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REVIEW TOPIC OF THE WEEK

Imaging Systemic Inflammatory Networks in Ischemic Heart Disease

Matthias Nahrendorf, MD,* Stefan Frantz, MD,†‡ Filip K. Swirski, PhD,* Willem J.M. Mulder, PhD,§‖ Gwendalyn Randolph, PhD,¶ Georg Ertl, MD,†# Vasilis Ntziachristos, PhD,** Jan J. Piek, MD,†† Erik S. Stroes, MD,‖ Markus Schwaiger, MD,‡‡ Douglas L. Mann, MD,§§ Zahi A. Fayad, PhD§

ABSTRACT

While acute myocardial infarction mortality declines, patients continue to face reinfarction and/or heart failure. The immune system, which intimately interacts with healthy and diseased tissues through resident and recruited leukocytes, is a central interface for a global host response to ischemia. Pathways that enhance the systemic leukocyte supply may be potential therapeutic targets. Pre-clinically, imaging helps to identify immunity's decision nodes, which may serve as such targets. In translating the rapidly-expanding pre-clinical data on immune activity, the difficulty of obtaining multiple clinical tissue samples from involved organs is an obstacle that whole-body imaging can help overcome. In patients, molecular and cellular imaging can be integrated with blood-based diagnostics to assess the translatability of discoveries, including the activation of hematopoietic tissues after myocardial infarction, and serve as an endpoint in clinical trials. In this review, we discuss these concepts while focusing on imaging immune activity in organs involved in ischemic heart disease. (J Am Coll Cardiol 2015;65:1583–91) © 2015 by the American College of Cardiology Foundation.

Although acute myocardial infarction (AMI) mortality has declined, cardiovascular patients increasingly face reinfarction and/or the development of heart failure. There are over 20 million patients with heart failure worldwide, underscoring the need to prevent heart failure. Basic science progress in the last decade revealed that local cardiac repair after ischemia is influenced by macrophage function and systemic leukocyte supply (1). Here, we argue that expanding knowledge of the immune system's role provides opportunities for improving ischemic heart disease management. Regulating immune activity may reduce post-myocardial infarction (MI) heart failure and reinfarction. Because extensive pre-clinical and translational research has shed light on immunity in

From the *Center for Systems Biology, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts; †Comprehensive Heart Failure Center, Universitätsklinikum Würzburg, Würzburg, Germany; ‡Universitätsklinik und Poliklinik für Innere Medizin III, Universitätsklinikum Halle, Halle (Saale), Germany; §Translational and Molecular Imaging Institute, Department of Radiology, Icahn School of Medicine at Mount Sinai, New York, New York; ‖Department of Vascular Medicine, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands; ¶Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, Missouri; #Medizinische Klinik und Poliklinik I, Universitätsklinikum Würzburg, Würzburg, Germany; **Institute for Biological and Medical Imaging, Technische Universität München and Helmholtz Zentrum München, Neuherberg, Germany; ††Department of Cardiology, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands; ‡‡Department of Nuclear Medicine, Klinikum rechts der Isar, Technische Universität München, Munich, Germany; and the §§Center for Cardiovascular Research, Division of Cardiology, Department of Medicine, Washington University School of Medicine, St. Louis, Missouri. Dr. Frantz has received a grant from the Federal Ministry of Education and Research (BMBF). Dr. Ertl has served on steering committees for Novartis, Bayer, and Pfizer. Dr. Ntziachristos is a shareholder in iThera Medical GmbH. Dr. Piek has served on the medical advisory board of Abbott Vascular; and has served as a consultant to Miracor. Dr. Schwaiger has received a research grant from the German Research Foundation. All other authors have reported that they have no relationships relevant to the contents of this paper to disclose. Drs. Nahrendorf and Frantz contributed equally to this work. James De Lemos, MD, served as Guest Editor for this paper.
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ABBREVIATIONS AND ACRONYMS

AMI = acute myocardial infarction

CMR = cardiac magnetic resonance

FDG = fluorodeoxyglucose

IVM = intravital microscopy

PET = positron emission tomography

USPIO = ultrasmall superparamagnetic iron oxide nanoparticles

atherosclerosis and its comorbidities, clinical progress now appears to be within reach. The first clinical trials investigating neutralizing interleukin-1β (2) and modulating cellular immune responses with low-dose methotrexate (3) are bellwethers for immune targeting strategies in cardiovascular medicine.

