Early Assessment of Pulmonary Inflammation by \textsuperscript{19}F MRI In Vivo

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\textbf{Background}—Emulsified perfluorocarbons (PFCs) are preferentially phagocytized by monocytes/macrophages and are readily detected by \textsuperscript{19}F MRI. This study tests the hypothesis that \textsuperscript{19}F MRI can be used to quantitate pulmonary inflammation by tracking of infiltrating PFC-loaded monocytes.

\textbf{Methods and Results}—Pneumonia was induced in mice by intratracheal instillation of lipopolysaccharides (LPS) followed by intravenous injection of PFCs. Whereas regular \textsuperscript{1}H MRI provided no evidence of lung injury 24 hours after LPS, the concurrent \textsuperscript{19}F images clearly show PFC accumulation in both pulmonary lobes. Imaging at 48 hours after LPS revealed signals in \textsuperscript{1}H images at the same location as the 24-hour \textsuperscript{19}F signals. Thus, progressive pneumonia was first predicted by \textsuperscript{19}F MRI early after PFC administration. Without LPS, at no time were \textsuperscript{19}F signals observed within the lung. Histology and fluorescence-activated cell sorting (FACS) combined with \textsuperscript{19}F MRI confirmed the presence of infiltrating PFC-loaded monocytes/macrophages after LPS challenge. Additional experiments with graded doses of LPS demonstrated that \textsuperscript{19}F signal intensity strongly correlated with both LPS dose and pathological markers of lung inflammation. In separate studies, dexamethasone and CGS21680 (adenosine 2A receptor agonist) were used to demonstrate the ability of \textsuperscript{19}F MRI to monitor anti-inflammatory therapies.

\textbf{Conclusions}—PFCs serve as a contrast agent for the prognostic and quantitative assessment of pulmonary inflammation by in vivo \textsuperscript{19}F MRI, which is characterized by a high degree of specificity due to the lack of any \textsuperscript{19}F background. Because PFCs are biochemically inert, this approach may also be suitable for human applications. (\textit{Circ Cardiovasc Imaging}. 2010;3:202-210.)

\textbf{Key Words:} MRI ■ lung ■ inflammation ■ leukocytes ■ perfluorocarbons

Inflammation is a crucial factor in many clinical lung disorders including ventilator-induced lung injury, chronic obstructive pulmonary disease, acute lung injury (ALI), and acute respiratory distress syndrome (ARDS).\textsuperscript{1,2} ALI and ARDS determine to a great extent morbidity and mortality in critically ill patients with septic, posttraumatic, hemorrhagic, or cardiogenic shock. State-of-the-art diagnosis of acute lung injury is performed by blood gas analysis, and supporting imaging information is typically provided by conventional chest radiography or rarely by computed tomography.\textsuperscript{3} Especially in cardiogenic shock or after high-volume treatment, data obtained by these modalities are sometimes difficult to interpret because images reveal bilateral opacities of the lung that can represent interstitial or alveolar fluid accumulation caused by cardiac congestion, fluid overload, or beginning pulmonary inflammation and ALI. Although serum markers such as acute phase proteins and inflammatory cytokines such as procalcitonin, C-reactive protein, interleukin-1\(\beta\), and tumor necrosis factor-\(\alpha\) can provide prognostic information in patients with ARDS,\textsuperscript{4-6} these are less useful to specify diagnosis or treatment. Therefore, new imaging techniques that provide noninvasive, early, and specific diagnostic information are definitely desirable and helpful in such patients.

\textbf{Clinical Perspective on p 210}

It is well known that during ALI, the response to pulmonary inflammation includes a profound infiltration of polymorphonuclear neutrophils (PMNs) and mononuclear phagocytes (MNPs), which are recruited to the lung in response to the release of chemokines such as MCP-1 and MIP-1\(\beta\) or RANTES.\textsuperscript{7} MNPs and PMNs appear to play decisive roles in many inflammatory states. In endotoxin-induced lung injury models, PMN invasion occurs between 6 to 24 hours followed by MNP infiltration between 12 to 48 hours.\textsuperscript{8} Also in the pathogenesis of ventilator-induced lung injury, ALI, and ARDS, MNPs have been reported to be critically involved.\textsuperscript{9}
Once established in the lung, monocytes rapidly differentiate into macrophages initiated by a complex process after transendothelial migration out of the bloodstream. Because recruitment of MNPs and PMNs is an early event within the inflammatory cascade, a specific tracking of these cells would be an ideal target for a molecular imaging approach to visualize pulmonary inflammation in the initial stage.

MRI is not currently used to assess patients with acute lung diseases because conventional proton-based (1H) MRI does not provide more specific data than conventional chest radiographs, which would justify the effort of performing MRI in critically ill patients. Additionally, the lung is not typically appraised by MRI because of respiratory motion and sparse proton density due to large amounts of air with relatively small amounts of tissue. The latter factor exacerbates the imaging challenge faced by MRI because the many air-tissue interfaces in the lung substantially reduce the apparent T2 relaxation time and give rise to pronounced susceptibility artifacts. To date, MRI of the lung airways is possible with high contrast agents and of the lung parenchyma with image acquisition strategies that use ultrashort echo times. However, ultrashort echo time MRI of the lung tissue mainly relies on the accumulation of water protons as consequence of tissue damage and leaky membranes, thereby enabling a diagnosis only in advanced stages of disease. The early visualization of cell infiltration associated with inflammatory processes by MRI up until now used spin-echo imaging with 1.5T MRI in critically ill patients. Additionally, very short echo time MRI of the lung is not currently used to assess patients with acute lung inflammation essentially as previously described. Data were acquired on a Bruker 9.4-T NMR spectrometer using a 30-mm birdcage resonator tuneable to 1H and 19F. After acquisition of the morphological 1H images, the resonator was tuned to 19F and anatomically matching 19F images were recorded (see data supplement for a more detailed description of MRI setup, acquisition parameters, and quantification procedures).

