A method for long-term live imaging of tissue macrophages in adipose tissue explants

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Gericke M, Weyer U, Braune J, Bechmann I, Eilers J. A method for long-term live imaging of tissue macrophages in adipose tissue explants. Am J Physiol Endocrinol Metab 308: E1023–E1033, 2015.—Obesity is frequently associated with a chronic low-grade inflammation within adipose tissue (AT). Although classical signs of inflammation are missing in AT inflammation, there is a significant increase in macrophages and, to a lesser extent, other immune cells, such as T cells, B cells, mast cells, and neutrophils. The spatial and temporal activation of these cells as well as their accumulation in the AT seem to be tightly linked to so-called crown-like structures (CLS). CLS are accumulations of adipose tissue macrophages (ATMs) around dead adipocytes and are thought to reflect a scavenger response. At present, data on the life cycle of CLS are missing. To better understand the cellular events underlying AT inflammation, we developed an approach that allows long-term imaging of ATMs, adipocytes, and CLS within live AT explants. We tested three putative reporter mouse lines for myeloid cells in regard to their suitability for live imaging. Thereby, we identified ATMs from CSF1R-eGFP mice to exhibit the most robust expression of eGFP. AT explants from these mice allowed stable live imaging for more than 7 days without significant phototoxicity. Long-term imaging thus revealed the accumulation of ATMs around dying adipocytes, migration of ATMs within AT, and also the degradation of the lipid remnants of perishing adipocytes. The observed behavior of ATMs in the context of AT inflammation is in line with previous studies but for the first time provides data on the specific behavior of individual ATMs and on the life cycle of CLS with unprecedented spatiotemporal resolution.

Obesity and diabetes are associated diseases, but the molecular link between them is still ill-defined. In 2003, the first evidence was reported that obesity is frequently associated with a chronic low-grade inflammation within white adipose tissue (AT) (43), which represents a crucial step in the development of type 2 diabetes (20). In the past decade, many immunological characteristics of obesity-associated AT inflammation have been studied. To date, a well-accepted concept of the pathogenesis of the obesity-associated AT inflammation is that adipocyte hypertrophy and cellular stress in adipocytes cause adipocyte death. Subsequently, adipose tissue macrophages (ATMs) infiltrate the AT to accumulate around dead adipocytes (3, 30). This formation of so-called crown-like structures (CLS) is a unique hallmark of AT inflammation, and the number of CLS increases with obesity (13). However, apoptotic (12) and necrotic (6) adipocyte death has been described in obesity. Furthermore, adipocyte death can be induced in a genetic model of lipodystrophy (30) or by mechanical alteration using a needle injury (22). However, all these models of adipocyte death cause comparable morphological characteristics, including macrophages acting as scavenger cells accumulating around the dead adipocyte and thereby forming characteristic CLS. Interestingly, a small number of CLS are also found in AT from lean mice, suggesting that they also play a physiological role in AT homeostasis (13). Most importantly, CLS are the primary site of adipocyte precursor and macrophage proliferation (13, 22), antigen presentation (29), and immune cell activation (25). However, despite the functional importance of CLS, our understanding of the cellular dynamics after adipocyte death is limited.

To study AT inflammation, CLS can be induced by feeding mice a high-caloric fat-enriched diet (HFD) for several months. In this model, treatment of AT inflammation by anti-inflammatory drugs or depletion of immune cells reduces inflammation and increases insulin sensitivity (9, 24, 31). Applying this strategy to several conditional knockout models, recruitment and activation of ATMs has been studied, revealing potential pharmacological targets for treatment of diabetes, such as the chemokine receptor 2 (19, 42). Furthermore, different subsets of macrophages have been characterized based on the expression of surface markers (4). Accordingly, different functions of distinct ATM subsets have been implicated by expression analyses for proinflammatory cytokines, costimulatory molecules, or scavenger receptors (26, 46). Finally, correlation of distinct subsets of ATMs to the onset of insulin resistance has also supported a functional heterogeneity of these cells. However, since anti-inflammatory ATMs also contribute to proinflammatory cytokine secretion and monocyte recruitment (28, 44), a clear-cut discrimination of diabetogenic and protective ATM subsets is impossible.

