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The Latest Advances in Imaging Crosstalk Between the Immune System and Fibrosis in Cardiovascular Disease

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Inflammation and fibrosis are hallmarks of tissue repair process and organ failure progression in cardiovascular diseases. Paradigm-shifting research on diverse immune cell populations within the cardiovascular system have enabled discovery of new biomarkers fostering development of diagnostic and therapeutic agents at the molecular level to better manage cardiovascular diseases. To date, a variety of molecular imaging agents have been developed to visualize the biomarkers expressed on immune cells and fibroblasts within their crosstalk network, which drives the pathogenesis of fibrosis triggered by both innate and adaptive immunity. Herein, key biomarkers up-regulated in the immune-fibrosis axis are discussed. The promising molecular imaging agents to reveal this critical pathological process are summarized.

Key Words: Immune system; inflammation; fibrosis; cardiovascular diseases; molecular imaging

Fibrosis, a scarring process, is defined as an uncontrolled accumulation of extracellular matrix (ECM) molecules on injured tissues, and ultimately leads to adverse tissue remodeling, organ damage, and failure. Inflammatory and immunological reactions involving both innate and adaptive immune systems are the underlying players driving fibrosis (*1,2*). In cardiovascular diseases (CVDs), vascular remodeling triggered by inflammatory stimuli is significantly associated with atherogenesis, deposition of ECM proteins on the arterial wall, and eventually vascular fibrosis. It is known that cardiac fibrosis is a major contributor to many CVDs including myocardial infarction (MI) (*3*). Clinical diagnostics mostly provide anatomic characterization of fibrotic scars at the stage where disease is irreversible and irreparable. The limited options of anti-fibrotic medications in clinic compel an urgent search for novel diagnosis using molecular imaging to identify new biomarkers overexpressed during immune response and tissue repair within the immune-fibrosis network at early stage for potential intervention and theranostics (*4,5*).

Till now, many molecular probes have been developed for inflammation and fibrosis imaging in CVDs (*6,7*). However, the mechanism of crosstalk between immune cells and fibroblasts is not fully understood. Detection of the early onset of immune response and wound healing process, such as activation of immune cells and subsequent fibrotic response, is underexplored. Herein, we briefly discuss current imaging research on targets upregulated in the molecular and cellular pathways of the immune-fibrosis crosstalk network. We focus on positron emission tomography (PET) and single photon emission computed tomography (SPECT) radiotracers due to their high sensitivity, quantitative measurement, and well-established translational strategies (*4,8,9*).

IMMUNE CELLS INVOLVED IN INFLAMMATION, TISSUE REPAIR AND FIBROGENESIS

Cardiovascular diseases arise from various types of injurious stimuli on heart and/or blood vessels, either acute (e.g. ischemia/reperfusion (I/R) injury in MI) or chronic (e.g. cholesterol deposition for atherosclerosis). Following injury, the immune system is activated and initiates a wound healing process to minimize damage and restore function to injured tissues (Fig. 1) (*1-3,10-12*). Within minutes of injury, damaged, stressed, and dying cells release damage-associated molecular patterns, which bind to pattern recognition receptors (PRRs), including toll-like receptors and receptors for advanced glycation end products, which are expressed on surviving adjacent cells and leukocytes. Stimulation of these PRRs activates complementary signaling pathways for not only pro-inflammatory cytokines and chemokines, but also cell adhesion molecules. These inflammatory mediators promote the recruitment of leukocytes, including neutrophils and proinflammatory monocytes expressing high levels of Ly6C ($Ly6C^{high}$ monocytes in mice), to remove damaged cells by efferocytosis and release enzymes (proteases and oxidases) for tissue digestion. Following the clearance of neutrophils, monocytes expressing low levels of Ly6C ($Ly6C^{low}$ monocytes in mice) are recruited to the lesion and differentiate into reparative macrophages, which secret anti-inflammatory mediators such as TGF-β and IL-10 to promote myofibroblast and vascular cell infiltration for tissue repair and regeneration. Macrophages interact with fibroblasts via secreting cytokines, chemokines and other factors such as high levels of MMPs, which cause extensive matrix breakdown, altering the mechanical properties of the tissues to increase