Blood Analysis
Blood was obtained from the inferior vena cava, and mononuclear cells were isolated from the blood samples by centrifugation over Histopaque density gradients (for details, see Data Supplement).

Lung Preparation for Wet-to-Dry Weight Measurements or Flow Cytometric Analysis
Mice were deeply anesthetized with ketamine/xylazine i.p. After opening of the thorax, the heart and lungs were excised and blood was removed. After surgical preparation, lungs were used for determination of wet-to-dry (W/D) weight ratios or processed for flow cytometric analysis according to standard procedures (see data supplement for details).

Immunohistochemistry
Frozen sections (10 µm) were cut from organs of animals that received LPS and PFCs after 24 or 48 hours. Lung sections were fixed and processed for immunohistochemistry according to standard procedures (see data supplement for details).

Statistics
All data are given as mean±SEM. A level of P<0.05 was considered statistically significant (see data supplement for additional details on the statistical analysis).

Results
Progressive Pneumonia Is Predicted by 19F MRI After PFC Administration
Pulmonary inflammation was induced by intratracheal instillation of LPS, which is well known to result in an acute inflammatory response and subsequent infiltration of PMNs.
and MNPs into lung tissue.\textsuperscript{15,16} Six hours after LPS application, 500 μL of 10% perfluoro-15-crown-5 ether emulsion (average size, 130 nm; potential, \(-31.3 \pm 1.5 \text{ mV}\)) was injected via the tail vein. Animals were imaged 24 and 48 hours after LPS challenge. \textsuperscript{1}H fast gradient echo (GE) and fast spin echo (RARE) MRI were acquired to assess structural alterations in the lung via developing hyperintense areas. Subsequently, anatomically matching \textsuperscript{19}F images were recorded for localizing the injected PFCs.

A typical example of consecutively recorded \textsuperscript{1}H and \textsuperscript{19}F images obtained 24 and 48 hours after induction of pulmonary inflammation with 0.3 μg/g BW LPS is illustrated in Figure 1. GE images of the thorax acquired after LPS (Figure 1A, left) show some signals arising from flowing blood within pulmonary vessels next to the heart, which were also detectable under control conditions (data not shown). The anatomic matching RARE images—where signals from protons in motion (eg, blood vessels and heart) are not visible and thus allow a more specific attribution of evolving hyperintense regions—also gave no evidence for any alterations within the lung at 24 hours after LPS (Figure 1A and 1B, left). There were as well no differences detected between control and sham-instilled animals (Figure 2A). However, the corresponding \textsuperscript{19}F image clearly demonstrates the accumulation of PFCs in both lobes of the lung (Figure 1C and 1D, left). Because the fast spin echo pulse sequence (RARE) used to acquire the \textsuperscript{19}F images was similar to that used for \textsuperscript{1}H images, signals from circulating fluorine are also suppressed and the \textsuperscript{19}F signals detected by this method can therefore be assigned unambiguously to PFCs deposited in lung tissue. Only at 48 hours after LPS could anatomically corresponding signals be detected by \textsuperscript{1}H MRI (Figure 1A and 1B, right). Note that the \textsuperscript{19}F signals obviously preceded the colocalized hyperintense regions in GE and RARE images detected one day later by \textsuperscript{1}H MRI (Figures 1 and 2). Without LPS, at no time were \textsuperscript{19}F signals observed within the lung (supplemental Figure 2).

\textbf{Figure 1.} \textsuperscript{19}F signals precede structural changes in LPS-induced pneumonia. Representative respiratory-gated \textsuperscript{1}H (A, gradient echo; B, fast spin echo) and \textsuperscript{19}F (C) MRI of the mouse thorax 24 and 48 hours after intratracheal administration of LPS (0.3 μg/g BW). For merging of data sets (D), a hot iron color table was applied to the \textsuperscript{19}F images. On day 1, \textsuperscript{19}F MRI clearly detected the accumulation of PFCs in both lung lobes, whereas no hyperintense signals were observed in \textsuperscript{1}H-derived images. Follow-up \textsuperscript{1}H MRI investigation on day 2 revealed bilateral infiltrates in the same anatomic region where \textsuperscript{19}F signals were observed at day 1.

\textbf{Figure 2.} Observed \textsuperscript{19}F signals are dose dependent. Quantification of \textsuperscript{1}H and \textsuperscript{19}F MR signals in the lung 24 hours (A) and 48 hours (B) after induction of pneumonia by intratracheal LPS application. Black circles indicate \textsuperscript{1}H-derived area (% of total lung volume); red circles, \textsuperscript{19}F-derived signal integrals (corrected for total lung volumes). Values are mean±SEM, \(n=5\) per dose investigated (\textsuperscript{19}F: \(*P<0.05\), **\(P<0.001\) compared with saline application; \textsuperscript{1}H: #\(P<0.05\), ##\(P<0.001\)).
a strong correlation with the applied LPS dose over a wide concentration range (0.06 to 1.5 μg/g BW LPS, n=5 per group) and exceeded background noise levels already at only 0.15 μg/g BW (Figure 2A, P<0.05). At the same time, 1H MRI only revealed signs of lung injury in the group treated with the highest dose tested (1.5 μg/g BW), which was also the sole group to exhibit a transient alteration in body temperature (Δtmean 1.7±0.5°C). After 48 hours, data pattern obtained from both nuclei did not differ any longer from each other (Figure 2B). Note that the 19F signal at 0.15 μg/g BW LPS after 48 hours was significantly lower than the respective value at 24 hours (P<0.05), suggesting washout of the PFC label (Figure 2A and 2B). Determination of lung W/D weight ratios after 48 hours revealed LPS dose-dependent increases in W/D ratio that reached statistical significance at 0.3 μg/g BW LPS as compared with sham application of saline (5.49±0.38 mg/mg, n=6 each, P<0.01).