THE CELLULAR IMMUNE SYSTEM IN HEALTHY CARDIOVASCULAR ORGANS

The cardiovascular system is in constant, intimate contact with immune cells, and does far more than transport circulating leukocytes and cytokines. The vascular endothelial layer regulates cell recruitment, signaling the stroma's status to circulating leukocytes via expression of adhesion molecules and chemokines. Some immune cells, including monocytes and neutrophils, patrol the endothelium by crawling along or below its surface (4–7). The endothelium is less of a barrier than previously thought: even in steady state, leukocytes extravasate. Extravasation is subject to circadian rhythms and may be partly regulated by nervous signals (8). There is a surprisingly dense network of tissue-resident leukocytes (9) in healthy vascular (10) and myocardial tissue (11–14). Tissue-resident macrophages are numerous and create a close-knit network interspersed within the stroma. The high number and organization of the cells, whose delicate dendrites increase their reach and surface area, could only be observed using the recently-devised technique of applying fluorescence microscopic imaging to thicker cardiac tissue sections (**Figure 1**). Expression of bright fluorescence reporters in tissue-resident macrophages revealed their number and organization in the hearts of transgenic mice (12,14). These cells are assumed to pursue sentinel functions and support stromal cells' tissue-specific tasks.

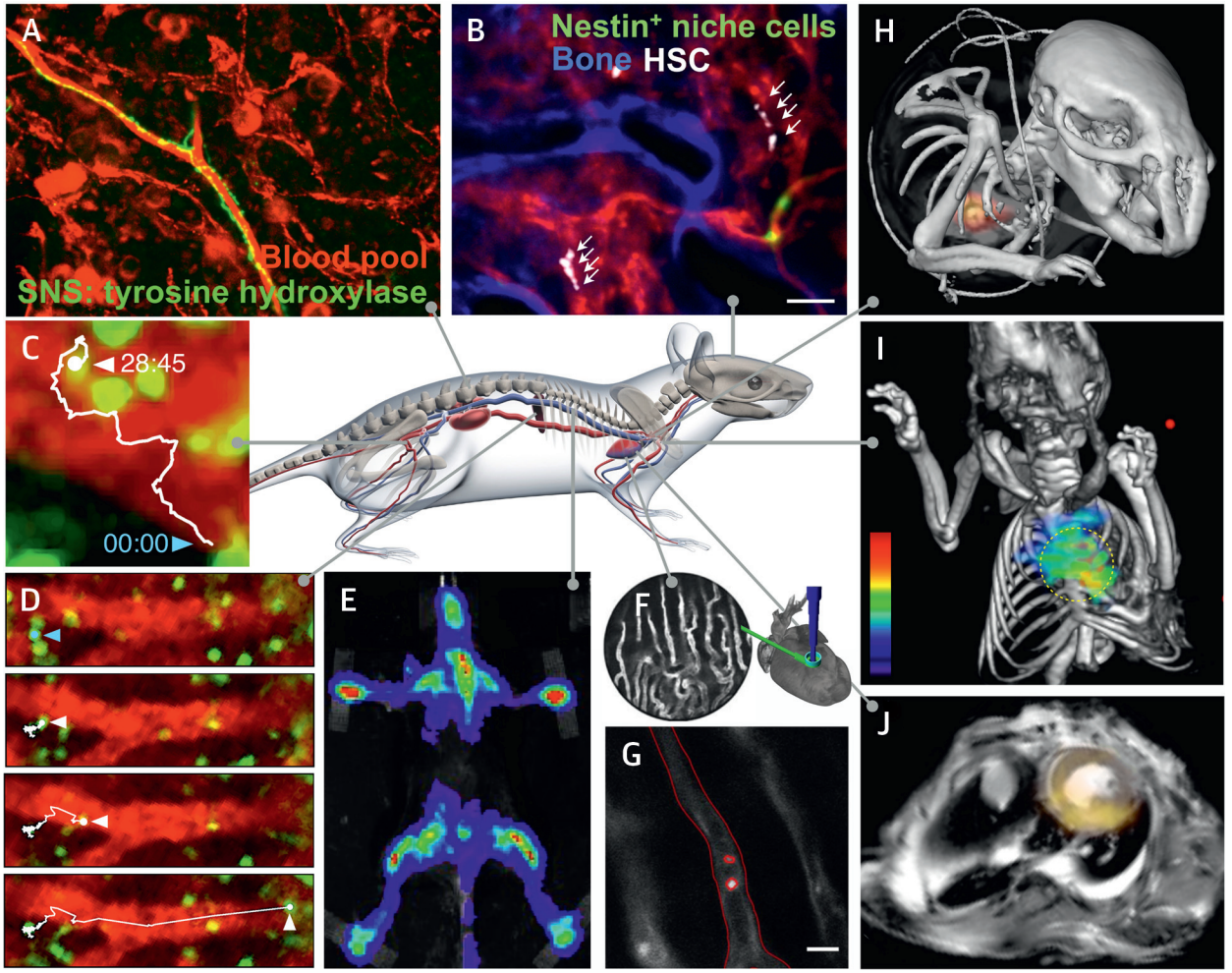
THE CELLULAR IMMUNE SYSTEM IN ATHEROSCLEROSIS

Immune cell phenotype, number, and function in the arterial wall change drastically in atherosclerosis. Macrophages increase 20-fold in apolipoprotein $E^{-/-}$ mice on a Western diet (10). Pre-clinical (15,16) and clinical (17) studies show that activated endothelium recruits monocytes, neutrophils, and lymphocytes, even in the early stages of atherosclerosis (1,18,19). Innate immune cells should remove cholesterol deposits from the intima, but they fail to do so, and instead give rise to inflamed plaques. Monocytes adopting inflammatory phenotypes differentiate into macrophages and foam cells and engage in tissue destruction by releasing cytokines and proteases that weaken the stromal architecture (20). Prototypical sequelae include plaque rupture and erosion, with subsequent thrombotic stenosis or occlusion of arteries and tissue ischemia. Dendritic cells and lymphocytes also participate in disease progression or regulation (21). Presentation of autoantigens, including oxidized lipoproteins, to lymphocytes may trigger their proliferation and activation (18). B cells pursue regulatory functions (21), for instance, by instructing increased innate immune cell production (22). In addition, regulatory T cells have protective roles (23). Imaging data support many of these insights. Pre-clinical optical imaging, especially intravital microscopy (IVM), shows immune cell interactions with stromal cells and with each other. In the past, the rigorous and rapid motion of cardiovascular organs posed a problem for IVM; however, recent progress in tissue stabilization, gating image acquisition, and/or reconstruction has overcome these hurdles (24,25) (**Figure 1**).