New imaging technologies have broadened our understanding of cell-cell interactions in many fundamental processes in health and disease (1, 21). Hence, live-imaging strategies could also give new insights into AT inflammation. For instance, intravital live imaging of AT has confirmed an enhanced immune cell recruitment into AT in obese mice (32). A less invasive, bioluminescence-based imaging approach allowed studying the progress and expansion of AT inflammation in mice during a HFD (10). However, as of today, direct cellular interactions of ATMs and adipocytes have never been studied. Furthermore, the dynamic behavior of individual ATMs, potentially belonging to distinct functional groups, within healthy or inflamed AT is unknown.

We have established long-term imaging of ATMs in live AT explants, allowing the analysis of cell dynamics for more than 7 days. For the first time, we were able to observe the
accumulation of ATMs around a dying adipocyte (formation of CLS), the migration of ATMs toward the CLS and out of the CLS, different migratory behaviors of ATMs, and the degradation of dead adipocytes. Our approach can be used for studying the impact of various physiological and pathophysiological conditions on adipocyte death, on scavenging of perishing adipocytes by ATMs, and on the life cycle of CLS. Direct observation of cellular behavior in situ may spur pharmacological and genetic studies in the field of AT immunology.

MATERIALS AND METHODS

**Experimental animals.** Mouse strains were maintained in the local animal facility on a 12:12-h light-dark cycle with free access to food and water. We studied three different commonly used reporter mouse lines: CD11c-DTR/GFP (18), CX3CR1-eGFP (17), and CSF1R-eGFP (37) mice as well as wild-type mice (C57BL/6J) serving as controls. All mice were male. Reporter mice were homozygous on a C57BL/6J background. Littermates were fed a chow diet (chow, 9% kcal fat; Sniff-Spezialdiäten; Soest, Germany) or a HFD (60% kcal fat; Sniff) for 24 wk starting at 6 wk of age. Body weight was monitored weekly.

**Flow cytometry.** Flow cytometry of mouse epididymal AT was done as described previously (13). Briefly, after dissection and digestion, the cell suspension was filtered through a 75-μm mesh. Next, Fc receptors of the cells were blocked using anti-CD16/32 (1:100; eBioscience, San Diego, CA) for 15 min on ice. Subsequently, cells were stained with anti-CD45-e780 (1:200), anti-F4/80-PE-Cy7, anti-CD11c-PE, (all 1:100, eBioscience), and anti-CD206-Alexa 647 (1:50; AbD Serotec, Kidlington, UK). Finally, 7-AAD (BD Pharmingen, Heidelberg, Germany) staining was used to exclude dead cells. Analysis was performed using an LSR II (BD Pharmingen) equipped with FACS Diva software v. 8.0. Quantification was performed using FlowJo software v. 10.0.5 (Tree Star, Ashland, OR).

