In Vivo Fluorescence-mediated Tomography for Quantification of Urokinase Receptor-dependent Leukocyte Trafficking in Inflammation

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ABSTRACT

Background: Inflammation is characterized by leukocyte recruitment. Macrophages and neutrophils contribute to tissue damage and organ dysfunction. Modulating leukocyte invasion can protect from these adverse effects. Leukocyte recruitment critically depends on the urokinase-type plasminogen activator receptor (u-PAR). We here use a novel technique to longitudinally quantify cell trafficking in inflammatory models in live animals.

Methods: Near-infrared fluorophore-labeled leukocytes were adoptively transferred to mice with thioglycollate peritonitis to study leukocyte trafficking to sites of inflammation. Macrophage and neutrophil trafficking was followed with three-dimensional fluorescence-mediated tomography. u-PAR+/− and wild-type macrophage recruitment was studied by cross-over adoptive cell transfer to elucidate the role of leukocytic versus u-PAR expressed on other cells. Endotoxic shock-induced pulmonary inflammation was used to study u-PARs role for pulmonary neutrophil recruitment.

Results: Mice experiencing peritonitis showed a significant increase in mean fluorescence intensity because of enhanced macrophage (315%, n = 9–10), P < 0.05) or neutrophil (194%, n = 6, P < 0.02) recruitment. Fluorescence-mediated-tomography uncovered a macrophage recruitment defect in the peritonitis model for u-PAR+/− mice (147% of baseline) compared with control mice (335% of baseline, n = 8–9, P < 0.05). When u-PAR+/− macrophages were transferred to wild-type mice fluorescence intensity increased to 145% while wild-type macrophage transfer into u-PAR+/− resulted in 192% increase compared with baseline (n = 6, P < 0.05). Reduced neutrophil recruitment in pulmonary inflammation in u-PAR+/− mice was accompanied by improved pulmonary gas exchange.

Conclusion: Using noninvasive in vivo fluorescence-mediated tomography to image leukocyte recruitment in inflammatory mouse models, we describe a novel macrophage recruitment defect in u-PAR+/− mice. Targeting u-PAR for modulation of leukocyte recruitment is a promising therapeutic strategy to ameliorate leukocyte induced tissue damage.

What We Already Know about This Topic

- Inflammation is described by the appearance of leukocytes in infected or injured tissues in post mortem samples.

What This Article Tells Us That Is New

- Using novel fluorescence-labeled macrophages and neutrophils, the leukocytes were shown in real time to migrate to experimentally inflamed sites if urokinase-type plasminogen activator receptors were present in the tissues.

- This article is accompanied by an Editorial View. Please see: Carles M, Pittet J-F: Leukocyte chemotaxis and migration: Can we follow the cells? ANESTHESIOLOGY 2010; 113:512–3.

- Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of this article on the Journal’s Web site (www.anesthesiology.org).
LEUKOCYTES are the immune system’s most powerful tool to fight infections, but they also contribute to tissue damage and organ failure in response to noxious stimuli. Therefore, the molecular mechanisms of leukocyte trafficking have been investigated over the past decades in great detail.1–14

An important ligand pair governing the recruitment to and egress of leukocytes from sites of inflammatory processes is intercellular adhesion molecule-1 (ICAM-1),15 engaging the β2-integrin CD11c/CD18 on leukocytes. β2-Integrin function critically depends on their direct interaction with the urokinase-type plasminogen activator receptor (u-PAR, CD87),16 which, mediated through direct interaction with the β2-propeller, allows activation of the integrin.19 Integrin activation describes the necessary conformational change of the molecule that will increase affinity of the integrin for binding of its ligand.2 u-PAR has been described as a central orchestrator for cell-cell and cell-matrix interactions (reviewed by Blasi et al.20). Its function in vivo, and particularly the role of leukocytic u-PAR versus u-PAR expressed on other cells has, however, not been elucidated because of the lack of tools for a detailed analysis of the migratory behavior of leukocyte subpopulations in vivo. The vast majority of studies investigating the molecular mechanisms of leukocyte endothelial interactions have been performed using in vitro adhesion assays under static or dynamic conditions.4,12,13,21–23 Other investigators have used intravital microscopy techniques to study the interaction of individual leukocytes with the endothelial vascular lining, including inhibitors and blocking antibodies in animal models of local inflammation.24,25 Leukocyte content of circumscribed inflammatory lesions at a defined point in time can be estimated using peptides directed against integrins specific for certain leukocyte populations.26 However, in vivo studies of bulk movement and recruitment of leukocytes into inflamed body compartments over longer periods of time have been hampered by the lack of tools to follow leukocyte traffic in live animals.

