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Longitudinal ¹⁸F-FDG PET imaging in a rat model of autoimmune myocarditis

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Aims

Although mortality rate is very high, diagnosis of acute myocarditis remains challenging with conventional tests. We aimed to elucidate the potential role of longitudinal 2-Deoxy-2-¹⁸F-fluoro-D-glucose (¹⁸F-FDG) positron emission tomography (PET) inflammation monitoring in a rat model of experimental autoimmune myocarditis.

Methods and results

Autoimmune myocarditis was induced in Lewis rats by immunizing with porcine cardiac myosin emulsified in complete Freund's adjuvant. Time course of disease was assessed by longitudinal ¹⁸F-FDG PET imaging. A correlative analysis between *in*- and *ex vivo* ¹⁸F-FDG signalling and macrophage infiltration using CD68 staining was conducted. Finally, immunohistochemistry analysis of the cell-adhesion markers CD34 and CD44 was performed at different disease stages determined by longitudinal ¹⁸F-FDG PET imaging. After immunization, myocarditis rats revealed a temporal increase in ¹⁸F-FDG uptake (peaked at week 3), which was followed by a rapid decline thereafter. Localization of CD68 positive cells was well correlated with *in vivo* ¹⁸F-FDG PET signalling ($R^2 = 0.92$) as well as with *ex vivo* ¹⁸F-FDG autoradiography ($R^2 = 0.9$, $P < 0.001$, respectively). CD44 positivity was primarily observed at tissue samples obtained at acute phase (i.e. at peak ¹⁸F-FDG uptake), while CD34-positive staining areas were predominantly identified in samples harvested at both sub-acute and chronic phases (i.e. at ¹⁸F-FDG decrease).

Conclusion

¹⁸F-FDG PET imaging can provide non-invasive serial monitoring of cardiac inflammation in a rat model of acute myocarditis.

Keywords

myocarditis • inflammation • ¹⁸F-FDG • PET • personalized treatment

Introduction

Myocarditis is defined as myocardial infection in combination with autoimmunity finally resulting in the inflammatory destruction of cardiac myocytes.¹ Silent myocarditis represents a major cause of unexpected deaths among children and is one of the main reasons of

sudden cardiac death in athletes under 35 years of age.^{2,3} To identify high-risk patients eventually developing chronic dilated cardiomyopathy, disease activity should be closely monitored.⁴ Several non-invasive imaging approaches have been advocated to provide evidence of active myocardial inflammation and to differentiate between acute and post-inflammatory reaction, but none of the current

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methodology in place can solve this issue successfully^{4,5}. For instance, cardiac magnetic resonance imaging (cMRI) only allows for visualization of indirect signs of myocardial inflammation, i.e. tissue oedema, capillary leakage or necrosis indicated by late gadolinium enhancement (LGE). Apart from that, the diagnostic accuracy of cMRI might be hampered in challenging borderline cases.⁶

2-Deoxy-2-¹⁸F-fluoro-D-glucose (¹⁸F-FDG) positron emission tomography (PET) has emerged as a tool for imaging of inflammatory cardiovascular diseases such as myocardial infarction or atherosclerosis.⁷ Activated leucocytes, especially macrophages, are known to express high levels of glucose transporters, which result in rapid accumulation of ¹⁸F-FDG at the site of inflammation.^{8,9} In the present study, we aimed to elucidate the potential role of longitudinal ¹⁸F-FDG PET imaging in an experimental autoimmune myocarditis rat model.

Methods

Animal protocols were approved by the local Animal Care and Use Committee and conducted in accordance to the Guide for the Care and Use of Laboratory Animals.¹⁰ Described reagents were commercially available and used without further purification unless otherwise specified.