**Live imaging of AT explants.** Male reporter mice were fed either a chow diet or a HFD for 24 wk for inducing an increase in CLS formation. After the mice were euthanized, the rostral part of the epididymal white adipose tissue (EWAT) was dissected under sterile conditions and placed in a culture dish filled with PBS (Life Technologies, Darmstadt, Germany). Using a sterile razor blade, AT was cut into tissue blocks of ~1 mm³. These AT explants were stained with 0.1 μg/ml Bodipy 558/568 C12 4,4-[difluoro-5(2-thienyl)-4-bora-3a,4a-diazas-indacene-3-dodecanoic acid, Life Technologies] in 1 ml of RPMI medium (Life Technologies) supplemented with 1% insulin-transferrin-selenium mixture [1.0 mg/ml bovine insulin, 0.55 mg/ml human transferrin (iron-free), and 0.5 μg/ml sodium selenite; Sigma-Aldrich] and antibiotics [100 U/ml penicillin and streptomycin; Sigma-Aldrich] in 2 ml Eppendorf tubes for 1 h at continuous rotation (~8 turns per minute) at room temperature. For visualization of blood vessels and for distinguishing between M1 and M2 macrophages, Alexa fluor 647-conjugated isoelectin Griffonia simplicifolia IB4 (IB4, Life Technologies) was applied at a final concentration of 10 μg/ml in a subset of experiments. Subsequently, AT explants were washed once again in PBS and transferred to a six-well plate filled with 2 ml of the same supplemented RPMI medium. AT explants were immobilized at the bottom of the wells by placing a sterile cell culture insert (Millipore, Billerica, MA) on top of the explant (see Fig. 2, B and C). For long-term imaging (~72 h), the culture medium was substituted every other day. AT explants were kept overnight at 5% CO₂-21% O₂ and 37°C to allow for flattening and stabilization of the tissue. Starting on the next day (day 0), live imaging was performed using an inverted FV300 confocal microscope (Olympus, Hamburg, Germany; Fig. 2B). GFP and Bodipy 558/568 were excited by using a 488-nm argon laser or a helium-neon 543-nm laser, respectively (Fig. 2D). Emission was collected through appropriate narrow band-pass filters (AHF Analysetechnik, Tübingen, Germany). A computer-controlled motorized scanning stage (Prior Scientific, Jena, Germany) allowed imaging of multiple positions within the same explant, from different explants in the same dish or from explants in neighboring dishes. During continuous live imaging, AT explants were maintained at 5% CO₂-21% O₂ and 37°C temperature (CTI-Controller 3700 Digital, an O₂-Controller and a Tempcontrol 37-2 Digital 2-Channel; all from PeCon, Erbach, Germany). At each position, Z-stacks (20–50 μm in height) of images were collected at 2-μm intervals. Stacks were recorded every 10 min for studying cell migration (see Fig. 5) or every hour to assess morphological changes such as CLS formation (see Figs. 3, 4, and 6). Time lapse movies were calculated by custom-written routines using ImageJ software v. 1.47 (39). Briefly, for each position and each fluorescence channel, Z-planes of single images were flattened to an XY image using the “sum slices” function. Single-channel movies were obtained by concatenating XY images. Finally, movies of each fluorescence channel were merged to a time lapse multicolor movie. Movements in X or Y were adjusted using the StackReg plugin (40).

For stitching of multiple images, image tiles were automatically recorded at XY positions having 5% overlap as calculated by Fluoview300 software (Olympus). Images were combined by using the Fiji software 2.0 (38) and a Fiji stitching plugin (35). Cell tracking of individual ATMs was performed using ImageJ and the Manual Tracking plugin. Migration plots were generated using the Chemotaxis and Migration Tool V2.0 (ibidi, Martinsried, Germany).

**Troubleshooting and optimization of the imaging procedure.** While establishing our method, we faced three major problems: 1) mechanical instability of the explant, 2) outgrowth of ATMs from the explant onto the plastic surface of the cell culture dish, and 3) bleaching of the lipid staining. To increase mechanical stability and to circumvent imaging problems due to a shift in XY- or Z-axis, the explants were prepared the day before the start of the imaging procedure. For optimal imaging condition, the entire imaging set-up was prewarmed overnight to 37°C, avoiding shifts due to temperature differences. We learned that, during use of a motorized scanning stage to image multiple spots, glass coverslips should be avoided in order to evade movement-related shifts of the explants. In general, however, better mechanical stability was often combined with a higher tendency of macrophages to migrate out of the explant onto the plastic surface. In our experience, this cannot be totally avoided. Using serum-free conditions, outgrowth of ATMs could be limited to a minimum. Moreover, after overnight incubation spots or explants with ATM outgrowth were already visible and could be avoided for the imaging procedure. Photobleaching, a common problem of long-term live imaging, affected our lipid staining. Constant supplementation of 0.1 μg/ml Bodipy 558/568 C12 to the cell culture medium, however, perfectly balanced the dye fading, allowing for more than 300 stacks of images to be obtained at a relatively constant brightness.