In vivo near-infrared fluorescence imaging is an emerging imaging modality that allows highly sensitive detection and quantitation of fluorescent signatures in vivo.27 Because fluorophores emitting in this spectrum penetrate the tissue very efficiently and tissue background fluorescence is limited, near-infrared fluorophore-labeled molecular and cellular targets can be detected with exquisite sensitivity in vivo using novel three-dimensional fluorescence-mediated tomography.28,29

We used in vivo fluorescence-mediated tomography imaging of labeled macrophages and neutrophils to demonstrate alterations in phagocyte trafficking in u-PAR-deficient (u-PAR−/−) mice experiencing chemically induced peritonitis and in a model for endotoxic shock-induced pulmonary inflammation, by devising an in vivo longitudinal imaging modality that allows to track leukocyte trafficking.18,30

Materials and Methods

Materials

Cell culture materials were purchased from PAA (Coelbe, Germany). 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindotri-carbocyanine iodide (DiR) (Invitrogen, Karlsruhe, Germany) was dissolved in 99% ethanol to 10 mg/ml and stored at 4°C until use at 20 μg/ml. Unless otherwise noted all other reagents are from Sigma Chemical (Munich, Germany).

Cell Harvesting and DiR-staining of Murine Leukocytes

Leukocytes from sex-matched enhanced green fluorescent protein (eGFP) transgenic mice (C57BL/6) were harvested as described previously.31 Macrophages and neutrophils were elicited by intraperitoneal injection of 500 μl Brewer thioglycollate medium, 4%. Animals were killed, and peritoneal lavage was collected in ice-cold Hanks’ buffered salt solution (HBSS) without Ca2+/Mg2+ after 4 h for harvesting neutrophils and on day 3 for macrophages. Leukocytes were counted, resuspended at 106/ml in HBSS, stained with DiR for 15 min at room temperature, and washed twice in HBSS. The resulting cell suspension after 4 h was 86 ± 3% (n = 5) pure resting neutrophils (MCA771G; AbD Serotec, Oxford, United Kingdom) and, on day 3, 84 ± 2% (n = 5) peritoneal macrophages (MAC-2, clone: M3/38; Cedarlane, Burlington, ON, Canada), as assessed by flow cytometric analysis. These cells are referred to as double-labeled leukocytes because they carry the eGFP and the DiR signal. Successful double labeling was proven by fluorescence microscopy (Eclipse TE300; Nikon, Düsseldorf, Germany), and cell viability was assessed using propidium iodide in a flow cytometry assay.

Leukocyte Adhesion and Transmigration Assays for Assessment of Cell Function

To examine the effect of the double label on leukocyte adhesive and migratory function in vitro endothelial cell adhesion and transmigration assays were carried out using the immortalized murine endothelial cell line f.End5.32 In brief, confluent serum-starved endothelial cells were activated with tumor necrosis factor-α (100 ng/ml; BioSource International, Camarillo, CA). Double- and single-labeled (eGFP) macrophages or neutrophils were allowed to adhere to endothelium on a rocking plate (20/min) for 30 min before decanting and two buffer washes. Adhering leukocytes were counted by fluorescence microscopy in 15 preselected high-power fields by a blinded investigator. For transmigration assays 5 × 105 double- or single-labeled macrophages or neutrophils were placed on top of the endothelial monolayer preincubated with 100 ng/ml tumor necrosis factor-α or vehicle for 12 h. Transmigration through Transwell inserts (5-μm pore; Corning, Brussels, Belgium) was allowed for 4 h before leukocytes in the lower compartment were counted by their eGFP label using flow cytometry.

