, 180–189.
- (43) Geiser, M.; Rothen-Rutishauser, B.; Kapp, N.; Schurch, S.; Kreyling, W.; Schulz, H.; Semmler, M.; Hof, V. I.; Heyder, J.; Gehr, P. Ultrafine Particles Cross Cellular Membranes by Nonphagocytic Mechanisms in Lungs and in Cultured Cells. *Environ. Health Perspect.* **2005**, *113*, 1555–1560.
- (44) Markus, M. A.; Dullin, C.; Mitkovski, M.; Prieschl-Grassauer, E.; Epstein, M. M.; Alves, F. Non-Invasive Optical Imaging of Eosinophilia during the Course of an Experimental Allergic Airways Disease Model and in Response to Therapy. *PloS One* **2014**, *9*, e90017.
- (45) Cortez-Retamozo, V.; Swirski, F. K.; Waterman, P.; Yuan, H.; Figueiredo, J. L.; Newton, A. P.; Upadhyay, R.; Vinegoni, C.; Kohler, R.; Blois, J.; *et al.* Real-Time Assessment of Inflammation and Treatment Response in a Mouse Model of Allergic Airway Inflammation. *J. Clin. Invest.* **2008**, *118*, 4058–4066.
- (46) Biffi, S.; Dal Monego, S.; Dullin, C.; Garrovo, C.; Bosnjak, B.; Licha, K.; Welker, P.; Epstein, M. M.; Alves, F. Dendritic Polyglycerolsulfate Near Infrared Fluorescent (NIRF) Dye Conjugate for Non-Invasively Monitoring of Inflammation in an Allergic Asthma Mouse Model. *PloS One* **2013**, *8*, e57150.
- (47) Varna, M.; Ratajczak, P.; Ferreira, I.; Leboeuf, C.; Bousquet, G.; Janin, A. *In vivo* Distribution of Inorganic Nanoparticles in Preclinical Models. *J. Biomater. Nanobiotechnology* **2012**, *3*, 269.

- (48) Beckmann, N.; Cannet, C.; Babin, A. L.; Blé, F.-X.; Zurbrugg, S.; Kneuer, R.; Dousset, V. *In vivo* Visualization of Macrophage Infiltration and Activity in Inflammation Using Magnetic Resonance Imaging. *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* **2009**, *1*, 272–298.
- (49) Weissleder, R.; Nahrendorf, M.; Pittet, M. J. Imaging Macrophages with Nanoparticles. *Nat. Mater.* **2014**, *13*, 125–138.
- (50) Madsen, S. J.; Gach, H. M.; Hong, S. J.; Uzal, F. A.; Peng, Q.; Hirschberg, H. Increased Nanoparticle-Loaded Exogenous Macrophage Migration into the Brain Following PDT-Induced Blood-Brain Barrier Disruption. *Lasers Surg. Med.* **2013**, *45*, 524–532.
- (51) Dullin, C.; Dal Monego, S.; Larsson, E.; Mohammadi, S.; Krenkel, M.; Garrovo, C.; Biffi, S.; Lorenzon, A.; Markus, A.; Napp, J.; *et al.* Functionalized Synchrotron in-Line Phase-Contrast Computed Tomography: A Novel Approach for Simultaneous Quantification of Structural Alterations and Localization of Barium-Labelled Alveolar Macrophages within Mouse Lung Samples. *J. Synchrotron Radiat.* **2015**, *22*, 143–155.
- (52) Geiser, M.; Wigge, C.; Conrad, M. L.; Eigeldinger-Berthou, S.; Künzi, L.; Garn, H.; Renz, H.; Mall, M. A. Nanoparticle Uptake by Airway Phagocytes after Fungal Spore Challenge in Murine Allergic Asthma and Chronic Bronchitis. *BMC Pulm. Med.* **2014**, *14*, 116.
- (53) Dames, P.; Gleich, B.; Flemmer, A.; Hajek, K.; Seidl, N.; Wiekhorst, F.; Eberbeck, D.; Bittmann, I.; Bergemann, C.; Weyh, T.; *et al.* Targeted Delivery of Magnetic Aerosol Droplets to the Lung. *Nat. Nanotechnol.* **2007**, *2*, 495–499.
- (54) Boorsma, C. E.; Draijer, C.; Melgert, B. N. Macrophage Heterogeneity in Respiratory Diseases. *Mediators Inflamm.* **2013**, *2013*, 769214.
- (55) Robbe, P.; Draijer, C.; Borg, T. R.; Luinge, M.; Timens, W.; Wouters, I. M.; Melgert, B. N.; Hylkema, M. N. Distinct Macrophage Phenotypes in Allergic and Nonallergic Lung Inflammation. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **2014**, ajplung.00341.2014.
- (56) John-Schuster, G.; Hager, K.; Conlon, T. M.; Irmeler, M.; Beckers, J.; Eickelberg, O.; Yildirim, A. Ö. Cigarette Smoke-Induced iBALT Mediates Macrophage Activation in a B Cell-Dependent Manner in COPD. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **2014**.
- (57) Takenaka, S.; Karg, E.; Kreyling, W. G.; Lentner, B.; Möller, W.; Behnke-Semmler, M.; Jennen, L.; Walch, A.; Michalke, B.; Schramel, P.; *et al.* Distribution Pattern of Inhaled Ultrafine Gold Particles in the Rat Lung. *Inhal. Toxicol.* **2006**, *18*, 733–740.
- (58) Thorley, A. J.; Ruenraroengsak, P.; Potter, T. E.; Tetley, T. D. Critical Determinants of Uptake and Translocation of Nanoparticles by the Human Pulmonary Alveolar Epithelium. *ACS Nano* **2014**, *8*, 11778–11789.
- (59) Gupta, N.; Hatoum, H.; Dy, G. K. First Line Treatment of Advanced Non-Small-Cell Lung Cancer - Specific Focus on Albumin Bound Paclitaxel. *Int. J. Nanomedicine* **2014**, *9*, 209–221.
- (60) Jain, N. K.; Mishra, V.; Mehra, N. K. Targeted Drug Delivery to Macrophages. *Expert Opin. Drug Deliv.* **2013**, *10*, 353–367.
- (61) Mbawuike, I. N.; Herscowitz, H. B. MH-S, a Murine Alveolar Macrophage Cell Line: Morphological, Cytochemical, and Functional Characteristics. *J. Leukoc. Biol.* **1989**, *46*, 119–127.
- (62) Dullin, C.; Zientkowska, M.; Napp, J.; Missbach-Guentner, J.; Krell, H.-W.; Müller, F.; Grabbe, E.; Tietze, L.-F.; Alves, F. Semiautomatic Landmark-Based Two-Dimensional-Three-Dimensional Image Fusion in Living Mice: Correlation of near-Infrared