the expression of TNF-α, TGF-α, and TGF-β (*13*). Additionally, the loss of IL-1β and IL-10 expression during proliferative phase allows fibroblasts to transdifferentiate into myofibroblasts, which produce ECM proteins to help maintain the structural integrity of injured tissues (*14*). During this dynamic and phasic process, crosstalk between immune system and fibrosis plays crucial role in regulating the secretion of pro-inflammatory and anti-inflammatory mediators, fibrogenesis, remodeling and tissue repair. Therefore, the real-time detection of biomarkers overexpressed by immune cells and fibroblasts and targets elevated during their interactions may facilitate the comprehension of the underlying mechanism of the crosstalk and illuminate the discovery of targeted treatment for timely intervention to improve patient outcome (*3*).

MOLECULAR IMAGING OF THE CROSSTALK BETWEEN THE IMMUNE SYSTEM AND FIBROSIS IN CVDS

Immune Cell Imaging

Due to the elevated expression of CXCR4 on multiple leukocytes following cardiovascular/cardiac injury, much effort has been devoted to the development of CXCR4-targeting radiotracers. At day 3 post I/R injury in mice, ^{68}Ga-Pentixafor uptake was determined at the site of infarct, with signal proportional to leukocyte infiltration (*6*). In humans, ^{68}Ga-Pentixafor demonstrated heterogeneous PET signals in hearts between day 4 and 6 post MI, suggesting alternative regulation of chemokine signaling and inflammatory response (*15*). Though the combination with plerixafor for targeted

intervention, improved treatment efficacy was observed in MI mice when treatment was administrated at high ^{68}Ga-Pentixafor uptake compared to those at low PET signals. This was illustrated with improved LV remodeling and cardiac function measured at 6 weeks post MI, as well as less neutrophils and Ly6C^{high} monocytes in LV (*16*), which highlighted the importance of CXCR4 PET measuring the spatiotemporal distribution of CXCR4+ cells to optimize the treatment outcome.

Monocytes and macrophages are indispensable effector cells involved in tissue repair and remodeling. The remarkable heterogeneity of macrophage populations in CVDs is well-documented, and encompasses their distinct functions in promotion of inflammation, tissue repair and regeneration, and inflammation resolution (*1*). Due to the dynamic variation of macrophage lineage populations, spatiotemporal detection of macrophage subtypes could facilitate the understanding of their identities, origins, and functions along the initiation and progression of the inflammation-fibrosis axis.

Following MI in mice, the composition and ontogeny of macrophages are dramatically shifted. Ly6C^{high}, CCR2+ monocytes infiltrate the heart, replace resident cardiac macrophages (CCR2-), and differentiate into CCR2+ macrophages to stimulate proinflammatory responses, collateral tissue damage, and ultimately contribute to heart failure pathogenesis (*17*). In mice with acute autoimmune myocarditis, siRNA silencing of CCR2 significantly decreased the number of Ly6C^{high} monocytes in hearts and lead to a reduction of left ventricular fibrosis (*18*). These findings implicate the role of infiltrating CCR2+ monocytes and macrophages as important mediators of heart failure pathogenesis and the potential of CCR2-targeted therapies to improve outcomes of MI

patients. In mouse models of sterile cardiac injury, ^{68}Ga-DOTA-ECL1i specifically detected infiltrating CCR2+ monocytes and macrophages into the injured heart (Fig. 2) with a loss of signal in CCR2$^{-/-}$ mice. Tracer uptake in the injured myocardium at day 4 showed linear correlation with LV function and infarct size measured on day 28 after I/R injury, demonstrating its potential predictive value for adverse effects governed by CCR2+ leukocytes subsets (*19*). Moreover, ^{64}Cu-DOTA-ECL1i not only showed comparable imaging efficiency to ^{68}Ga-DOTA-ECL1i in mouse heart injury models (*20*), but also has been used to track CCR2+ monocytes and macrophages in atherosclerosis and other fibrotic diseases (*21,22*). Ongoing clinical studies will further evaluate the performance of ^{64}Cu-DOTA-ECL1i for tracking CCR2+ cells in humans (*23*). Recently, an ^{18}F radiolabeled small molecule was also developed for CCR2 preclinical imaging (*24*).