To demonstrate that the acquired 19F signals of LPS-challenged lungs relate to phagocyte invasion, tissue sections were processed for immunohistochemistry. Microscopic survey images after Cy3 staining for Mac-2 (also known as galectin-3) as marker for macrophages exhibited a fluorescence pattern comparable to that observed for the 19F signal in the corresponding MRI (Figure 3A and 3B). Higher magnification (Figure 3C) reveals that substantial numbers of Mac-2–positive cells were located in the lung parenchyma and the small airways (bronchioli).

**Uptake and Transport of PFCs by Phagocytes**

To define more closely the cell populations that transport PFCs to the lung, blood samples were subjected to density gradient centrifugation at different points in time (2 to 72 hours) after injection of PFCs. The time course of PFC uptake and their distribution in the different cell populations was then assessed by 1H and 19F MRI, as illustrated
in Figure 4 for a representative experiment. As can be seen, MRI-detectable $^{19}$F signals were observed from 2 to 12 hours after administration of the contrast agent to be preferentially associated with the MNP and PMN fractions, whereas after 24 and 48 hours the signal within the MNPs dominated. After 72 hours, however, the PFCs were completely cleared from the bloodstream. The temporal development of PFC distribution was similar in LPS-treated animals (Figure 4B) compared with untreated control animals (Figure 4A). From the quantified data reported in supplemental Table 1, it can be seen that peak $^{19}$F signal intensities were found in both groups 12 hours after PFC injection.

To further identify the cells within the inflamed tissue that carry the $^{19}$F label, LPS-challenged lungs (1.5 $\mu$g/g BW LPS) were excised and enzymatically digested, and the resulting cell populations were sorted by flow cytometry. After digestion approximately $150 \times 10^6$ cells were harvested per lung, and the expression levels of F4/80 (MNPs), clone 7/4 (PMNs), CD19 (B cells), and CD3 (T cells) were analyzed by fluorescence-activated cell sorting (FACS) (Figure 5A). Consistent with data from the literature, the most prominent leukocyte fraction within the lung after LPS challenge was found to be PMNs with $24.1 \pm 6.1\%$, followed by MNPs with $10.5 \pm 0.9\%$, whereas B and T cells comprised only $0.9 \pm 0.3\%$ and $1.5 \pm 0.4\%$, respectively (n=4). All sorted cell fractions were then analyzed by $^1$H/$^{19}$F MRI to determine the degree of $^{19}$F labeling. Figure 5B demonstrates that the most prominent $^{19}$F signals were found in the monocyte (MNP) fraction and to a lesser extent in neutrophils (PMNs), although the number of PMNs was almost 3-fold higher than that of MNPs (supplemental Table 2). To be able to evaluate PFC load at the level of individual cells, $^{19}$F signal intensities were calibrated with PFC concentration standards and related to the absolute numbers of the respective cell population as derived from the FACS analysis. This calculation revealed a PFC content of $12.8 \pm 3.7$ fmol/cell for MNPs and $1.5 \pm 0.7$ fmol/cell for PMNs, whereas T and B cells were not labeled.

**Figure 5.** FACS analysis of inflamed lungs: MNPs and PMNs transport $^{19}$F. Forty-eight hours after intratracheal LPS challenge (1.5 $\mu$g/g BW) lungs were processed for flow cytometric analysis of leukocytes. A, MNPs were identified as F4/80$^{high}$ and clone 7/4$^{low}$, PMNs as clone 7/4$^{high}$ and F4/80$^{low}$. T cells were defined as CD3$^+$ and B cells as CD19$^-$. Cell purity was determined after cell sorting by flow cytometry, and morphology was confirmed after azur and eosin staining using optical microscopy (see supplemental Figure 3). B, Sorted cells were analyzed using $^1$H and $^{19}$F MRI. Superimposing of the images revealed detectable amounts of PFCs in MNPs and to a lesser extent in PMNs (see text). The water-filled capillary shown in the figure served as geometric landmark for accurate sample identification (small white dot in $^1$H MR reference image). B indicates B cells; M, monocytes; N, neutrophils; T, T cells.

**Figure 6.** Monitoring therapy by $^{19}$F MRI. Anti-inflammatory treatment was performed with dexamethasone (DEX, 1 $\mu$g/g BW), the adenosine 2A receptor agonist CGS21680 (CGS, 2 $\mu$g/g BW), and vehicle (DMSO, 1 L/g BW) after pulmonary challenge with LPS (0.3 $\mu$g/g BW). Quantification of $^1$H and $^{19}$F MR signals in the lung 24 hours after induction of pneumonia revealed that only $^{19}$F MRI could differentiate between treated and untreated groups at this early point in time (n=6 each, $^{* * }P<0.001$ versus vehicle).
application of the PFC emulsion was carried out 6 hours thereafter followed by $^1$H/$^{19}$F MRI after 24 and 48 hours. Pharmacological treatment was continued twice daily within the observation period. Quantification of pulmonary $^{19}$F signals 24 hours after LPS revealed that both DEX and CGS treatment inhibited PFC accumulation by $\approx 80\%$ (Figure 6; $n=6$ per group, $P<0.001$). Consistent with our previous findings, $^1$H MRI failed to reveal significant alterations 24 hours after LPS.