Rat model of experimental autoimmune myocarditis

Female Lewis rats (Charles River Laboratories, 250–300 g) were immunized with minor modifications based on a previously described approach.^{11,12} In short, 0.5 mg/mL of antigen porcine cardiac myosin (0.25 mL, Sigma Aldrich, St. Louis, MO, USA) was emulsified in an equal volume of complete Freund's adjuvant (Difco, Becton Dickinson, Lawrence, KS, USA) supplemented with *Mycobacterium tuberculosis* (Difco). On day 0 and day 7, this mixture was injected subcutaneously (s.c.) into the rats' back at three different sites¹³ and time zero was defined as the time point of the second injection. Controls received saline and Freund's adjuvant alone ($n = 4$).

Study design

The first study was performed in a longitudinal setting to determine the temporal cardiac ¹⁸F-FDG PET signal in a rat model of acute myocarditis. Second, a correlative analysis between *in vivo* and *ex vivo* ¹⁸F-FDG results and CD68 staining was performed. Finally, histological analysis of the adhesion markers CD44 and CD34 at different inflammatory stages identified by serial ¹⁸F-FDG PET imaging was conducted. In total, 33 rats had been investigated (longitudinal ¹⁸F-FDG PET imaging study, $n = 8$; correlative analysis between ¹⁸F-FDG signal and CD68 histological staining, $n = 15$; and cell surface marker immunohistochemistry for CD34 and CD44, $n = 10$).

Longitudinal ¹⁸F-FDG PET imaging

A dedicated micro PET scanner (Inveon, Siemens Medical Solutions, Erlangen, Germany) was used.¹⁴ To determine the time course and feasibility of monitoring cardiac inflammation, serial ¹⁸F-FDG PET imaging (2, 3, 3.5, and 4 weeks after immunization) was performed in four myocarditis rats. Animals underwent prior fasting over 14 h and bedding was changed during the fasting period to avoid coprophagia or ingestion of bedding. Rats received water *ad libitum*.¹⁵ One hour after intraperitoneal (i.p.) administration of 37 MBq ¹⁸F-FDG, PET images were acquired over 7 min. Less than 5 min prior to PET acquisition, anaesthesia was started, and all animals were maintained under anaesthesia throughout the

imaging procedure with 2% isoflurane. List-mode data were reconstructed using ordered-subset expectation maximization with 16 subsets and four iterations. Three-dimensional region of interests of the entire heart were manually drawn using an imaging-processing application (AMIDE-bin 1.0.2).¹⁶ The cardiac ¹⁸F-FDG uptake was visualized as the percentage of the injected dose per tissue cubic centimetre (%ID/cm³) and the distribution pattern [left ventricle (LV) vs. right ventricle (RV)] was assessed visually over time. For the analysis of the regional most intense uptake, the average value (%ID/cm³) of the five segments (anterior, lateral, septal, inferior, and apex) was assessed. As a reference perfusion marker, ¹⁸F-fluorobenzyl triphenyl phosphonium PET was conducted subsequently (at acute phase of inflammation) and manual co-registration to the ¹⁸F-FDG images was carefully performed.¹⁷ Moreover, myocardial reference has also been obtained by a previously described protocol to enhance tracer uptake in the myocardium.¹⁸

Correlative analysis between ¹⁸F-FDG signal and CD68 histological staining

For autoradiography, ¹⁸F-FDG (37 MBq) was injected i.p. into myocarditis rats after a 14 h fasting period. Guided by longitudinal ¹⁸F-FDG imaging, myocardial tissue of nine rats was harvested at acute phase (i.e. at peak ¹⁸F-FDG uptake, 3 week post-immunization) and of six rats at subacute phase (i.e. at decrease of ¹⁸F-FDG uptake, 5 week post-immunization). Tracer distribution time was 60 min before euthanasia. Subsequently, the heart was extracted, frozen and cut into 20-μm short-axis slices using a cryostat (Leica, Nussloch, Germany). Immediately afterwards, the autoradiography plate (Multi Sensitive Phosphor Screens, PerkinElmer, Shelton) was exposed to the slices for 60 min for visualization of ¹⁸F-FDG distribution with a digital autoradiography system (CR 35 Bio, Raytest, Packard, Straubenhardt, Germany).