**Post hoc live/dead staining.** After completion of live imaging, AT explants were harvested and stained in 1 ml of prewarmed cell culture medium supplemented with Hoechst 33342 (1:2,000) and ToPro3 (1:500; both Life Technologies). AT explants were incubated for 30 min at continuous rotation. Subsequently, AT explants were placed in a six-well plate and fixed with a cell culture insert as described above. Hoechst and ToPro3 stainings were imaged at a confocal microscope using a LD405-nm or a 633-nm helium-neon laser, respectively.

**Statistical analysis.** Data are presented as means ± SE of 4–5 wild-type and 8–12 reporter mice per group. Statistical significance was evaluated by either Student’s t-test or, for multiple comparisons, the Student-Newman-Keuls method as indicated (GraphPad Prism 6.0; GraphPad Software, La Jolla, CA). P values of <0.05 were considered to be statistically significant, values of <0.0001 to be highly significant.
RESULTS

**CSF1r-eGFP reporter mice are well suited for live imaging of AT inflammation.** We compared three different mouse lines known to express GFP in subsets of macrophages [CD11c-DTR/GFP (18), CX3CR1-eGFP (17), and CSF1R-eGFP (37); heterozygous animals] for their susceptibility to diet-induced AT inflammation and their GFP expression. Wild-type littermates (C57BL/6J) were used as controls. Within 24 wk of a HFD, all strains gained significant body weight (50–60 g) compared with chow-fed littermates (~30 g, \( P < 0.001 \); Fig. 1A). The HFD induced a significant shift in the immune phenotype of ATMs from a preferential M2 polarization in chow-fed mice to a more classically activated M1 polarization in mice after HFD. In each mouse line, the percentage of M1-polarized ATMs (CD11c\(^+\)/CD206\(^-\)) significantly increased \( (P < 0.01) \) upon HFD (Fig. 1B, D, and F), whereas the percentage of M2-polarized ATMs (CD206\(^+\)/CD11c\(^-\)) decreased \( (P < 0.001) \) (Fig. 1B, D, and F). Hence, all mouse lines developed a substantial diet-induced AT inflammation.

For live imaging, GFP expression in ATMs should be strong and homogenous to allow visualization of more or less all ATMs within the tissue. We therefore quantified the fraction of GFP-positive cells in each strain of reporter mice by flow cytometry for the macrophage marker F4/80 and GFP (Fig. 1E). In CD11c-DTR/GFP\(^{+/+}\) mice, less than 10% of all ATMs in chow and HFD-fed animals expressed GFP, with no significant difference between the dietary groups (Fig. 1E). GFP-positive ATMs were more frequent in CX3CR1-eGFP\(^{+/+}\) mice, reaching 36 and 23% for chow- and HFD-fed animals, respectively. The difference in the two dietary groups was highly significant \( (P < 0.001) \). It may be explained by a relative decrease of M2 macrophages, which express CX3CR1 (44). The largest fraction of GFP-positive ATMs was found in CSF1R-eGFP\(^{+/+}\) mice, reaching more than 90% in the chow and HFD groups (Fig. 1F). Of note, in all reporter mice a fraction of GFP-expressing leukocytes did not express the macrophage marker F4/80, as shown for CSF1R-eGFP mice in Fig. 1E. In lean reporter mice, 34% (CD11c-DTR/GFP) or 21% (CX3CR1-eGFP and CSF1R-eGFP) of GFP-expressing cells did not express F4/80. In obese mice, GFP-expressing non-ATMs were less frequent. Here, 9, 15, and 15% of GFP cells in CD11c-DTR/GFP, CX3CR1-eGFP, and CSF1R-eGFP mice, respectively, could not be identified as macrophages. However, GFP expression in these F4/80-negative cells was substantially weaker than in ATMs (see, for example, Fig. 1E).

None of the wild-type mice showed GFP expression. Taken together, the CSF1R-eGFP\(^{+/+}\) mouse line represents an ideal reporter line for live-cell imaging of ATMs in both healthy and inflamed AT.