To relate the results obtained by in vivo $^{19}$F MRI to pathological markers of pulmonary inflammation, lungs were excised after MRI at 48 hours and processed for determination of lung W/D weight ratios and numbers of infiltrating leukocytes. From the data summarized in Figure 7, it can be seen that during continuing therapy $^{19}$F signals in both the DEX and CGS groups were still strongly depressed after 48 hours (Figure 7A; $n=6$, $P<0.001$). Similarly, both lung W/D weight ratios (Figure 7B) and numbers of PMNs and MNPs (Figure 7C and 7D) found within the lung were considerably diminished ($P<0.001$).

To exclude the possibility that the reduced $^{19}$F signal intensity observed during drug treatment was caused by an altered PFC loading of infiltrating cells, we also analyzed the $^{19}$F content of leukocytes in the blood after density gradient centrifugation similar to experiments shown in Figure 4. In DEX- and CGS-treated animals, $^{19}$F signal intensities from blood collected after LPS challenge were determined to be $119\pm 36$ and $96\pm 13$ arbitrary units, respectively, revealing no substantial differences in PFC loading between treated and untreated groups ($120\pm 10$ arbitrary units at 12 hours after LPS; see supplemental Table 1).

**Discussion**

In the present study, we demonstrate in a murine model of acute lung injury that nanoemulsions of PFCs can be used to sensitively localize foci of pulmonary inflammation as hot spots by simultaneous acquisition of morphologically matched proton ($^1$H) and fluorine ($^{19}$F) MRIs. Injected PFCs are primarily phagocytized by monocytes and to a lesser extent by neutrophils, resulting in early detectable $^{19}$F MRI signals as a consequence of progressive infiltration of the labeled immunocompetent cells into inflamed areas, at a time when $^1$H MRI provided no evidence of lung injury. Because of the lack of any $^{19}$F background in the
body, the observed signals are robust and exhibit an excellent degree of specificity. Quantification of \(^{19}\)F foci in experiments with stepwise increasing LPS doses and antiinflammatory treatment indicated that the severity of the induced pneumonia is reflected by the signal intensity in the \(^{19}\)F images and illustrated that this approach is also suitable to monitor therapeutic interventions.

The early assessment of pulmonary inflammation by the applied technique is based on the detection of PFC-loaded monocytes, which are known to be quickly recruited to the lung in response to the LPS-induced release of chemokines.\(^{19,20}\) In contrast, the identification of inflamed areas in the lung via hyperintense regions by conventional \(^{1}\)H MRI relies on an enhanced pulmonary proton density caused by plasma extravasation into the lung interstitium. In our LPS study, as in bacterial models of murine pneumonia, detection of pulmonary inflammation in conventional \(^{1}\)H images was not possible until 2 days after the induction of pneumonia,\(^{21,22}\) permitting only a delayed diagnosis at a time when the disease is already in an advanced state. Similarly, the diffuse opacities observed on radiographs are related to significant alterations in lung tissue density that develop only at advanced stages of pneumonia. The sensitivity of \(^{1}\)H MRI for pulmonary inflammation may be improved by loading circulating monocytes, similar to what we described for \(^{19}\)F, with \(^{1}\)H-detectable contrast agents, such as iron oxide- or gadolinium-containing compounds. However, a direct quantification of the observed signals, as is feasible for the background-free \(^{19}\)F images, may not be easily achieved, particularly in the case of signal-depleting iron oxide-based contrast agents. Even signal-amplifying gadolinium-based contrast agents may prove difficult to quantitate in absolute terms, because the cellular uptake of the contrast agent complicates appropriate calculations. For this, the effective relaxation enhancement by the gadolinium-containing compound is required, but, after internalization of the contrast agent, this essential parameter has recently been demonstrated not to be constant and critically dependent on both the intracellular localization and concentration of the gadolinium-containing compound.\(^{23}\) Furthermore, cellular uptake and prolonged persistence of gadolinium within the body may increase the risk of side effects such as nephrogenic systemic fibrosis.\(^{24}\)

There may be unwanted side effects with PFCs as well, in particular because the injectate volume used here was relatively large for a mouse (500 µL). However, contrast injection was well tolerated, and additional experiments in our laboratory have shown that inflammatory processes can be detected in mice with as little as 50 µL of a 10% PFC-containing emulsion (unpublished data). Furthermore, we and others so far have not observed any adverse reactions, neither on animals after PFC injection nor on the proliferation, function, or maturation of immune cells after incorporation of the fluorine label.\(^{14,25,26}\) The reasons underlying this inert behavior are probably related to (1) the strength of the C-F bond, which is resistant to any cleavage by endogeneous enzymes, and (2) the dense and repellent electron sheath that coats F-chains, resulting in extremely weak intermolecular interactions.\(^{27}\) The physiological inertness of PFCs is further supported by our data showing that both PFC-loaded monocytes and neutrophils are still capable of responding to pulmonary LPS challenge within a period of time, which is in good agreement with previous data suggesting an unaltered infiltration kinetics for the labeled cells.\(^{8}\) At this point, it is important to note that the LPS challenges used here were within a moderate range, because a transient alteration of body temperature was only observed at the highest dose tested. However, PFC deposition in the lung was also observed by \(^{19}\)F MRI at substantially lower doses of LPS emphasizing that (1) PFC-loaded leukocytes properly responded to threshold stimuli, and (2) \(^{19}\)F MRI can detect pulmonary inflammation well in advance of conventional physiological methods and proton MRI. The threshold dose of LPS at which PFC deposition in the lung became significant was 0.15 μg/g BW. Interestingly, at this dose \(^{19}\)F signal intensity peaked after 24 hours and substantially decreased from 24 to 48 hours (Figure 2A and 2B), which suggests that also transient adhesion of monocytes after minor challenges can be monitored with this approach.