Immunohistological CD68 analysis was performed using 7-μm slices adjacent to the short-axis slices utilized for autoradiographic analysis. Immunohistochemical staining with rabbit-anti CD68 (Abcam, Cambridge, U.K.) antibodies was conducted as previously described.¹³ In short, after fixation (acetone, 10 min) and blocking with 10% bovine serum albumin, the incubation with CD68 antibodies was performed for 12 h. As a secondary antibody, biotinylated goat anti-rabbit IgG (Thermo Fisher Scientific, Darmstadt, Germany) was used. Optical microscopy was performed using a Keyence BZ-9000 microscope (Keyence Corporation, Neu-Isenburg, Germany). Region of interests were set on the anterior, lateral, inferior, and septal wall of the LV and on the RV on midventricular short-axis slices. CD68 positivity in percentage was determined with ImageJ software (version 1.47v, National Institutes of Health, Bethesda, MD, USA) by using an intensity threshold that matched to the visually identified staining areas as closely as possible.

Subsequently, for correlation of CD68-positive areas with *in-* and *ex vivo* ¹⁸F-FDG uptake, region of interests were exclusively placed on areas of increased cell infiltration of adjacent slides of CD68 staining, *ex vivo* ¹⁸F-FDG autoradiography and *in vivo* PET imaging.

Cell surface marker immunohistochemistry for CD34 and CD44

Guided by longitudinal ¹⁸F-FDG imaging over time, the myocarditis rats were assigned to three different stages for harvesting of myocardial tissue: an acute phase at peak ¹⁸F-FDG uptake (3 week after immunization, $n = 4$) as well as at subacute (5 week post-immunization, $n = 3$) and chronic phases (10 week post-immunization, $n = 3$). The late phases were both accompanied by decrease of tracer accumulation. To differentiate between acute and post-inflammatory reaction, histological analysis at each stage was conducted for the adhesion markers CD44 and CD34. CD34 was shown to be expressed in vascular endothelial cells and