Reactive Oxygen Species Formation by Neutrophils

Oxidative radicals generated by double-labeled neutrophils were measured by oxidation of 2′,7′-dichlorofluorescin diacetate as described previously33 to assess whether the DiR label affects the capacity of neutrophils to produce reactive oxidative species. In brief, 2.5 × 105 double- or single-la-
beled neutrophils were incubated with 5 µm 2',7’-dichlo-rofluorescin diacetate in HBSS for 15 min at 37°C. The developed fluorescence intensity was measured before and after 160-min stimulation by 0.5 µM phorbol 12-myristate 13-acetate in a 96-well plate using a microplate reader (Spectrafluor Plus; Tecan, Crailsheim, Germany).

**Animals**

Experimental work on animals was performed after approval from the animal care committee of the University of Münster (Münster, Germany). Animals were handled according to the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Academy of Science. For each experiment, age- and sex-matched C57BL/6 mice, eGFP transgenic mice in the C57BL/6 background, u-PAR−/− mice, or their respective wild-type (WT) mice (25% SV129/75% C57BL/6) were used. eGFP mice were used as leukocyte donors to introduce a dual label for validation purposes and to make donor cells in the recipient accessible for flow cytometric analysis where indicated. Anesthesia was induced by intraperitoneal injection of 2% tribromoethanol (Avertine®; Sigma, Munich, Germany; 18 µl/g body weight). To guarantee optimal imaging conditions, chest or abdomens and backs of all mice were shaved.

**Fluorescence-mediated Tomography for Intraperitoneal Detection of Macrophages**

Fluorescence-mediated tomography is able to recover depth, size, and concentration of a fluorescence signature three dimensionally from tissue. Detection is strictly linear over a wide range of biologically relevant concentrations and detection sensitivities are in the order of femtomoles. Mice were injected with near-infrared (DiR) fluorescently labeled eGFP cells mixed with peritonitis at 21 days old. The development of the peritonitis model allows recruitment of both neutrophils and macrophages. For baseline and follow-up, the tomographic volume was placed in the center of the abdomen. Thirty sequential coronal images of the abdominal region were collected throughout the three-dimensional region of interest as an image stack. In u-PAR−/− mice, single-label (DiR) macrophages were used. To this end, 30 u-PAR−/− and WT mice peritoneal lavages, labeled with DiR, and injected intravenously into recipient mice. These mice received thioglycolate to induce a peritonitic reaction, and the label intensity of macrophages in lavage fluid (72 h) was examined by fluorescence microscopy and quantitatively compared with the intensity of the cells after labeling and before injection.

**Assessment of Label Retention**

To examine whether the DiR label remained in the leukocytes, the peritonitis model was used because it allows recovery of single-cell suspensions from the site of inflammation. Cells attracted to the peritonitic stimulus were harvested by peritoneal lavages, labeled with DiR, and injected intravenously into recipient mice. These mice received thioglycolate to induce a peritonitic reaction, and the label intensity of macrophages in lavage fluid (72 h) was examined by fluorescence microscopy and quantitatively compared with the intensity of the cells after labeling and before injection.