**Long-term imaging of AT inflammation in AT explants.** GFP-positive ATMs and Bodipy-stained adipocytes were imaged in live AT explants with a confocal laser scanning microscope (Fig. 2). In chow-fed mice, ATMs were homogenously distributed within the AT and exhibited a heteroge-
neous morphology, ranging from spheroid shaped to being highly ramified. Signs of adipocyte death with the typical accumulation of ATMs, so-called CLS, were rarely found (absent in Fig. 3A left). In sharp contrast, in AT from HFD-fed mice the vast majority of GFP-positive ATMs formed CLS (asterisks, Fig. 3A right), much fewer ATMs resided in the interstitial spaces between hypertrophic adipocytes, showing no visible connection to CLS.

Live-cell imaging of AT explants from HFD-fed mice was performed to study cellular dynamics of AT inflammation. We found live imaging with a frame rate of one image per hour to be stable for at least 7 days. The majority of CLS were also stable for such long time periods (asterisks, Fig. 3B) with no signs of shrinkage or cell death. Although most CLS were rather stable in size, ATMs within CLS were scanning the central adipocyte with a surprisingly high mobility (Supplementary Movie 1; supplemental materials are available, linked to the online version of this article).

Cell death of ATMs or adipocytes was easily detectable by the following morphological criteria. Cell death of adipocytes is frequently associated with the accumulation of ATMs and the formation of a CLS. Cell death of macrophages was detected by cell rounding, loss of motility, cell blebbing, and, finally, loss of GFP expression. In general, cell death of either adipocytes or ATMs was a rare observation. To further confirm the high viability of our AT explants, we performed a live/dead discrimination after 7 days ex vivo, using ToPro3 and Hoechst 33342, which stain nuclei of dying cells or nuclei of all cells, respectively (32). In line, post hoc live/dead staining detected only a small number of dead or dying (ToPro3-positive) cells even after 7 days of continuous imaging (Fig. 3C).

Formation of CLS can be observed by long-term live imaging. Our long-term live-imaging approach also allowed observation of the formation of newly developing CLS. Typically, one ATM recognized a presumably damaged adipocyte and subsequently became attached to it (arrow, Fig. 4, “15 h”). Subsequently, more ATMs were attracted to the dying adipocyte, forming a CLS (arrows, Fig. 4, “30 h” to “148 h”). Formation of new CLS typically took several days (Fig. 4 and Supplementary Movie 2).

Live imaging unravels heterogeneity of ATMs during AT inflammation. Our live-imaging approach allowed high-resolution tracking of the migration of individual ATMs in live AT, specifically in the vicinity of preexisting CLS. Increasing the imaging speed to one image per 10 min revealed that subsets of ATMs exhibit remarkably different migratory behaviors, suggesting that different ATMs subserve distinct functions. For instance, some ATMs resided at a given location for at least 24 h, even when in close proximity to a focal spot of inflammation, i.e., a CLS. Often, these cells were ramified and scanned their environment by using their cell protrusions (arrow, Fig. 5A). Patrolling ATMs were also observed; these cells migrated through the interstitial spaces between adipocytes without getting attached to CLS (Fig. 5B). CLS-associated ATMs that
emigrated out of the CLS were frequently observed (Fig. 5C). Our imaging approach allowed discrimination of emigrating ATMs from interstitial ATMs, which were not associated with CLS but occasionally entered a CLS. In the latter cases, ATMs interacted with the central adipocyte for only a short period of time, without signs of attachment to the CLS (Fig. 5D and Supplemental Movie 3). To further distinguish between M1 and M2 macrophages, IB4 was applied, which selectively