- Fluorescence Imaging of Cy5.5-Labeled Antibodies with Flat-Panel Volume Computed Tomography. *Mol. Imaging* **2009**, *8*, 2–14.
- (63) Amaral, E. P.; Ribeiro, S. C. M.; Lanes, V. R.; Almeida, F. M.; de Andrade, M. R. M.; Bomfim, C. C. B.; Salles, É. M.; Bortoluci, K. R.; Coutinho-Silva, R.; Hirata, M. H.; *et al.* Pulmonary Infection with Hypervirulent Mycobacteria Reveals a Crucial Role for the P2X7 Receptor in Aggressive Forms of Tuberculosis. *PLoS Pathog* **2014**, *10*, e1004188.
- (64) Schindelin, J.; Arganda-Carreras, I.; Frise, E.; Kaynig, V.; Longair, M.; Pietzsch, T.; Preibisch, S.; Rueden, C.; Saalfeld, S.; Schmid, B.; *et al.* Fiji: An Open-Source Platform for Biological-Image Analysis. *Nat. Methods* **2012**, *9*, 676–682.
- (65) Reiner, D.; Lee, R.-Y.; Dekan, G.; Epstein, M. M. No Adjuvant Effect of *Bacillus Thuringiensis*-Maize on Allergic Responses in Mice. *PLoS ONE* **2014**, *9*.
- (66) Hammer, Ø.; Harper, D. A. T. I Ryan, PD. PAST: Paleontological Statistics Software Package for Education and Data Analysis. *Palaeontol. Electron.* **2001**, *4*, 9.

Abbreviations

AFM, atomic force microscopy; BAL, broncho-alveolar lavage; COPD, Chronic Obstructive Pulmonary Disease; CT, computed tomography; DAPI, 4',6-diamidino-2-phenylindole; FRI, fluorescence reflectance imaging; MFI, mean fluorescence intensity; MRI, magnetic resonance imaging; NIRF, near infrared fluorescence; i.n., intranasal; NP, nanoparticles; NTA, nanoparticle tracking analysis; OCT, optical coherence tomography; OVA, ovalbumin; PET, positron emission tomography; RT, room temperature; SPECT, single-photon emission computed tomography; THF, tetrahydrofuran



TOC graphic
65x39mm (300 x 300 DPI)