**Tomographic in Vivo Imaging of Leukocyte Recruitment**

A small-animal fluorescence-mediated tomography system (FMT; VisEn Medical Inc., Woburn, MA) was used to image and quantify in vivo macrophage and neutrophil recruitment in the peritonitis model and neutrophil recruitment in the lipopolysaccharide-induced pulmonary inflammation model. Ten million double-labeled macrophages or 5 × 10⁶ DiR-labeled neutrophils in 300 µl HBSS were injected into the tail veins of 32 animals. Peritonitis was induced by intraperitoneal injection of 500 µl thioglycollate, 4% H₂O₂ after leukocyte injection. Isotonic sodium chloride solution was used as control. After a baseline abdominal tomography 4 h after intravenous injection of leukocytes but before induction of peritonitis, follow-up tomographies were performed at 24, 48, and 72 h after induction of peritonitis to quantify macrophage recruitment and at 4 h to assess neutrophil recruitment. For baseline and follow-up, the tomographic volume was placed in the center of the abdomen. Thirty sequential coronal images of the abdominal region were collected throughout the three-dimensional region of interest as an image stack. In u-PAR−/− mice, single-label (DiR) macrophages were used. To this end, 30 u-PAR−/− and WT mice peritoneal lavages, labeled with DiR, and injected intravenously into recipient mice. These mice received thioglycolate to induce a peritonitic reaction, and the label intensity of macrophages in lavage fluid (72 h) was examined by fluorescence microscopy and quantitatively compared with the intensity of the cells after labeling and before injection.

**Neutrophil Recruitment to the Lung in u-PAR-deficient Mice**

To assess whether the leukocyte recruitment defect is also present in clinically relevant models, neutrophil recruitment to the lungs of mice suffering from lipopolysaccharide-induced shock with accompanying pulmonary inflammation was examined by intravenous injection of 10⁶ near-infrared fluorescence-labeled neutrophils followed by intraperitoneal injection of 10 µg/g body weight lipopolysaccharide (type 0111:B4; Sigma; LD₅₀, 45 µg/g). Fluorescence-mediated tomography of the chest was performed 24 h after lipopolysaccharide injection. Before mice were killed, blood was collected from the infrarenal aorta of spontaneously breathing anesthetized (isoflurane in air) mice for blood gas analysis (Rapidlab 860; Siemens, Erlangen, Germany).

**Statistical Analysis**

Data were analyzed using Instat 3.0 (GraphPad Software, San Diego, CA). Because multiple data families were normally distributed but had significantly different SDs, data were nonparametrically tested throughout. For comparisons of data families with more than two groups, Kruskal–Wallis test followed by Mann–Whitney U test were used. Post hoc testing was limited to the indicated tests (lines in figures) to reduce errors that were due to multiple testing. Wherever an analysis was between two groups only, a Mann–Whitney U test was carried out. Because of their normal distribution, data are nevertheless reported as mean ± SEM. The regression coefficient for the number of injected DiR-macrophage and fluorescence-mediated tomography-measured fluorescence intensity was calculated by linear regression analysis. The nonparametric Spearman ρ test was
Fig. 1. 1,1'-Dioctadecyl-3,3',3'-tetramethylindocarbocyanine iodide (DiR) staining of macrophages and neutrophils is retained in vivo and does not interfere with adhesion to or transmigration across a monolayer of immortalized murine endothelial cells (fEnd5) in vitro. DiR-labeled and control leukocytes were tested on tumor necrosis factor-α (TNFα)–stimulated and control endothelial cells. (A) No differences in the number of adhering macrophages per high-power field (hpf) were detected in DiR-labeled and control macrophages on resting or activated endothelial cells (n = 6, P = not significant [n.s.]). (B) Double-labeled leukocytes were allowed to transmigrate across an endothelial cell monolayer grown on transwell inserts. DiR labeling did not affect macrophage transmigration across resting or tumor necrosis factor-α stimulated endothelial cells (n = 6, P = not significant). Similar results were obtained for DiR-labeled neutrophils. (C) The dye did not affect adhesion (n = 6, P = not significant) and no significant differences were seen for (D) neutrophil transmigration (n = 6, P = not significant). (E) Oxidative burst was detected in neutrophils by dichlorofluorescin diacetate staining and was measured in phorbol 12-myristate 13-acetate (PMA)–activated and control neutrophils after 160 min of stimulation. The mean fluorescence intensity (MFI) of dichlorofluorescein diacetate resulting from neutrophil-generated oxidative burst did not differ in DiR-labeled and control cells. AU = arbitrary units. (n = 4, P = not significant) (F) The staining procedure was highly efficient. Double label of enhanced green fluorescent protein (eGFP) and DiR was detected in more than 97% of the cells before injection. Double-labeled macrophages were detected in the lavage at day 3. (G) Only a minor fading of DiR fluorescent intensity was observed when cells were recovered from two lavages at day 3 and compared with cells before injection by fluorescence microscopy (n = 4, P < 0.05).
applied to calculate the correlation index for lavage results and fluorescence-mediated tomography signal intensity. $P < 0.05$ was considered significant.