Fig. 3. Stable long-term live imaging of inflammation in AT explants. A: representative images of live AT explants from CSF1R-eGFP+/− mice on either a chow (left) or a HFD (right). ATMs express eGFP (green); adipocytes are stained with Bodipy (blue). In this and the following figures, asterisks denote adipocytes surrounded by CLS. B: long-term live imaging of ATMs, adipocytes, and CLS with representative images taken on days 0, 2, 4, and 7 of the imaging session. The corresponding movie file recorded at one frame per hour is available online (Supplementary Movie 1). Note the stability of CLS and adipocytes and the concurrent surveillance activity of single ATMs. C, left: post hoc live/dead staining using Hoechst 33342 (nuclei of all cells, blue) and dead cell nuclear marker ToPro3 (red) of a CLS in an AT explant taken at day 7 of the imaging session. ATMs are in green, cell nuclei in blue (Hoechst staining), and adipocytes in gray. Right: the same image with nuclear and live/dead staining only. Arrow denotes a putative ToPro3-positive ATM. Scale bars, 50 μm.
binds M1 macrophages in CLS (25, 44) as well as endothelial cells. Hence, IB4-positive (M1; arrowhead, Fig. 5E) and IB4-negative (M2; arrow, Fig. 5E) ATMs can be studied. Since the number of IB4-positive ATMs increased during image acquisition (possibly due to uptake of IB4-stained cell debris by M2 macrophages) we defined M1- and M2-polarized ATMs based on the first image. Analyzed over the first 10 h, without overnight incubation, M2 macrophages exhibited a 10-fold higher migration velocity (0.1 μm/min vs. 1.4 μm/min) and a 5-fold higher accumulated migration distance (35 μm vs. 189 μm) than M1 macrophages as presented by migration plots (Fig. 5E, middle and right).

Live imaging of adipocyte degradation in CLS. Most CLS remained stable for up to 7 days in the explants, suggesting rather slow dynamics of the degradation of the dying adipocyte in the center of the CLS. However, we routinely observed smaller adipocytes or lipid remnants in CLS, which may represent later stages in the life cycle of CLS. In line with this interpretation, long-term live imaging of such structures revealed that the size of the small adipocyte in the center of the CLS was reduced over time (circle, Fig. 6B). Furthermore, surrounding ATMs exhibited strong Bodipy staining, indicating substantial lipid accumulation within these ATMs (arrow, Fig. 6, A and B). However, the amount of lipid...
Fig. 5. Live imaging of ATMs functional heterogeneity. A–D: AT explant from a CSF1R-eGFP+/− mouse on HFD. Representative images from a 1-day-long movie taken at high temporal resolution (1 frame per 10 min); time points are indicated in bottom right. ATMs express eGFP (green); adipocytes were stained with Bodipy (blue); asterisk denotes a CLS. A: white arrow marks an ATM that remained at a stable position in close proximity to the CLS. B: white arrow marks an ATM that patrolled (yellow arrows) the tissue. C: an ATM that emigrated out of the CLS. D: an ATM that immigrated into the CLS. The corresponding movie file is available online (Supplementary Movie 3).  

E: AT explant from a CSF1R-eGFP+/− mouse on HFD stained with IB4 (red) to distinguish between IB4-positive (M1, arrowhead) and IB4-negative (M2, arrow) ATMs (left). Migration plots are presented for M1 (middle) and M2 ATMs over 10 h; blue cross marks position of cells at the beginning of the imaging session. Scale bar, 50 µm.
accumulation varied between ATMs (arrowhead, Fig. 6, A and B).

**DISCUSSION**

CLS are dynamic accumulations of immune cells, preferentially macrophages, around dying adipocytes (6). Importantly, the number of CLS increases with obesity and is a robust sign of AT inflammation in mice and humans (2, 13). Hence, studying the life cycle of CLS as well as the cellular dynamics of ATMs and adipocytes in general may provide further insights into healthy and inflamed AT.