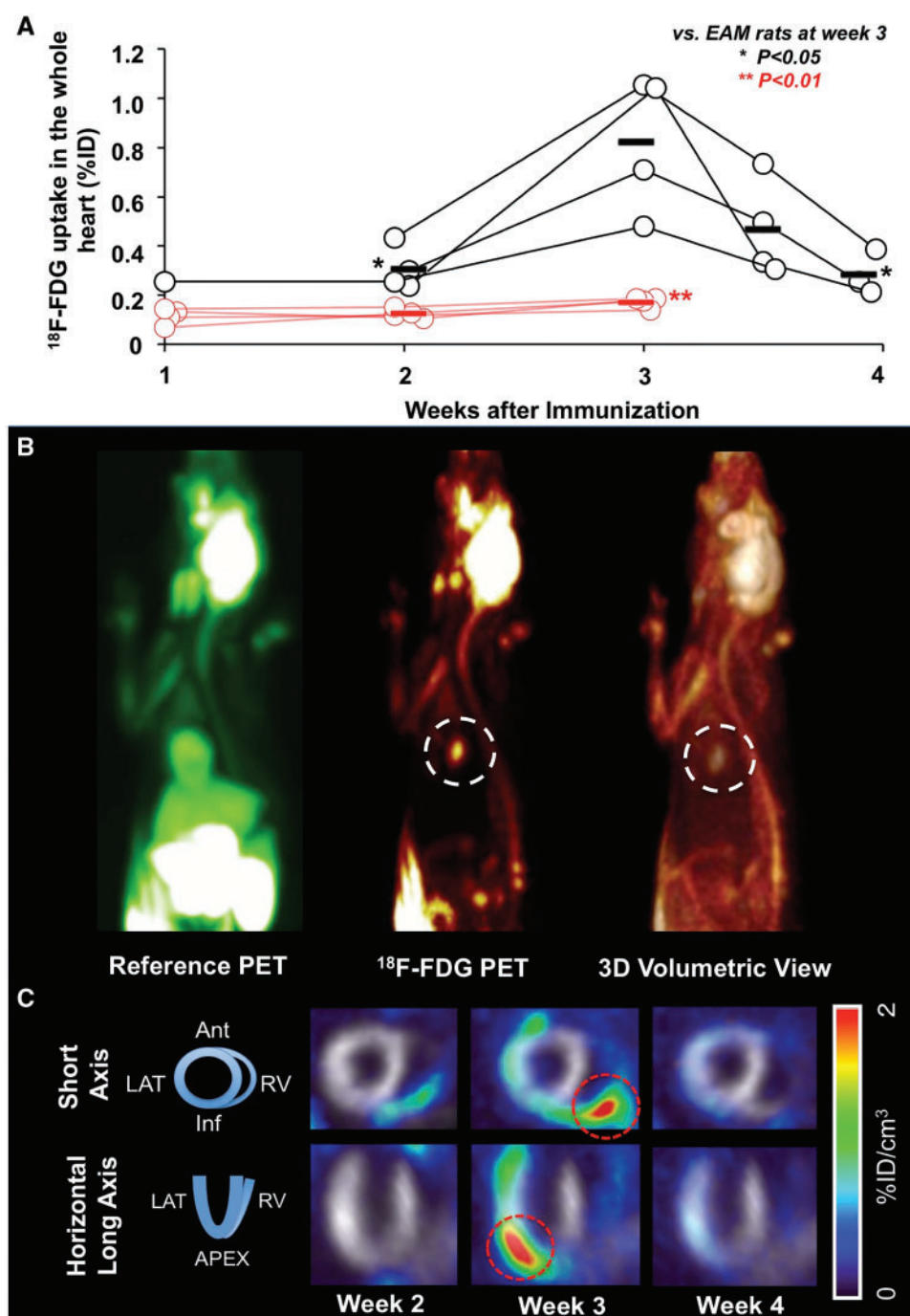


Figure 1 Longitudinal ^{18}F -FDG PET imaging. (A) Total ^{18}F -FDG uptake by PET given at week 1, 2, 3, 3.5, and 4. Uptake peaked at week 3, whereas a decrease could already be visualized at week 3.5. A good discrimination compared with controls could be observed. (B) *In vivo* PET imaging 3 weeks after immunization (acute phase). Myocardial reference PET (^{18}F -FDG under insulin stimulation, left), ^{18}F -FDG PET (middle) and 3D volume rendering view (right). Clear focal ^{18}F -FDG uptake signal in the heart can be observed (white dotted circles). (C) Serial ^{18}F -FDG imaging 2, 3, and 4 weeks after immunization with representative short-axis and horizontal long-axis PET images of a myocarditis rat. Inflammation indicated by ^{18}F -FDG starts at the right ventricle (week 2), whereas at week 3, the global heart is affected (red dotted circles). Grayscale images served as a myocardial reference. EAM, experimental autoimmune myocarditis.