THE IMMUNE SYSTEM IN AMI

Whereas atherosclerotic lesions develop due to chronic low-grade inflammation, the ischemic events triggered by atherosclerosis induce high-amplitude acute inflammatory responses. In patients and in mice, AMI triggers robust blood leukocytosis (1). These events have recently been studied in more detail, and we now understand which immune cells or cell subsets react to MI. The changes observed in blood are the "tip of the iceberg": circulating components mirror systems-wide increases in immune cell numbers and activity in the ischemic myocardium, the remote nonischemic myocardium, nonculprit plaques, heart-draining lymph nodes, the spleen, and bone marrow. Sampling blood reveals immune cell migration from storage or production sites to the atherosclerotic plaque or failing myocardium. Tissue is more difficult to assay than blood. Imaging and flow cytometry of digested myocardium and arteries enable quantitative approaches and provide the cell recruitment timeline after ischemic injury. The most numerous and fastest responders are innate immune cells. Neutrophils and the inflammatory monocyte subset begin to infiltrate the distressed myocardium within the first 30 min after ischemia onset (24), while resident macrophages disappear (14), most likely due to local death or emigration. The neutrophil response wanes quickly, and inflammatory monocytes continue to infiltrate at high rates for the first 4 days after ischemia (11). The number of

FIGURE 1 Preclinical Cardiovascular Immune Imaging



(A) Confocal microscopy of sympathetic nerve fibers in the bone marrow (48). **(B)** Intravital microscopy (IVM) of adoptively-transferred, fluorescently-labeled hematopoietic progenitor cells in the bone marrow (48). **(C)** IVM of a patrolling monocyte (74). **(D)** IVM of a departing splenic monocyte (43). **(E)** Bioluminescence imaging of cell cycling in the bone marrow after ischemic injury (48). **(F and G)** IVM of the beating mouse heart visualizes leukocytes in myocardial capillary. A tissue stabilizer is also shown (25). **(H)** Positron emission tomography / magnetic resonance imaging of macrophages in atherosclerotic plaque using isotope-labeled nanoparticles (75). **(I)** Protease fluorescence tomography in a mouse with atherosclerosis (37). **(J)** Positron emission tomography/magnetic resonance imaging of macrophages in a mouse with myocardial infarction using isotope-labeled nanoparticles (Nahrendorf, unpublished data, 2012). HSC = hematopoietic stem cells; SNS = sympathetic nervous system.