Matrix metalloproteinases (MMPs) are a multi-gene family of endopeptidases that selectively digest individual components of ECM. Their activities are associated with tissue remodeling including recruitment and migration of immune cells and promoting angiogenesis and apoptosis, making them attractive targets for inflammation and fibrosis imaging (*25*). An MMP-2 inhibitor, RP805 and a pan-MMP inhibitor, RYM1 were both radiolabeled with ^{99m}Tc for CVDs imaging in preclinical models using SPECT (*5,26*). In contrast to ^{99m}Tc-RP805, ^{99m}Tc-RYM1 had desirable pharmacokinetics and low blood retention. In a mouse abdominal aortic aneurysm model, ^{99m}Tc-RYM1 uptake at aneurysm correlated with CD68 macrophage and activated MMP activity, indicating its potential for inflammation and fibrosis imaging.

Besides CCR2 and MMP, a variety of radiotracers have been developed for macrophage by targeting other chemokine receptors, somatostatin receptors, translocator proteins and mannose receptors (*6,27*). Further studies are warranted using these radiotracers to image the subtypes of macrophages, shedding light on their varied roles in the inflammation-fibrosis axis.

Fibroblasts and Myofibroblasts Imaging

Fibroblasts not only modulate the recruitment of immune cells, but also regulate their behavior, retention, and survival in damaged tissue. Cardiac fibroblasts contribute to myocardial homeostasis by synthesizing and maintaining the ECM network critical for structural and functional integrity. When activated, fibroblasts express cytoplasmic actin and adhesion complexes, permitting migration to injury site. Upon differentiation, fibroblast become a phenotypically distinct cell referred as a myofibroblast, which is the key cellular effector for tissue repair and fibrogenesis (*28*). Myofibroblasts produce and deposit structural ECM proteins including collagen, fibronectin, and elastin in injured tissues. They release proteases like MMPs and their inhibitors regulating matrix remodeling. Therefore, activated fibroblasts/myofibroblasts are undisputable target cell populations for molecular imaging to predict outcomes of tissue repair and remodeling process in CVDs.

Fibroblast activation protein (FAP) exhibits a specific expression on activated fibroblasts, making it a promising cell surface biomarker for targeted imaging of fibrotic diseases. Due to its upregulation on cancer-associated fibroblasts, various radiolabeled

FAP inhibitors have been developed for tumor imaging (*29*). In a mouse model of hypertensive cardiac injury and fibrosis, depletion of FAP+ fibroblasts reduced myocardial fibrosis and restored cardiac function, indicating the potential of FAP for CVDs imaging and therapy (*29,30*). Through ^{68}Ga radiolabeling of a FAP inhibitor, ^{68}Ga-FAPI-04 specifically determined the activated fibroblasts in injured heart in a rat MI model (Fig. 3) (*31*). In humans, a retrospective analysis of ^{68}Ga-FAPI-04 imaging in cancer patients revealed an association between tracer heart uptake and LV ejection fraction, indicating its potential for risk stratification regarding early detection or progression of LV remodeling (*7,32*). Therefore, molecular imaging of activated fibroblasts and myofibroblasts have great potential for assessing the probability and complications of fibrosis in CVDs, providing information to optimize treatment, and monitoring treatment response for better management.