**Results**

**DIR Staining Does Not Interfere with Leukocyte Adhesion or Transmigration**

Adhesion of double-labeled macrophages was similar to single-labeled (eGFP transgenic) leukocytes (fig. 1A). Transmigratory ability of double-labeled macrophages was not different from that of control cells—not on resting endothelial cells nor on tumor necrosis factor-α–activated endothelial cells (fig. 1B). Similar results were obtained for neutrophil adhesion (fig. 1C) and transmigration (fig. 1D). The capacity of double-labeled neutrophils compared with single-labeled neutrophils to generate oxidative species was unaltered in stimulated and unstimulated neutrophils (fig. 1E). A meaningful difference in viability and transmigratory or adhesive capacity would have been accepted if differences had been larger than 15%. With the observed effect sizes and variabilities, detection of a statistically significant difference would have been expected ($\alpha$ error 0.05, power 95%) with groups of six animals, suggesting that a meaningful difference is highly unlikely.$^{32}$

**Assessment of Label Retention**

Fluorescence microscopy proved successful double-labeling of macrophages before intravenous injection (fig. 1F). Viability was more than 97% and was not affected by DiR-staining. Three days after injection, the intensity of the DiR label showed minor fading of only 9.3% compared with freshly isolated and labeled macrophages as assessed by near-infrared fluorescence microscopy (fig. 1G).

**Homing of Macrophages and Neutrophils to the Inflamed Peritoneum Can Be Detected by Fluorescence-mediated Tomography**

Fluorescence-mediated tomography can detect the DiR signal of macrophages (fig. 2A) and neutrophils that were recruited into the peritoneum. The detection limit of DiR-labeled cells inside the abdomen is exquisite (Supplemental

**Fig. 2.** In vivo homing of macrophages and neutrophils to the inflamed peritoneum can be detected by fluorescence-mediated tomography. Four hours before induction of peritonitis, mice received an intravenous (IV) injection of $10^7$ double-labeled enhanced green fluorescent protein (eGFP) transgenic and 1,1'-dioctadecyl-3,3',3'-tetramethylindotricarbocyanine iodide (DiR)-stained macrophages or $5 \times 10^6$ DIR-labeled neutrophils. (A) To detect indicator leukocytes that had homed into the peritoneum, baseline fluorescence-mediated tomography (FMT) scans were performed after intravenous injection of indicator cells but before thioglycollate (thio) injection (d0). (B) Four of 30 sequential tomographic images (Z3–Z6) per time point are displayed for two animals that received indicator macrophages. (C) Consistent with flow cytometry and microscopic cell counts (see Supplemental Digital Content 1, Flow Cytometry Confirms Fluorescence-mediated Tomography Measured Cell Recruitment section, http://links.lww.com/ALN/A606), fluorescence-mediated tomography scans revealed a significantly enhanced macrophage derived mean fluorescence intensity (MFI) in thioglycollate-injected mice (open square) compared with controls (cont., closed circles) ($n = 9/10$; * $P < 0.05$). (D) Mice that received DiR-labeled neutrophils intravenously demonstrated a significant increase in abdominal fluorescence intensity 4 h after thioglycollate injection, whereas no difference could be detected in control animals ($n = 6$; * $P < 0.02$).
u-PAR-−/− mice recruit fewer GR-1-, CD11b/CD18-, and CD3-expressing leukocytes in response to an infectious stimulus compared with WT control mice. To corroborate this observation, expand it to macrophages, and elucidate whether u-PAR expressed on the leukocyte or on other cells would contribute equally to this phenotype, we subjected u-PAR-−/− or WT mice to thioglycollate-induced peritonitis. We adoptively transferred macrophages harvested from WT or u-PAR-−/− mice before peritonitis induction in a cross-over design. At day 3, significantly fewer macrophages had been recruited into the peritoneal fluid of u-PAR-−/− compared with WT mice (fig. 3A). Although WT mice transferred with WT macrophages exhibited fluorescence intensities comparable with those of C57BL/6 mice, u-PAR-−/− mice injected with u-PAR-−/− macrophages displayed significantly attenuated fluorescence. No increase of macrophage homing was detected when u-PAR-−/− macrophages were injected into WT recipients, whereas significantly more WT macrophages were recruited into the peritoneum of u-PAR-−/− mice compared with the complete knockout, indicating a more prominent role for u-PAR expression on the leukocyte surface compared with tissue-bound u-PAR expression (fig. 3B). The number of macrophages harvested by peritoneal lavage was strictly correlated to values measured by fluorescence-mediated tomography (Spearman rank correlation coefficient: R = 0.8 [P < 0.01, n = 17]).