immune cells increases by 2 orders of magnitude in ischemic versus steady-state myocardium. Interestingly, recruited myeloid cells only survive in ischemic tissue for an average of 20 h in the mouse (26), highlighting the high turnover of cells in the infarct. Inflammatory monocytes, which differentiate to M1-like macrophages, pursue proteolysis of pre-existing extracellular matrix and phagocytosis of dying cells. After 4 days, there is a transition to a second, less inflammatory phase (11), during which low numbers of patrolling Ly6C^{low} monocytes are also recruited, and Ly6C^{high} monocytes give rise to macrophages with fewer inflammatory functions (27). Macrophages now support tissue rebuilding; crosstalk to stromal cells via vascular endothelial growth factor and transforming growth factor β supports angiogenesis and production of new extracellular matrix, respectively. These 2 phases were discovered in the mouse (11,28), but have since been observed in the blood (29) and heart (30) of humans with AMI. If 1 of these phases is compromised, for example, by an overzealous supply of inflammatory cells delaying the

transition to inflammation resolution, infarct healing deteriorates and heart failure occurs (31). In patients, post-MI blood monocyte levels predict cardiovascular mortality (32). When viewed together with the pre-clinical data, this association underscores the putative role of innate immune cells as a therapeutic target. Lymphocytes also participate in the post-MI immune response, albeit at lower numbers. Regulatory T cells hasten the transition from the inflammatory to the resolution phase by supporting a switch toward M2-type macrophage phenotypes (33), whereas B cells regulate the migratory patterns of myeloid cells, including their release from bone marrow (34).

SOURCES OF INNATE IMMUNE CELLS

Neutrophils and monocytes, the progenitors of inflammatory macrophages in the infarct and atherosclerotic lesions, are made in the bone marrow (35). The spleen also has myelopoietic functions in mice with atherosclerosis (36) and after MI (26). Before birth, the spleen and liver are sources of blood cells, but thereafter, they cease hematopoietic activity in the steady state. In certain diseases (at least in rodent models), the spleen regains the capacity to produce innate immune cells (1). Fluorescence imaging indicates that this occurs after hematopoietic progenitor and stem cells transfer from the bone marrow to the spleen (37). Although we are beginning to understand the signals that lead to bone marrow release and splenic seeding of hematopoietic stem cells in mice, translational data remain scarce, and are mostly limited to cell numbers in blood (38,39). Interestingly, recent data obtained on tissue-resident macrophages, that is, the cells that inhabit the myocardium and vascular wall in the steady state, suggest that many of these cells derive not from blood monocytes, but rather from proliferating macrophages in situ (13,14). Likewise, plaque macrophages, which derive from recruited blood monocytes, proliferate locally (40). The signals that regulate cell supply are of interest for future therapeutic strategies because locally-sourced tissue-resident macrophages may have different functions than macrophages derived from the bone marrow or spleen. Once we understand the pathways involved in the information transfer from ischemic tissue to the sites of leukocyte production, we can begin to explore whether these pathways provide opportunities to therapeutically target inflammation in ischemic heart disease. One such pathway may be the nervous system, which instructs hematopoietic tissues in AMI (37).

CHANCES AND CHALLENGES

Pre-clinical data provide a convincing rationale for the causal role of innate immunity in disease progression, including atherosclerotic lesion growth and post-ischemic complications, such as reinfarction and heart failure. However, this knowledge has not yet been harnessed to develop therapeutics. Distinct components of the immune system may either preserve health (e.g., tissue-resident macrophages, regulatory T cells) or promote disease (e.g., inflammatory monocyte-derived plaque macrophages, innate response activator B cells). Whether their functions are beneficial or not may also depend on the disease phase. Thus, broad immune pathway targeting is unlikely to confer therapeutic benefits, a lesson learned from trials inhibiting tumor necrosis factor in heart failure (e.g., the RECOVER and RENAISSANCE trials [41]). In addition, immune functions that are harmful for cardiovascular health may be essential for defense against microbes. Developing immune-targeted therapeutics in ischemic heart disease involves identifying suitable pathways in mice, pre-clinical testing of whether the pathway can be neutralized without causing collateral damage, translational studies identifying similarly relevant pathways in humans, and, finally, clinical trials.

We argue that this long drug discovery path can be negotiated more efficiently with the help of immune system imaging, especially of immune cells, their subsets, and their migration, production, and function. More specifically, imaging can report on stromal immune cell activity in multiple tissues in living patients. In addition to coronary imaging (recently reviewed by Garcia-Garcia et al. [42]), as comorbidities aggravate cardiovascular disease via inflammatory crosstalk, noncardiovascular organs may also be of interest and, therefore, constitute another putative therapeutic target. To understand systems-wide immune action after MI, particularly connections of cardiovascular with immune and hematopoietic organs, future imaging studies should focus not only on the ischemic heart, but also on remote myocardium, local lymph nodes, nonculprit atherosclerotic lesions, spleen, and bone marrow, and integrate these data with analysis of blood. Ideally, molecular imaging could also probe modes of information transfer, such as the nervous or endocrine systems, which regulate immune cell production, storage, migration, and function. While it is impossible to attain these comprehensive data through patient biopsies, cellular and molecular whole-body imaging reports on many tissues simultaneously. These methods can

translate pre-clinical findings, serve as companion imaging in clinical trials, and guide individualized therapy.