Owing to the pivotal role of angiotensin-converting enzyme (ACE) inhibitors in the treatment of CVDs, ACE inhibitor-based tracers are of interest for monitoring disease progression and the effectiveness of therapeutic interventions. Many ACE inhibitors, such as ^{18}F-captopril have been used to image ventricular remodeling post-MI in animal models (*9*). Moreover, the expression of angiotensin II receptor type 1 (AT1R) on fibroblasts and myofibroblasts triggered the radiolabeling of AT1R antagonists such as ^{11}C-KR31173 for post-MI remodeling and fibrosis imaging (*9*). Due to the upregulation of $\alpha_v\beta_3$ integrin on activated fibroblasts, several radiotracers have been developed (*4,11*). However, its expression on other cells, including macrophages and endothelial cells, warrants further investigation to ascertain its value for imaging activated fibroblasts.

Activated Platelets

Besides their role in hemostasis, additional functions of platelets have been uncovered in regeneration and remodeling of injured tissue, including immune cell recruitment, apoptosis, angiogenesis, and ECM formation (*33*). Activated platelets are involved in immune responses through expression of a variety of membrane receptors (e.g. CD40L) and the release of soluble inflammatory mediators (e.g. TGF-β1, CCL5, CXCL12), which further promote the production of ECM from myofibroblasts. In ST-elevation myocardial infarction patients, platelet activities were associated with adverse LV remodeling and fibrosis, indicating their potential not only as an imaging biomarker for the early assessment of tissue repair process but also therapeutic targets (*34*). Moreover, upon activation, the major platelet integrin glycoprotein GPIIb/IIIa ($\alpha_{IIb}/\beta_{IIIa}$; CD41/CD61) undergoes a conformation change, making the altered conformation a unique targeting epitope for the detection of activated platelets. Through ^{64}Cu radiolabeling, the single-chain antibody tracer ($scFv_{anti\text{-}GPIIb/IIIa}$-^{64}CuMeCOSar) revealed significantly higher uptake in the ischemic myocardium compared to the non-ischemic region in an I/R injury mouse model, suggesting its further evaluation to predict outcomes of subsequent tissue repair processes (*35*).

Targets Expressed on Thrombus

Thrombosis is a common pathology underlying ischemic heart disease, ischemic stroke, and venous thromboembolism triggered by either a mechanical injury or the rupture of an atherosclerotic plaque. Molecular imaging of the components involved in thrombus formation may afford accurate and early detection of thrombosis to minimize

the risk of complications for improved treatment (*36*). Blood coagulation factor XIII (FXIII) is an enzyme (tissue transglutaminase) that modulates fibrin crosslinking to form stable blood clots, making it a potential biomarker for cross-linked thrombi. Through ^{99m}Tc radiolabeling, the peptide-based tracer ^{99m}Tc-NC100668 revealed specific detection of active FXIII signals in the lesion of coronary microvascular disease (MVD) mouse model. The relative retention of ^{99m}Tc-NC100668 (MVD/septal region ratio) determined at 2 h was approximately 3-12-fold higher than those acquired from 3 d to 14 d post MVD, suggesting its potential for the early detection of coronary MVD associated with thrombus (*37*). Fibrin is typically upregulated in fresh thrombi and gradually replaced by collagen and other fibrotic protein, making the detection of fibrin an attractive strategy for identification of thrombosis and fibrosis. A ^{64}Cu radiolabeled fibrin binding probe (^{64}Cu-FBP8) demonstrated favorable thrombus uptake, background clearance, and imaging efficacy in preclinical models, and has been translated for human imaging (*36*).

UNDEREXPLORED MOLECULAR IMAGING OF ADAPTIVE IMMUNE SYSTEM IN CVDS

In addition to the innate immune system, the adaptive immune system also plays critical roles in tissue repair processes and fibrosis (*2,38*). The pivotal role of T cells modulating cardiac fibroblasts and MMP activity have been demonstrated in CVDs including heart failure, myocardial fibrosis, ischemia, and MI (*39*). The recent popularity of cancer immunotherapy has prompted the development of a range of T cell imaging

probes (*40*), which could be utilized to image subset T cells to investigate the underlying mechanisms of tissue repair and fibrosis.