Our study clearly delineates the mechanisms that are involved in inflammation imaging by \(^{19}\)F MRI. Experiments combining blood density gradient centrifugation, FACS analysis, and \(^{1}\)H/\(^{19}\)F MRI revealed that intravenously applied PFCs are phagocytized predominantly by MNPs and are thereafter transported to the area of inflammation. Our study cannot exclude the possibility that under LPS challenge, resident alveolar macrophages or activated leukocytes adhering to the venular endothelium after pulmonary injury\(^{28}\) also incorporated some circulating PFCs and may have contributed to the observed \(^{19}\)F signals. However, because we never observed fluorine signals above the background noise level in lungs without LPS application, at least under control conditions, this uptake would appear to be negligible. Consistent with the short half-life of monocytes,\(^{29}\) the PFC label persisted for approximately 48 hours within the circulation, as we also reported previously.\(^{14}\) In follow-up to our prior study, here we also noted a minor PFC uptake by PMNs, which is probably related to the different mouse strains used. In the present study, we used NMRI mice, whereas our previous investigation was conducted using C57BL/6 mice. On the other hand, this observation indicates that exploiting differences in surface properties of target cells and retargeting of liposomes by coupling with antibodies may be a promising future option to extend the diagnostic utility of \(^{19}\)F MRI.

In the current study, our initial approach to image inflammation in the heart and brain\(^{13}\) is now extended to the lung—an organ that poses serious challenges to all imaging modalities because it generally appears dark and without contrast. As discussed in detail above, both conventional chest radiography and \(^{1}\)H MRI require substantial alterations in pulmonary tissue density before inflammatory processes can be recognized, thereby providing a diagnosis only in advanced stages of the disease. The identification of pulmonary inflammation by \(^{19}\)F MRI—at
a time when the inflammatory cascade is just being initiated by chemokine release and the recruitment of monocytes—opens the possibility of earlier detection and more timely therapeutic intervention, provided that the early infiltration kinetics of inflammatory cells in human pulmonary diseases is similar to that of mice.

Because of the biochemical inertness of PFCs and the high MR sensitivity of the fluorine nucleus, 19F MRI may be applicable for clinical inflammation imaging. Fluorine coils can readily be interfaced with clinical scanners and the development of dedicated 19F phased-array multichannel coils, as used for coronary angiography by 1H MRI, should overcome signal-to-noise problems associated with the transition from small-animal MRI at high field to larger MRI scanners for human use.31 The PFC used in this study (perfluoro-15-crown-5 ether) exhibits ideal MR properties (to Drs French, Schrader, and Flögel).

These compounds have the disadvantage that when 19F exhalation by the lungs. Viewed from the MRI perspective, MRI scanners for human use.31 The PFC used in this study (perfluoro-15-crown-5 ether) exhibits ideal MR properties (to Drs French, Schrader, and Flögel).

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Disclosures
None.

References
Bilateral opacities in x-ray–based imaging of the lung are a common finding in patients with cardiogenic shock or acute respiratory distress syndrome. However, it remains difficult to resolve whether these opacities are caused by interstitial or alveolar fluid accumulation due to cardiac congestion, fluid overload, or beginning pneumonia and acute lung injury. $^{19}$F MRI using biochemically inert perfluorocarbons could help to distinguish whether these changes are related rather to cardiac failure resulting in lung edema or to acute pulmonary inflammation caused by leukocyte invasion. Injected perfluorocarbons are primarily phagocytized by monocytes, and, as a consequence of progressive infiltration of the labeled immunocompetent cells into inflamed areas, foci of pulmonary inflammation can be localized as hot spots by simultaneous acquisition of morphologically matched proton ($^1$H) and fluorine ($^{19}$F) MRI. The identification of pulmonary inflammation by $^{19}$F MRI—at a time when the inflammatory cascade is just being initiated by chemokine release and the recruitment of monocytes—opens the possibility of earlier detection and more timely therapeutic intervention. Because signal intensity in the $^{19}$F images reflects the severity of inflammation, this approach is also suitable to monitor therapeutic interventions. Because of the biochemical inertness of perfluorocarbons and the high MR sensitivity of the fluorine nucleus, $^{19}$F MRI may be applicable for clinical inflammation imaging.
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SUPPLEMENTAL MATERIAL

METHODS

Preparation of the PFC emulsion

The PFC emulsion containing of 10% wt/wt perfluoro-15-crown-5 ether (Chempur; Karlsruhe, Germany) and 4% wt/wt purified egg lecithin E 80 S (Lipoid; Ludwigshafen, Germany) in isotonic buffer (10 mM HEPES, 2.5% glycerol, pH 7.4) was prepared essentially as previously reported. High pressure homogenization (75 MPa, 10 cycles) with a Emulsiflex C5 homogenizer (Avestin; Mannheim, Germany) resulted in a particle size of ≈ 130 nm as determined by dynamic light scattering using a Zetatrac™ (Particle Metrix; Meerbusch, Germany) which was also used to measure the zeta potential of the particles as the average of at least ten runs at the stationary level. The resulting nanoemulsion was sterilized by autoclaving and stored until administration at 6 °C.

Animal experiments

All experiments were performed in accordance with the national guidelines on animal care and were approved by the Bezirksregierung Düsseldorf. NMRI mice (20-30 g body weight, 8-12 weeks of age) were bred at the Tierversuchsanlage of Heinrich-Heine-Universität, Düsseldorf, Germany. Animals were fed with a standard chow diet and received tap water ad libitum.