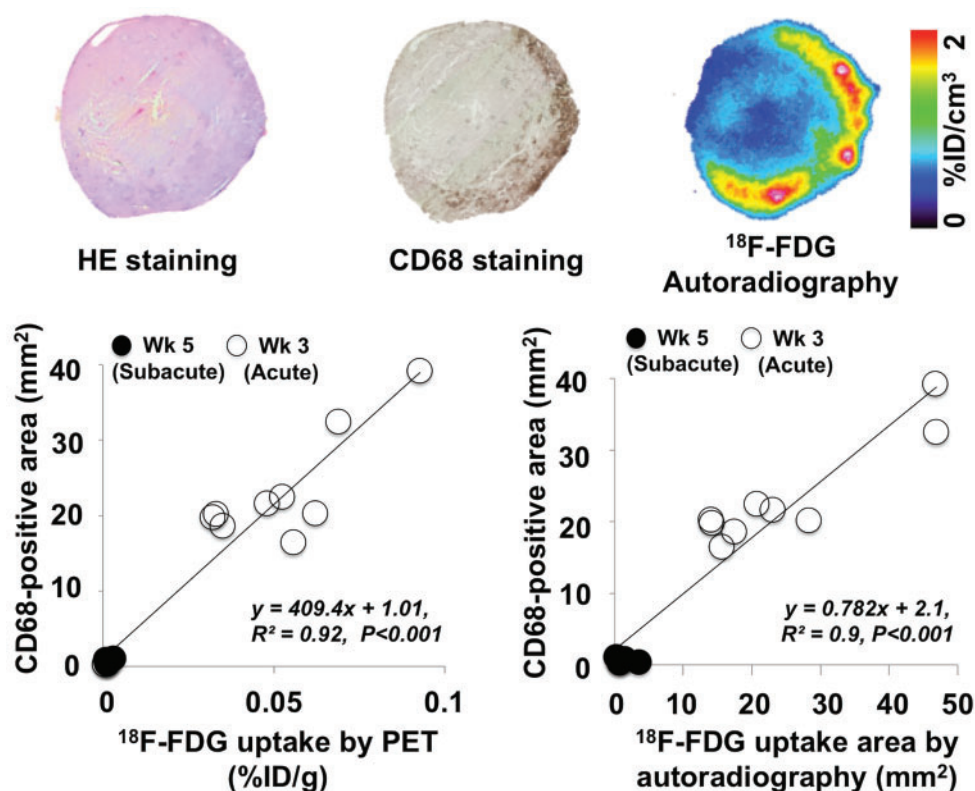


Figure 2 Upper row: HE-, CD68-staining, and ^{18}F -FDG autoradiography images of a myocarditis rat. Lower row: *In vivo* correlation of ^{18}F -FDG (%ID/g) by PET as well as *ex vivo* ^{18}F -FDG uptake by autoradiography (mm^2) with CD68 positive stained myocardial areas (mm^2). Guided by ^{18}F -FDG *in vivo* PET imaging, rats were selected at peak ^{18}F -FDG uptake (3 weeks post-immunization, acute phase) and at time-point of ^{18}F -FDG decrease thereafter (5 weeks post-immunization, subacute phase). A good correlation for *in vivo* ^{18}F -FDG ($R^2 = 0.92$) as well as for autoradiography findings ($R^2 = 0.9$) with CD68 positive areas was detected ($P < 0.001$, respectively). White dots indicate animals, in which myocardial tissue had been harvested at week 3 (acute phase) and black dots indicate animals, in which cardiac tissue had been harvested at week 5 (subacute phase). Wk, week.

reaction: The adhesion transmembrane receptor CD44 is critically involved in acute inflammation, as it promotes the recruitment of macrophages.^{25,26} On the other hand, CD34 positive cells contribute to post-inflammatory reaction.^{27,28}

CD44 is expressed on both endothelial and inflammatory cells and is known to play a key role for acute inflammatory response: it moderates adhesion of T lymphocytes to the endothelium²⁹ and releases mediator components from macrophages.³⁰ Yoshida *et al.*²⁶ investigated the same experimental autoimmune myocarditis model and analogous to our findings, a time-dependent expression of CD44 in the rat heart could be demonstrated: CD44 peaked 3 weeks after immunization, followed by a remarkable decline at chronic phase (12 weeks). However, CD44 in myocarditis is also prone to species-specific variations: Abel *et al.*³¹ used a murine myocarditis model and showed that CD44 seemed to be rather absent in the mice myocardium. Hence, extrapolations for the inflammatory regulation of CD44 from one species to another may have to be drawn with extreme caution. Also adding to the complexity of targeting CD44, its function might even vary among different tissues in the same species: in contrast to the negligible role in the infected mice myocardium,³¹ CD44 was critically involved in a murine model of thyroid gland

inflammation.³² Nonetheless, analogous to our findings of CD34 contribution to post-inflammatory reaction in myocarditis rats, an increased cardiac CD34+ cell mobilization at chronic phase has been recently demonstrated in a Coxsackie virus myocarditis model.²⁸ However, depending on the used species, one might have to expect different peaks of inflammatory response: contrary to our findings with EAM rats, SWR/J mice infected with coxsackievirus B3 developed acute myocarditis 6–12 days post-injection.²⁸