CONCLUSIONS AND FUTURE DIRECTIONS

Tissue repair and fibrosis are governed by the immune system. Balance between inflammatory and preparative immune responses guides the optimal tissue repair process. Thus, the immune-fibrosis axis is an unquestionable target for molecular imaging and immunomodulatory therapy. PET and SPECT imaging has shown great promise for visualizing signatures of the immune system, allowing insight into whether injured tissue will be properly repaired or subject to subsequent pathological fibrosis. To date, a variety of radiotracers have been developed to detect the immune response and fibrosis in CVDs. Additional research needs to focus on the sensitivity and specificity of these radiotracers detecting the subtype of immune cells and fibroblasts. Moreover, longitudinal studies are required to uncover the connection between measured immune system activity and resulting fibrosis. Through the combination of multiple imaging agents targeting a range of biomarkers upregulated during the immune-fibrosis network, these studies will provide quantitative measurement of early onset immune response, fibroblast activity, and subsequent pathological fibrosis to elucidate the mechanism of crosstalk between the two systems and highlight the predictive value of molecular imaging. The early, sensitive, and specific detection of malfunctioning pathways causing pathological fibrosis within the crosstalk network will enable the identification of potential therapeutic targets and provide real-time guidance to anti-fibrotic or targeted immunomodulatory therapy. The

multimodality imaging using PET/MR has great potential to differentiate subtypes of fibrosis (e.g. replacement vs reactive fibrosis) and provide information on cellular and molecular profiles in those fibrotic lesions for better management. Following establish pathway for radiotracer translation for first-in-human study (*22*), These imaging strategies may hold the potential to decipher the heterogeneity of fibrotic diseases in patients for individualized treatment.

Taken together, we envision a critical role of molecular imaging within the immune-fibrosis network to delineate the functions and interaction of immune cells and fibroblasts along the pathogenesis of fibrotic processes to better elucidate the mechanisms of CVDs. The information we gather from on-going clinical studies or future translational research will not only facilitate the development of diagnostic agents to phenotype and risk stratify patients, but also promote the discovery of novel therapeutic agents for targeted treatment.

DISCLOSURE

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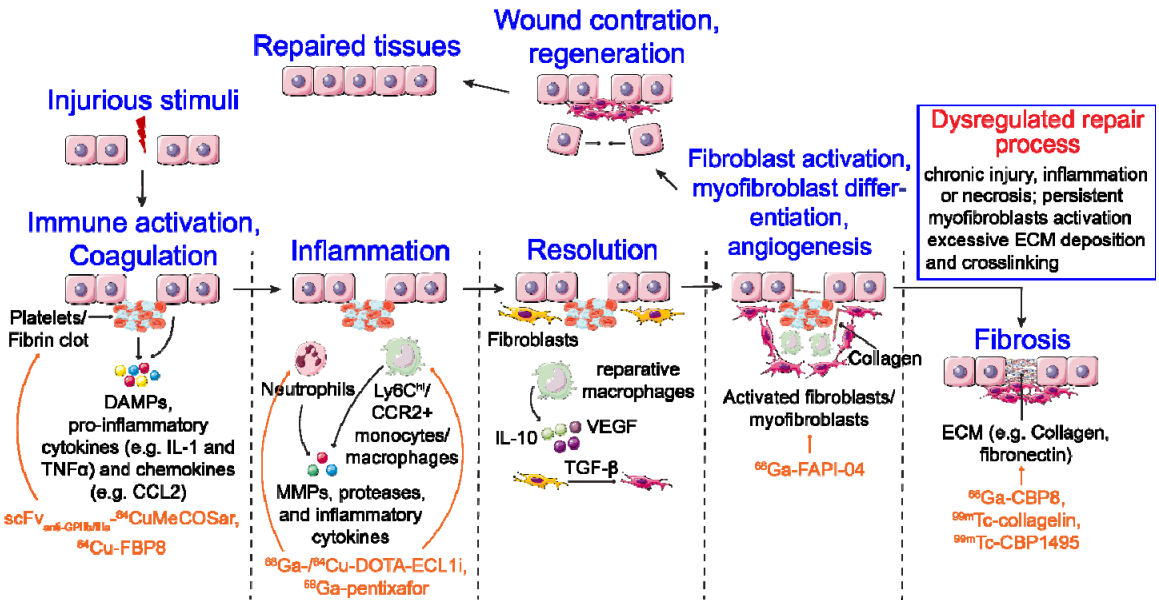