Induction of LPS-mediated pulmonary inflammation

LPS from S. enteriditis (Sigma) was dissolved at 1 mg/ml in sterile 0.9% saline and diluted to the concentrations reported for the individual experiments. Mice were anesthetized by application of isoflurane (2.5%) with a home-built nose cone and intubated under direct visualization with a 20G i.v. catheter. For intratracheal instillation, the LPS solution (1.5 µl/g BW) was gently introduced at the distal end of the tube. To avoid volume-induced damage to the lung no pressure was applied and the solution was instead instilled by the spontaneous
respiration of the mouse. Sham mice were instilled intratracheally with 1.5 µl/g BW LPS-free sterile 0.9% saline, whereas control mice received no instillation. For dose relation measurements gender-, weight-, and age-matched animals were used and LPS doses of 0 (n=6), 0.06 (n=6), 0.15 (n=5), 0.3 (n=8), and 1.5 µg/g BW (n=7) in 1.5 µl/g BW of 0.9% sterile saline were applied. Six hrs after the induction of pneumonia 500 µl of the PFC emulsion were injected through the tail vein. Weight and body temperature of mice were recorded after 0, 24, and 48 hrs.

**Anti-inflammatory treatment**

For therapy studies, groups of mice were weight, age and gender matched, and animals received 0.3 µg/g BW LPS as described above. Drugs were dissolved in DMSO and the respective solutions were injected with a microsyringe twice daily (1 µl/g BW) into the subcutaneous tissue beginning 30 min before LPS challenge. Mice were administered dexamethasone (1 µg/g BW per day), CGS21680 (2 µg/g BW per day) or vehicle control (DMSO 1 µl/g BW). The application of the PFC emulsion was performed intravenously 6 hrs after induction of pneumonia as described above.

**Magnetic resonance imaging**

Magnetic resonance imaging (MRI) was performed 24 and 48 hrs after induction of pulmonary inflammation essentially as previously described. Data were acquired on a Bruker DRX 9.4 Tesla Wide Bore (89 mm) NMR spectrometer operating at frequencies of 400.13 MHz for ¹H and 376.46 MHz for ¹⁹F measurements. Experiments were performed and analyzed using ParaVision 4 (Bruker) as operating software. A Bruker microimaging unit (Mini 0.5) equipped with an actively shielded 57-mm gradient set (capable of 200 mT/m maximum gradient strength and 110 µs rise time at 100% gradient switching) and a 30-mm birdcage resonator tuneable to ¹H or ¹⁹F was used. After acquisition of the morphological ¹H images, the resonator was tuned to ¹⁹F, and anatomically matching ¹⁹F images were recorded.
For superimposing images from the two nuclei, the “hot iron” color table provided by ParaVision was applied to $^{19}$F images. To fade out the background noise from $^{19}$F images a constant threshold was applied to $^{19}$F data.

Mice were anesthetized with 1.5% isoflurane in a water-saturated gas mixture of 20% oxygen in nitrogen applied at a rate of 75 ml/min by manually restraining the animal and placing its head in a home-built nose cone. Respiration was monitored from a pneumatic pillow positioned at the animal’s back using an M1025 system (SA Instruments, Stony Brook) and was used to synchronize data acquisition with respiratory motion. Throughout the experiment, mice were respiring spontaneously at a rate of approximately 100 breaths min$^{-1}$ and were kept at 37 °C.

**Lung imaging:** For anatomical analysis $^1$H images of murine lungs were acquired using a respiratory-triggered multislice fast gradient echo (GE) sequence. A flip angle of 15°, echo time (TE) of 1.8 ms, and a repetition time (TR) of 120 ms were used. The pixel size after zero filling was 117·117 μm$^2$ (field of view (FOV) 30·30 mm$^2$, matrix 128·128, slice thickness (TH) 1 mm, acquisition time 1-2 min). Fast spin echo images were acquired using multislice rapid acquisition with relaxation enhancement (RARE) from the same FOV: 16 slices, RARE factor 8, matrix 256·192 resulting in a pixel size after zero filling of 117·117 μm$^2$, TH 1 mm, TR 3.5 s, TE 10.83 ms, acquisition time 2 min. $^{19}$F images were recorded using a multislice RARE sequence (8 slices, RARE factor 64, FOV 30·30 mm$^2$, matrix 64·64 resulting in a pixel size after zero filling of 234·234 μm$^2$, TH 2 mm, TR 4.5 s, TE 3.38 ms, 256 averages, acquisition time, 19.12 min). No respiratory gating was applied for $^{19}$F MRI. For fusion with $^{19}$F images, additional $^1$H datasets with a TH of 2 mm were acquired.

**Volumetric analysis of inflamed areas:** Regions of interest (ROIs) were defined for mean $^{19}$F background noise levels in every dataset. These ROIs were placed in an area outside the animal and the determined background noise level was substracted in each voxel used for
analysis. Inflamed lung regions were quantified from anatomical matching multislice $^1$H and $^{19}$F MR data sets. Affected volumes and signal integrals (corrected for background noise as described above) were calculated from $^{19}$F images by digital planimetric analysis of ROIs within the lung, multiplication with the slice thickness, and summation over all slices covering the lung. In order to account for differences in organ size obtained values were related to total lung volumes as assessed from the corresponding GE $^1$H images. Alterations as a consequence of LPS treatment detectable by normal $^1$H MRI were quantified from RARE images, where spins from blood and heart are almost not visible and cannot be mistaken for signals arising from pulmonary edema. Hyperintense areas were determined by planimetric analysis using the ROI tool of ParaVision from each set of images. The affected lung volume in the RARE images was related to the total lung volume as “hyperintense to total lung volume”.