Aiming at establishing a live-imaging approach to study AT inflammation, we first characterized three different reporter lines for myeloid cells (CD11c-DTR/GFP, CX3CR1-eGFP and CSF1R-eGFP), which were studied as heterozygotes. We here show that CSR1R-eGFP mice have a strong eGFP expression by 43 h. Images on the left (A) show GFP and Bodipy fluorescence; images on the right (B) show the Bodipy signal only, highlighting the degradation of the dying adipocyte within the CLS (delineated by a yellow dashed oval) over time. Arrow indicates an example of a lipid-laden ATM (foam cell), identifiable by its substantially higher lipid content (Bodipy signal) compared with other ATMs (example marked by arrowhead). Scale bars, 20 μm. The corresponding movie file (showing a wider field of view) is available online (Supplementary Movie 4).
in over 90% of all ATMs independent of the state of inflammation; hence, behavioral properties and functionality of ATMs can be studied without a bias of a selective GFP expression. Of note, GFP expression in neutrophils has also been described in this mouse model (15). In line with this, in lean and obese CSF1R-eGFP mice, we also found between 20 and 15% of GFP-expressing cells, respectively, which could not be identified as macrophages. In comparison, the numbers of GFP-expressing non-ATMs in obese CD11c-DTR/GFP and CX3CR1-eGFP mice were similar (9 and 15%), and both promoters have been shown to be expressed in other leukocytes such as T cells (16, 41). Selective genetic targeting of macrophages is difficult, as has been reviewed before (15). However, we here report that over 90% of ATMs express GFP, and over 80% of GFP-expressing cells could be identified as mature F4/80-positive macrophages. In addition, GFP-expressing cells exhibit macrophage morphology with typical cell protrusions, suggesting that CSF1R-eGFP mice represent a valuable tool for studying ATMs in vivo and ex vivo.

Although CD11c-DTR/GFP and CX3CR1-eGFP mice also develop a diet-induced AT inflammation, overall GFP expression in ATMs was low. However, these mouse strains may be used to further distinguish between different subsets of ATMs. In fact, CX3CR1 has been reported to be most prominent in M0-polarized (negative for CD206 and CD11c) ATMs (47), whereas CD11c expression is one of the hallmarks of M1 macrophages and increases during obesity (26). Despite a relative increase in CD11c-positive-stained ATMs in obese CD11c-DTR/GFP mice, GFP expression did not significantly increase upon HFD feeding. Furthermore, GFP was not consistently expressed in CD11c-positive-stained ATMs in this mouse strain. This might be explained by 1) a low expression of the transgene, 2) the expression of GFP instead of eGFP in these mice, or 3) the fusion of GFP with the diphtheria toxin receptor in this mouse model, which could also cause rapid degradation of the fusion protein (18). To circumvent these issues, a CD11c-eYFP mouse line had been developed, which is now widely used for live-imaging approaches (23) and might also be suitable for live imaging of CD11c-positive cells in AT. Importantly, other immune cells, such as T cells (31), B cells (45), NK cells (33), and mast cells (8) also contribute to AT inflammation in obesity. Reporter lines for these cells could easily be used with our imaging approach. However, the increase of ATMs and the formation of CLS are common end points of obesity-associated AT inflammation. Furthermore, depletion or pharmacological targeting of other immune cells also affects ATMs and CLS density (31, 33).

We here introduced a long-term imaging approach using AT explants. Organotypic slice cultures or embryonic organ cultures have been used before to study cell behavior continuously with single-cell resolution over several days (11, 34). Of note, the lack of physiological blood perfusion is a major disadvantage of these ex vivo models. In contrast, intravital imaging in anesthetized animals with intact tissue perfusion is limited to \(-4\) h (27, 36). Although both approaches have specific benefits, the cellular dynamics of AT inflammation in obesity ultimately demand the possibility of performing high-resolution microscopic imaging in well-preserved tissue from adult animals or even humans over several days. Our approach allows cell tracking of individual cells in living AT over at least one week. By applying IB4 staining, we were also able to distinguish between M1 (IB4-positive) and M2 (IB4-negative) ATMs, as reported before (25, 44), and could analyze differences between these macrophage subsets in regard to migration velocity and migrated distance. IB4 binding could also impair AT migration; however, a few IB4-positive ATMs were able to migrate as fast as the IB4-negative ATMs. Moreover, our finding that most IB4-positive ATMs lack significant motility is in agreement with Cho et al. (5), who studied ATMs from a CD11c-mCherry reporter mouse line over 20 min in vivo. A double-reporter mouse using CD11c-mCherry and the CSF1R-eGFP mouse would allow the study of the migratory behavior of ATMs subsets without the need of additional staining.