In an attempt to assess the capability of fluorescence-mediated tomography to track adoptively transferred leukocyte subsets to solid organ inflammation, we used an endotoxin-induced systemic inflammation model, where pulmonary inflammation incurs secondary to peritoneal injection of *Escherichia coli* endotoxin. Mice were injected with DiR-labeled neutrophils before induction of endotoxic shock. Cell homing to the lungs was then tracked by fluorescence-mediated tomography. u-PAR-−/− neutrophils exhibited significantly reduced homing to the lung of u-PAR-−/− mice compared with WT neutrophils in WT mice as assessed by fluorescence-mediated tomography (fig. 3C). Diminished neutrophil recruitment to the lungs was accompanied by improved pulmonary gas exchange. $P_{02}$ was increased in u-PAR-−/− mice ($91.22 ± 12.44$ vs. $53.71 ±$...
2.93 mmHg, n = 9, P < 0.01) and PCO₂ was significantly ameliorated (39.04 ± 5.15 vs. 55.17 ± 4.01 mmHg, n = 9, P < 0.05), whereas pH was unaltered (7.08 ± 0.03 vs. 7.11 ± 0.02, n = 9, P = not significant).

Discussion

We developed a novel imaging technique using fluorescence-mediated tomography for the noninvasive in vivo characterization of macrophage and neutrophil recruitment. Using fluorescence-mediated tomography, we demonstrated that the previously described neutrophil recruitment defect in u-PAR-deficient mice extends to macrophages and, more importantly, resides on the lack of leukocytic rather than tissue-expressed u-PAR. In addition, we were able to show that u-PAR-dependent modulation of neutrophil invasion benefits pulmonary gas exchange in a clinically relevant lung inflammation model.

Because of its paramount role in various disease entities as well as physiologic processes, leukocyte trafficking and its molecular and cellular pathways have been a major focus of inflammation research over the past few decades. These events have been studied using a wide variety of in vitro assays, intravital microscopy, and in vivo homing assays. In the past, homing assays to assess tissue distribution and targeted recruitment of larger populations of leukocytes in response to inflammatory stimuli were carried out in animals, mostly mice, by injecting populations of leukocytes, lymphocytes, or macrophages, and homing was analyzed post mortem on histologic sections or homogenates of lymphatic organs by flow cytometry.

Different approaches have been pursued to directly visualize molecular targets (e.g., matrix metalloproteinases, myeloperoxidase, cathepsins, vascular cell adhesion molecule) in inflammatory processes using target-specific probes. Adhesion molecule expression is a major target for inflammatory events. However, leukocyte content itself will not necessarily deliver sufficient information on the dynamics of an inflammatory process (i.e., the actual recruitment process of cells). In contrast, fluorescence-mediated tomography employed to longitudinally follow fluxes of exogenous indicator leukocytes is capable of detecting even low numbers of exogenous cells that have been attracted to the peritoneal cavity by the relatively modest thioglycollate stimulus.