**Ex vivo imaging:** Immediately after separation of blood components by centrifugation over a Histopaque density gradient (see below), the tube was fixed in a home-built adapter and inserted into the resonator. Coronal $^1$H and $^{19}$F MR image sets with a FOV of 30·30 mm$^2$ were acquired with the same parameters as given above with the exception that $^1$H images were recorded with a non-triggered GE sequence.

For MRI of FACS-sorted cell populations, counted cell suspensions were transferred into 200-µl microfuge tubes and spun to the bottom of the vials. They were fixed in a home-built adapter equipped with a water-filled capillary as geometric landmark and capable of holding up to four samples. Transversal sections were acquired from the basal part of the tubes with a slice thickness of 1 mm and an acquisition time of 120 min for $^{19}$F. For absolute quantification of the PFC content within cells, concentration standards were prepared by dilution of the PFC stock emulsion. The known amount of PFC per voxel from the concentration standards was used to calibrate $^{19}$F signal intensities. This correlation was then utilized to calculate the
average PFC-loading per cell from the $^{19}$F signal intensity per voxel and the corresponding number of cells per voxel for each sample.

**Blood analysis**

Blood was obtained from the *vena cava inferior* at various times after injection of the PFC emulsion as indicated in the different experiments. Mononuclear cells were isolated from the blood samples by centrifugation over Histopaque density gradients (2.5 ml layers of both 1083 and 1119 (Sigma), 25 min, 700 g at room temperature). Thereafter, the tube was immediately transferred into the NMR spectrometer for MRI (see above) followed by collection of mononuclear cells from the interface of the layers and cell counting.

**Lung preparation for wet to dry weight measurements**

Mice were deeply anesthetized with ketamine/xylazine i.p. After opening of the thorax heart and lungs were excised and blood was removed by injection of 10 ml 0.9% saline through the right ventricle at a pressure of 25 cm H$_2$O. The pressure was kept constant to avoid excess diffusion of fluid into the tissue. After surgical preparation and removal of superficial fluid, lungs were weighed and then immediately frozen. Thereafter, lungs were lyophilized for 24 hrs and weighed again to determine dry weight.

**Flow cytometric analysis of leukocytes within the lung**

Lungs were excised as described above. After removal of the heart and surrounding connective tissue, lungs were minced and digested for 1 hr at 37 °C in 5 ml PBS containing 0.4 mg collagenase XI, 1 mg hyaluronidase, and 300 U DNase II (all Sigma). Digested lungs were filtered through a 70 µm sterile nylon mesh, the resulting cells were washed with PBS, and erythrocytes were lysed with a buffered NH$_4$Cl solution.

For the cell sorting experiments, 25 million cells were used and stained according to standard procedures. To prevent non-specific binding of primary antibodies, cells were first incubated
with anti-FcγII/IIIR monoclonal antibodies (mAb) 2.4G2 (BD PharMingen) in ice-cold PBS containing 1% BSA and 0.1% sodium azide. Subsequently, cells were incubated for 20 min on ice with directly labelled mAb: anti-F4/80 mAb PE (Biolegend), anti-neutrophil-mAb clone 7/4 FITC (Acris), anti-CD19 mAb PerCPCy5.5 (BD PharMingen), anti-CD3 APC (Biolegend) or isotype-matched control antibodies (all from eBioscience). Cells were immediately analyzed on a FACSaria flow cytometer (Becton Dickinson). For each sample at least 50,000 live events were acquired and analyzed with the FCSExpress software. In the sorting assays, cells were separated into four vials as MNPs, (F4/80\textsuperscript{high}, clone 7/4\textsuperscript{low}), PMNs (clone 7/4\textsuperscript{high}, F4/80\textsuperscript{low}), B-cells (CD19\textsuperscript{+}), and T-cells (CD3\textsuperscript{+}). Sorted cells were verified microscopically (Online Data Supplement Figure 3) after conventional staining with a Hemacolor kit (Merck). The sorted cells were then resuspended and centrifuged into 200-µl microfuge tubes for further MRI analysis (see above).

Analytic FACS assays of lungs from pharmacologically treated animals were performed on a FACSCalibur (BD Biosciences). The digested lung cells were counted and 1 million cells were stained with directly labelled mAb: anti-F4/80 mAb PE (Biolegend), anti-neutrophil-mAb clone 7/4 FITC (Acris), and anti-CD11b mAb APC (BDPharmingen). For quantification of individual cell types 20,000 cells per lung were analyzed with WINMDI 2.9 software. The relative cell content so determined was afterwards multiplied by the cell number per lung to obtain total values.

**Immunohistochemistry**

Frozen sections (10 µm) were cut from organs of animals which received LPS and PFCs after 24 or 48 hrs. Lung sections were fixed for 10 min in Zamboni’s fixative and rinsed thrice with PBS. Subsequently, slides were transferred for 10 min into blocking solution (10% normal goat serum (Linaris) in PBS containing 0.1% saponin). Incubation with the primary Ab (goat anti-mouse galectin-3 (Mac-2, polyclonal Ab (RD Systems), 1:50, 2% normal horse serum in
PBS with 0.1% saponin) was performed overnight at 4 °C. After three washing steps with PBS containing 0.1% saponin, sections were protected from light and incubated for 4 hrs with the secondary Ab (purified donkey anti-goat IgG, Cy3-conjugated (Jackson Immunoresearch), 1:400, 2% normal goat serum in PBS with 0.1% saponin). Thereafter, slides were washed 4 times and mounted in ProLong Gold antifade reagent with DAPI (Invitrogen, Eugene Oregon). Slides were viewed with an Olympus BX50 fluorescence microscope. Images were captured with a 12-bit CCD monochrome camera driven by CellF software.