FIGURE 1. Molecular and cellular processes of immune cells involved in inflammation, tissue repair and fibrosis. (Edited with permission of (*4*).)

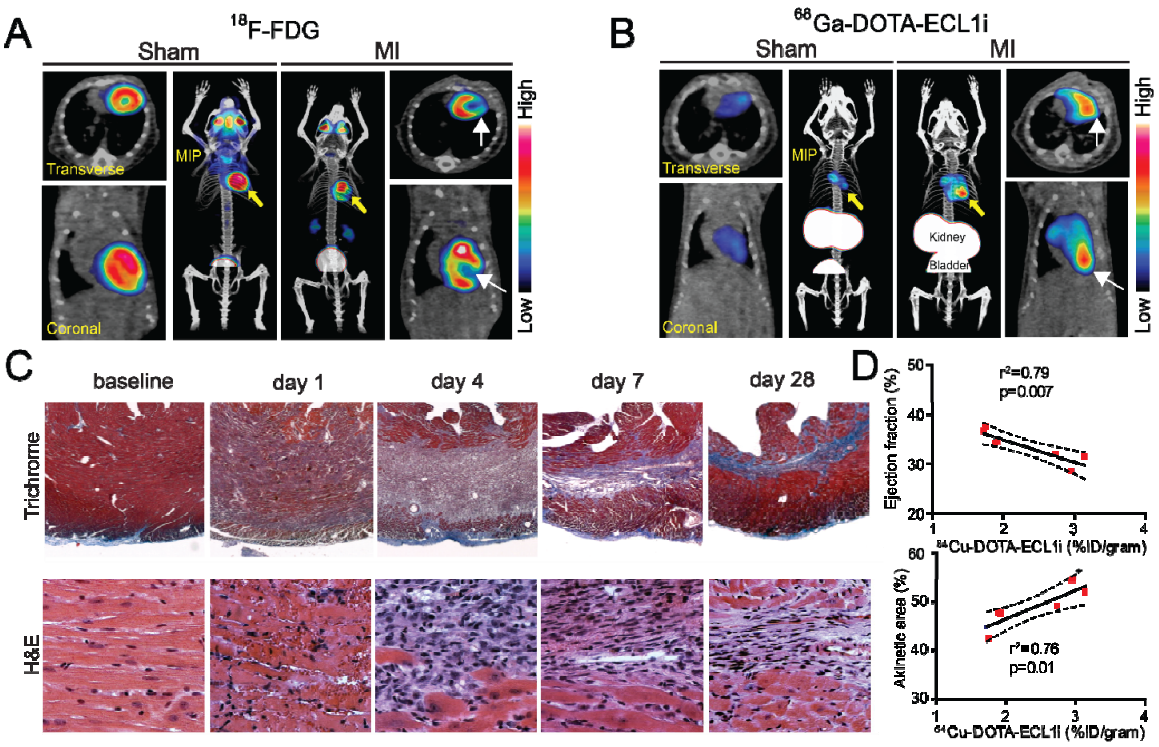

FIGURE 2. PET of ^{68}Ga-DOTA -ECL1i in a mouse model of closed-chest I/R injury. (A) Representative ^{18}F-fluorodeoxyglucose (^{18}F-FDG) PET/CT images obtained 5 d after 90 min of I/R injury identifying the infarct region in mice that underwent I/R compared with sham controls. Transverse, coronal, and maximal-intensity projected (MIP) views are shown, and white arrows denote the infarct area. (B) ^{68}Ga-DOTA-ECL1i PET/CT images showing regional accumulation of ^{68}Ga-DOTA-ECL1i signal in the infarct and border zone 4 d after I/R injury. Yellow arrow identifies tracer uptake in hearts that underwent I/R injury compared with sham controls. White arrows denote the infarct area as determined by ^{18}F-FDG imaging. (C) Trichrome and hematoxylin and eosin (H&E) staining show the evolution of fibrosis (trichrome-blue, ×40) and cell infiltration (H&E, ×200) over time in the closed-chest I/R injury model. Note the dense accumulation of cells within the infarct 4 d after I/R injury. (D) Linear regression