Most importantly, our data indicate that the turnover of CLS (formation of new CLS with subsequent degradation of the central adipocytes) takes several days. Therefore, a long-term imaging approach seems mandatory. Although these processes have never been directly observed before, some in vivo data also point to slow dynamics in CLS turnover. For instance, after induction of adipocyte death in a transgenic mouse model of lipodystrophy, an increase of CLS was first detectable after 2 days in mesenteric AT and after 3 days in epididymal AT. Interestingly, after the occurrence of dead adipocytes in this model, the formation of CLS was delayed by at least 24 h (30). These data suggest that our findings on the formation of new CLS in AT explants reflect the physiological situation after adipocyte death in vivo. Interestingly, the inflammatory response characterized by the occurrence of a high number of CLS was most prominent 3 days after the induction of adipocyte death (30). A similar spatiotemporal response has been reported after localized tissue damage of AT by mechanical needle injury. Here, the inflammatory response was still evident after 3 and 10 days (22). Both studies suggest that the degradation of dead adipocytes also takes several days in vivo, a time course that is easy to study with our imaging approach.

Besides its advantages, continuous long-term imaging has the potential to damage the cells under study due to phototoxicity. Phototoxicity is a major concern in long-term live imaging due to production of reactive oxygen species and free radicals (7). In our experience, short-wavelength light, especially using a 405-nm laser, also injures living cells. However, using 488-nm and 543-nm laser light to excite eGFP and Bodipy 558/568, respectively, did not lead to detectable cell death over 7 days of live imaging (at a rate of one frame per hour). To further decrease phototoxic damage, Bodipy 558/568 can also be excited by a 488-nm laser, allowing simultaneous detection of eGFP and Bodipy 558/568 with appropriate emission filters. In general, fluorescent proteins or dyes with excitation spectra of longer wavelength, such as red or far-red, would be favorable over GFP, since long-wavelength light is less scattered in tissue and therefore would allow for a better depth resolution. Similarly, two-photon excitation may significantly enhance the depth resolution as well as the long-term stability of our imaging approach (14). Two-photon microscopy will also be the gold standard for translating our technical approach to live animals.

Taken together, our long-term imaging approach of AT explants represents a viable and stable method for analyzing AT inflammation on the single-cell level over a period of several days. Our method can easily be adapted to other mouse reporter lines for studying different immune cells and specific cell-cell interactions in the context of obesity-induced AT inflammation; hence, behavioral properties and functionality of ATMs can be studied without a bias of a selective GFP expression. Of note, GFP expression in neutrophils has also been described in this mouse model (15). In line with this, in lean and obese CSF1R-eGFP mice, we also found between 20 and 15% of GFP-expressing cells, respectively, which could not be identified as macrophages. In comparison, the numbers of GFP-expressing non-ATMs in obese CD11c-DTR/GFP and CX3CR1-eGFP mice were similar (9 and 15%), and both promoters have been shown to be expressed in other leukocytes such as T cells (16, 41). Selective genetic targeting of macrophages is difficult, as has been reviewed before (15). However, we here report that over 90% of ATMs express GFP, and over 80% of GFP-expressing cells could be identified as mature F4/80-positive macrophages. In addition, GFP-expressing cells exhibit macrophage morphology with typical cell protrusions, suggesting that CSF1R-eGFP mice represent a valuable tool for studying ATMs in vivo and ex vivo.
inflammation. For instance, the phagocytosis of dead cells or free macrolipids can be analyzed in living AT, which represents a fundamental function of ATMs. Studying these processes may shed light on the underrepresented beneficial aspects of an AT inflammation in obesity. Furthermore, combining our approach with second messenger imaging (e.g., for Ca\(^{2+}\) or cAMP) in adipocytes and ATMs may boost our understanding of the molecular events of cell-cell interactions in health and obesity.

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**DISCLOSURES**

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**AUTHOR CONTRIBUTIONS**

Author contributions: M.G., I.B., and J.E. conception and design of research; M.G., U.W., J.B., and J.E. performed experiments; M.G., U.W., J.B., and J.E. analyzed data; M.G., U.W., J.B., and J.E. interpreted results of experiments; M.G. and J.E. prepared figures; M.G. and J.E. drafted manuscript; M.G., U.W., J.B., and J.E. edited and revised manuscript; M.G., U.W., J.B., and J.E. approved final version of manuscript.

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