Statistics

All data are given as mean ± SEM. Data were analysed for normal distribution by Kolmogorov-Smirnov test and for homogeneity of variances according to Levene using SPSS (Version 10; Chicago, IL). Statistical significance for $^{19}$F and $^1$H data was assessed using two-way ANOVA, and the values at 24 and 48 hrs were verified by repeated measures analysis. A level of $P < 0.05$ was considered statistically significant.
Table 1: Quantification of $^{19}$F signal intensities in individual cell populations after density gradient centrifugation of blood samples collected at different times after PFC injection (values are given as $^{19}$F signal integrals in arbitrary units, n = 3 each, n.d. = not detectable). Maximum fluorine content was found 12 hrs after administration of the PFC emulsion. At the time points investigated, similar $^{19}$F signal intensities were observed for the two labelled cell populations (MNP or PMN) in the LPS- (0.3 µg/g BW in 1.5 µl saline/g BW) and vehicle-treated (1.5 µl saline/g BW) groups, respectively.

<table>
<thead>
<tr>
<th>hrs</th>
<th>2</th>
<th>12</th>
<th>24</th>
<th>48</th>
<th>72</th>
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<tr>
<td></td>
<td>Saline</td>
<td></td>
<td></td>
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<tr>
<td>MNP</td>
<td>29 ± 5 83 ± 4 57 ± 5 31 ± 0.1 n.d.</td>
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<tr>
<td>PMN</td>
<td>18 ± 5 27 ± 6 5 ± 0.1 n.d. n.d.</td>
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<tr>
<td>Σ</td>
<td>47 ± 19 110 ± 11 63 ± 5 31 ± 0.1 n.d.</td>
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<tr>
<td></td>
<td>LPS</td>
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<tr>
<td>MNP</td>
<td>46 ± 12 99 ± 8 56 ± 5 38 ± 3 n.d.</td>
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<tr>
<td>PMN</td>
<td>7 ± 4 21 ± 9 1.0 ± 0.6 n.d. n.d.</td>
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<tr>
<td>Σ</td>
<td>53 ± 13 120 ± 10 57 ± 4 38 ± 3 n.d.</td>
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</table>
Table 2: FACS analysis of leukocytes within the lung after high-dose LPS challenge (1.5 µg/g BW). Distribution of subpopulations and PFC contents (n = 4, total cell count/lung was 154.3 ± 25.9).

<table>
<thead>
<tr>
<th>Cells</th>
<th>Cells [10^6]</th>
<th>PFC/cell [fmol]</th>
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<tbody>
<tr>
<td>PMNs</td>
<td>37.3 ± 9.5</td>
<td>1.5 ± 0.7</td>
</tr>
<tr>
<td>MNPs</td>
<td>16.0 ± 1.5</td>
<td>12.8 ± 3.7</td>
</tr>
<tr>
<td>B cells</td>
<td>1.1 ± 0.5</td>
<td>n.d.</td>
</tr>
<tr>
<td>T cells</td>
<td>2.3 ± 0.7</td>
<td>n.d.</td>
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</tbody>
</table>
REFERENCES


Experimental Protocol

Pneumonia was induced by intratracheal instillation of lipopolysaccharide from *Salmonella enteritidis* dissolved in 0.9% saline. LPS doses of 0, 0.06, 0.15, 0.3, and 1.5 μg/g BW in 1.5 μl/g BW of sterile saline were applied. Six hrs after the induction of pneumonia 500 μl of the PFC emulsion were injected through the tail vein.

For therapy studies animals received 0.3 μg/g BW LPS. Drugs were dissolved in DMSO and the respective solutions were injected with a microsyringe twice daily (1 μl/g BW) into the subcutaneous tissue beginning 30 min before LPS challenge. Mice were administered dexamethasone (1 μg/g BW per day), CGS21680 (2 μg/g BW per day) or vehicle control (DMSO 1 μl/g BW).

**Figure 1**

- LPS
- PFC
- \(^1\)H/\(^19\)F MRI
- \(^1\)H/\(^19\)F MRI

<table>
<thead>
<tr>
<th>day</th>
<th>0</th>
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<tbody>
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<td>LPS</td>
<td>⏳ 6h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PFC</td>
<td>6h</td>
<td>⏳</td>
<td></td>
</tr>
<tr>
<td>(^1)H/(^19)F MRI</td>
<td></td>
<td></td>
<td>⏳</td>
</tr>
<tr>
<td>(^1)H/(^19)F MRI</td>
<td></td>
<td></td>
<td>⏳</td>
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</tbody>
</table>

**Treatment:**
- Dexamethasone
- CGS21680
- DMSO (vehicle)

Blood analysis
Immunohistology
Lung weight
Lung digestion → FACS
PFC deposition in the lung of mice under control conditions (bottom) and LPS challenge (top)
Anatomical matching $^1$H and $^{19}$F MR images were acquired 24 hrs after LPS (top) or saline (bottom) instillation (FOV 30x30 mm$^2$). PFC injection was carried out 6 hrs after instillation and 18 hrs before MRI.
Microscopic verification of sorted cell populations carrying the $^{19}$F label
Leukocytes sorted by flow cytometry after digestion of LPS-challenged lungs were verified microscopically after Hemacolor staining. Cell populations containing the $^{19}$F label were identified via their surface markers (Figure 5) as well as via their typical morphology and staining as granulocytes/neutrophils (top) and monocytes (bottom), respectively. MNPs were identified by their dove-blue, agranular cytoplasm and round or indented nuclei. PMNs showed typical cytoplasmatic granules and multi-lobulated nuclei.