analyses showing the relationship between ^{68}Ga-DOTA-ECL1i heart uptake measured on day 4 and echocardiographic assessment of LV ejection fraction and akinetic area measured on day 28 after I/R injury. (Reprinted with permission of (*19*).)

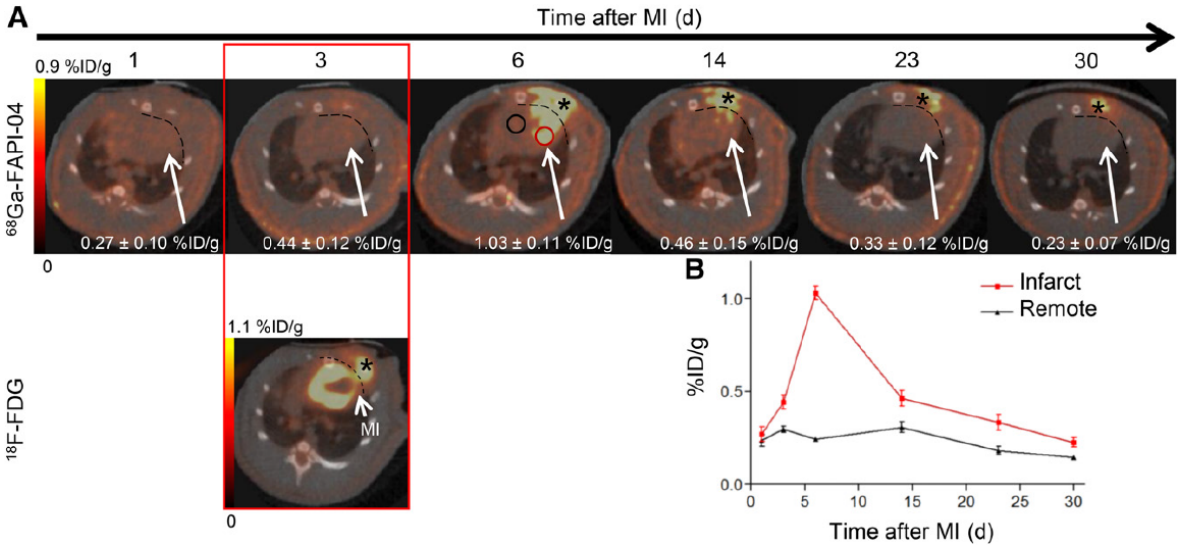

FIGURE 3. ^{68}Ga-FAPI-04 PET/CT in a rat MI model. (A) Static PET/CT matched axial slices in same rat subjected to coronary ligation and scanned 1 h after injection of ^{68}Ga-FAPI-04 (1, 3, 6, 14, 23, and 30 d after MI) and ^{18}F-FDG (3 d after MI). Dashed lines separate tracer uptake in myocardium from uptake in surgical wounds. At day 6, representative regions of interest drawn over infarct border zone and remote myocardium are illustrated as red and black circles, respectively. (B) Corresponding time–activity curves for infarcted and noninfarcted heart tissue (mean±SD, n = 3). ^{68}Ga-FAPI-04 and ^{18}F-FDG exhibited elevated uptake in scars from operation (asterisk). (Reprinted with permission of (*31*).)