Apart from that, CD68 for the assessment of activated macrophages has been extensively used for endomyocardial biopsy in a clinical setting.³³ In our study, CD68 staining confirmed that macrophages represented the majority of infiltrative cells at peak ¹⁸F-FDG uptake. Although linear regression might have predominantly been driven by animals with intense reaction to immunization (Figure 2), correlative analysis with *in-* and *ex vivo* ¹⁸F-FDG signalling demonstrated that the radiotracer accumulation matched well with macrophage infiltration. Albeit this might not reflect the variance in inflammation pattern and severity induced by myocarditis, even exclusion of those two animals with intensive reaction still led to considerably high R^2 values of ≥ 0.86 . Hence, imaging with ¹⁸F-FDG could improve the accuracy of endomyocardial biopsy by reducing the risk

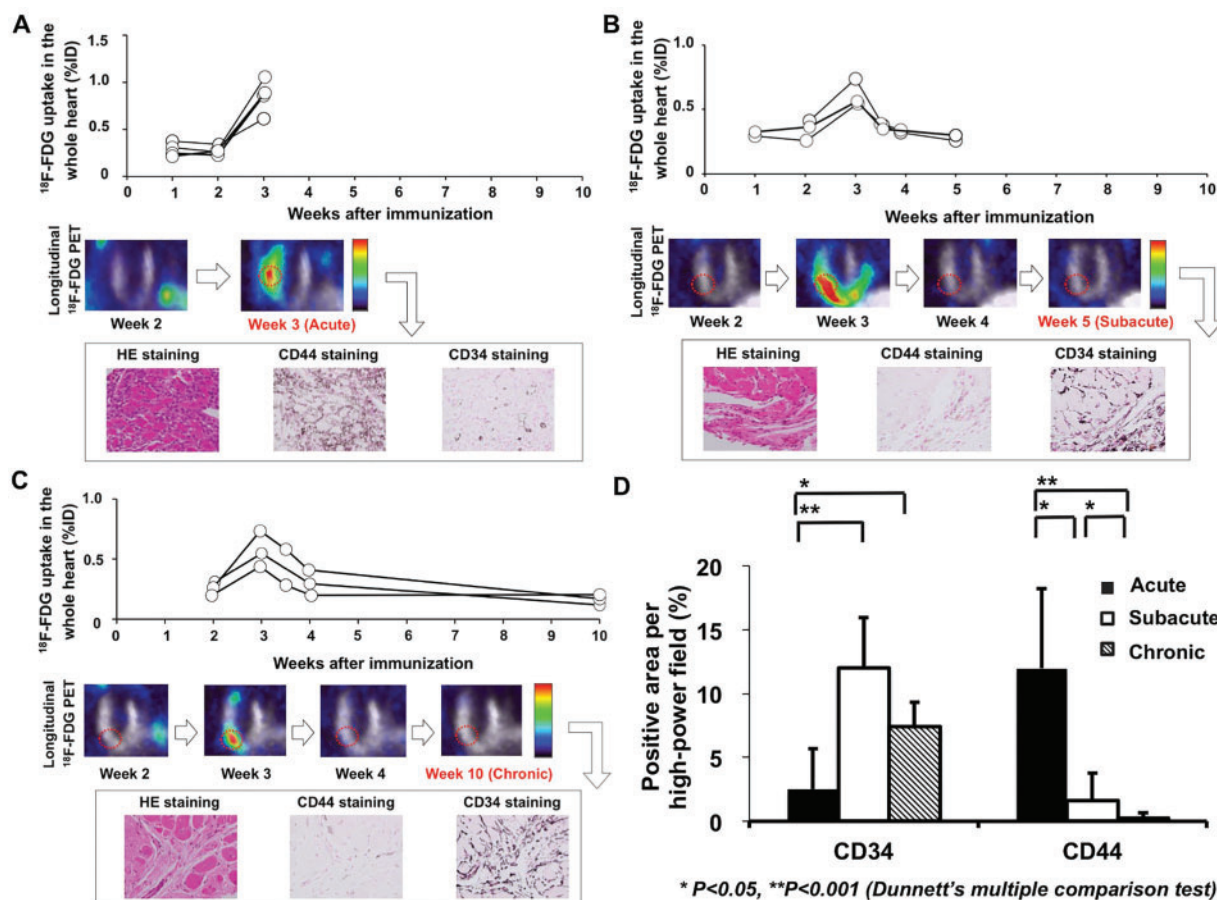


Figure 3 Exchange of adhesion molecules CD34 and CD44, guided by longitudinal *in vivo* PET imaging. (A) At acute phase (3 weeks after immunization), a peak ^{18}F -FDG uptake was recorded, with corresponding CD44 positive stained myocardial areas. (B) At subacute phase (5 weeks post-immunization) and (C) chronic phase (10 weeks post-immunization), PET revealed a decline of cardiac tracer uptake: An increase in CD34 positivity was noted, whereas a further decrease of CD44 positively stained cells could be identified. (D) Quantitative analysis of adhesion markers at different phases revealed a low CD34 and—conversely—an increased CD44 positivity (in %) at acute phase. At both subacute and chronic phases, opposite findings with CD34 positively stained areas and a further decrease in CD44 positivity were recorded.

of sampling errors. In addition to that, the potential of ^{18}F -FDG for PET-guided biopsies has been recently confirmed in patients with clinically suspected active myocarditis.³⁴