Modulating leukocyte recruitment for therapeutic reasons has been proposed for many disease entities. u-PAR functions as a versatile cell-cell and cell-matrix signaling molecule. May et al. were the first to report that a u-PAR/B₂ integrin interaction is responsible for a neutrophil invasion defect, the major detectable phenotype of u-PAR−/− mice. We have demonstrated in this study that u-PAR−/− mice (in addition to their known neutrophil recruitment defect, which we corroborate in a model of pulmonary inflammation caused by systemic shock) also exhibit reduced in vivo macrophage recruitment to the inflamed peritoneum and that this phenotype depends on u-PAR expressed on leukocytes rather than on other cells.

As we demonstrate in the u-PAR-deficient animals, fluorescence-mediated tomography in conjunction with near-infrared fluorescently labeled indicator leukocytes allows quantitative assessment of reduced leukocyte homing. This technique proved to be more sensitive than peritoneal lavage, the accepted standard, has been to date, because we are able to show that a significant amount of signal is retained in the peritoneum after the lavage. Mean fluorescence intensities impressively correlated with data obtained by lavage counts from the same animal.

Besides being useful as a research tool, which we demonstrate here, clinical usefulness of optical imaging to track leukocyte traffic can be clearly envisioned. In particular, fluorescence reflectance imaging techniques can easily be miniaturized, and hand-held optical devices are currently under development that may be applied similarly to ultrasound probes, thus allowing scanning of the abdomen to yield information on leukocyte recruitment to the peritoneal cavity or any other inflammatory site at the surface of the body.

We recognize several limitations of the present study. Apoptotic labeled cells may have been taken up by macrophages, and in this way the DiR label may have been transferred to cells other than the injected cells. But transmigratory function was unaffected by DiR, and viability of indicator macrophages was excellent after homing to the peritoneum. Dissociation of the dye to other cells is therefore unlikely. In addition, we used a double labeling strategy (eGFP-transgene + DiR) and were able to prove histologically that injected eGFP/DiR-labeled cells were identified in organs and the peritoneal fluid carrying a double fluorescence signature (see Supplemental Digital Content 1, fig. 2, http://links.lww.com/ALN/A606).

We used peritoneal leukocytes because of their similarity, with regard to adhesive and migratory function, to peripheral and bone marrow-derived leukocytes. For potential future clinical applications, the use of peripheral blood mononuclear cells would be an attractive alternative, but their use in this application has yet to be characterized. The adoptively transferred cells constitute only a very small proportion of cells that are recruited to the inflamed site (less than 1% of macrophages in the peritoneal lavages; see Supplemental Digital Content 1, Flow Cytometry Confirms Fluorescence-mediated Tomography Measured Cell Recruitment section, http://links.lww.com/ALN/A606) and are therefore unlikely to exert notable biologic effects. We have therefore refrained from fully characterizing the cells used in this study with respect to their phagocytic or secretory properties because similar cell populations have been used in the past to analyze leukocyte homing. Despite an a priori selection of meaningful comparisons between groups, the occurrence of type I error cannot be fully excluded.

In conclusion, we here demonstrated that optical imaging techniques such as fluorescence reflectance imaging and fluorescence-mediated tomography are capable of noninva-
successfully investigating leukocyte traffic between different organs over time and, in addition, if fluorescence-mediated tomography is used, are also able to detect macrophage or neutrophil influx into the inflamed peritoneum or the lungs. It can be used to longitudinally image and quantify the intensity of an inflammatory process. Employing fluorescence-mediated tomography for the characterization of different inflammatory models in u-PAR−/− and WT mice, we demonstrate that u-PAR functions as important modulator of inflammation in vivo, making it a promising therapeutic target for attenuation of leukocyte-induced tissue damage and organ dysfunction.

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Larmann et al.
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