Analogous to the findings of the present study using PET, the area of LGE obtained by cMRI matched with histologically proven myocarditis at day 21 in Lewis rats.³⁵ MRI sequences can monitor structural alterations, i.e. tissue oedema, capillary leakage, or necrosis.⁴ However, functional imaging modalities offer several key advantages in non-invasive inflammatory imaging, e.g. direct interrogation of infiltrating immune cells on a subcellular level.⁷ Consequently, the combination of PET and MRI could provide incremental information about myocardial injury and inflammatory activity: in a small cohort of ten patients, simultaneous PET/MRI using ^{18}F -FDG was feasible to diagnose both cardiac sarcoidosis and myocarditis.³⁶ Thus, such an imaging approach could also potentially be applied in the herein presented experimental setting.

An extensive body of evidence reported on the suitability of gamma emitting compounds to localize inflammatory sites in the

human heart.^{37,38} However, even promising candidates, such as ^{67}Ga citrate showed a lack of sensitivity in detecting myocardial infiltration.³⁷ To overcome limitations of conventional scintigraphy studies, PET offers improved spatial and temporal resolution along with the possibility of quantification approaches. By visualizing infiltration of mannose receptor-positive macrophages, Lee *et al.*³⁹ have recently introduced the PET compound ^{68}Ga -2-(p-isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid mannosylated human serum albumin (^{68}Ga -NOTA-MSA): Compared with its novel ^{68}Ga -labelled counterpart, the sensitivity of ^{18}F -FDG to detect cardiac inflammatory cell infiltration was reduced. However, an ^{18}F -labelled PET imaging agent such as FDG inherits all advantages of ^{18}F -radionuclides, i.e. lower positron energy along with higher positron yield, logistical benefits (longer physical half-life), cost-effectiveness (use of delivering system), availability at almost every PET centre, as well as the potential of delayed imaging protocols.^{40,41} In addition, glucose can be seen as the backbone of monitoring inflammatory processes, as neutrophils up-regulate GLUT1/3 transporters as well