Currently, large-scale cardiovascular trials use survival as a hard endpoint. Although this is the ultimate test of a therapy's value, it is so expensive that these trials often become unaffordable (43). Each failed trial increases research and development costs, ultimately reducing the capacity to pursue such projects. Companion imaging may provide faster, much more cost-effective surrogate endpoints that also report on drug action. Although survival endpoint data are likely necessary, imaging trials, which can be much smaller, may provide a cost-effective intermediate step with a higher tolerance for failure. In the following text, we describe immune system imaging that could facilitate development of ischemic heart disease therapeutics.

HOW CAN PRE-CLINICAL IMAGING ACCELERATE PROGRESS?

Over the last 2 decades, pre-clinical imaging has made astounding progress. Optical imaging led to many catalyst tools that allowed us to uncover immune cell birth, activity, and function. Specifically, the combination of fluorescent reporters with IVM-facilitated discoveries such as the splenic monocyte reservoir (44) and patrolling leukocytes (4,7), i.e., monocytes and neutrophils that crawl along the endothelium in small vessels. Multicolor imaging revealed the interaction of leukocytes with each other during antigen presentation (45) or with stromal cells and pathogens. These tools were originally developed for observing cells in steady state or malignant disease, but are now being adapted for cardiovascular applications. Even in immobile solid tumors, small movements, for instance due to respiration, are amplified during cellular-scale imaging. The rigorous cardiovascular motion caused by cardiac contractions and pulsatile blood flow is a formidable hurdle that has been overcome by combining tissue stabilizers, electrocardiographic gating, and post-processing algorithms. The result is that for the first time, we can watch cells rolling though myocardial capillaries, infiltrating the infarct or atherosclerotic plaque, and interacting with each other in the vascular wall (24,25,45–47) (**Figure 1**). This is an important step toward the goal of therapeutically interfering with immune cell migration or antigen presentation. Another example of innovation through imaging involves serial observation of hematopoietic stem cells (48) that give rise to inflammatory leukocytes and do so at increased rates after organ ischemia (49) (**Figure 1**). On the whole-animal scale, optical imaging is used to study the time course of pathway activation (50) and disease progression, for instance, by serially probing protease activity, adhesion molecules, or immune cell abundance in inflamed tissue (37). Of particular value are quantitative methods, such as fluorescence tomography and bioluminescence imaging. Both detect photons that arise from either laser-excited fluorochromes (51) or reporter gene activation of bioluminescent luciferin (52). Pre-clinical work on translatable imaging methods, mostly magnetic resonance and nuclear imaging, has led to a number of promising candidate technologies (53) now awaiting first-in-human trials. Typical imaging targets in studying ischemic heart disease are macrophages, adhesion molecules, and leukocyte receptors (54). Cell-specific expression of fluorescent or bioluminescent reporters enables the study of specific immune cells over time and, in the case of dual reporters, cell interactions.

HOW CAN CURRENT CLINICAL IMAGING ACCELERATE TRANSLATION?

Several agents that target immune activity have already been translated, and other agents initially developed for different applications are being repurposed successfully. One repurposed agent is ultrasmall superparamagnetic iron oxide nanoparticles (USPIO), which are sensitive because 1 particle drastically changes the behavior of many surrounding protons that give rise to magnetic resonance imaging signal. Extensive testing in mice showed that USPIO localize within plaque and infarct macrophages (55), a result later confirmed in patients after carotid endarterectomy (56). Two recent trials used USPIO to image the inflammatory response in AMI patients. Before and 48 h after injection of ferumoxytol, T2* value assessed in the infarcted and remote myocardium indicated significant nanoparticle accumulation (57,58). One study also reported a signal change in the spleen (57). In conjunction with earlier mouse studies (59), these results indicate that ferumoxytol is useful for imaging macrophages in the arterial wall and myocardium. Low signal on T2*-weighted CMR can also arise from hemorrhage, motion, and flow-related artifacts. These limitations can be addressed with pre-injection scans and positive-contrast iron imaging techniques (55).

The nuclear imaging modality positron emission tomography (PET) has a quantitative advantage and is the most sensitive. A widely-used agent, ^{18}F-fluorodeoxyglucose (FDG), accumulates

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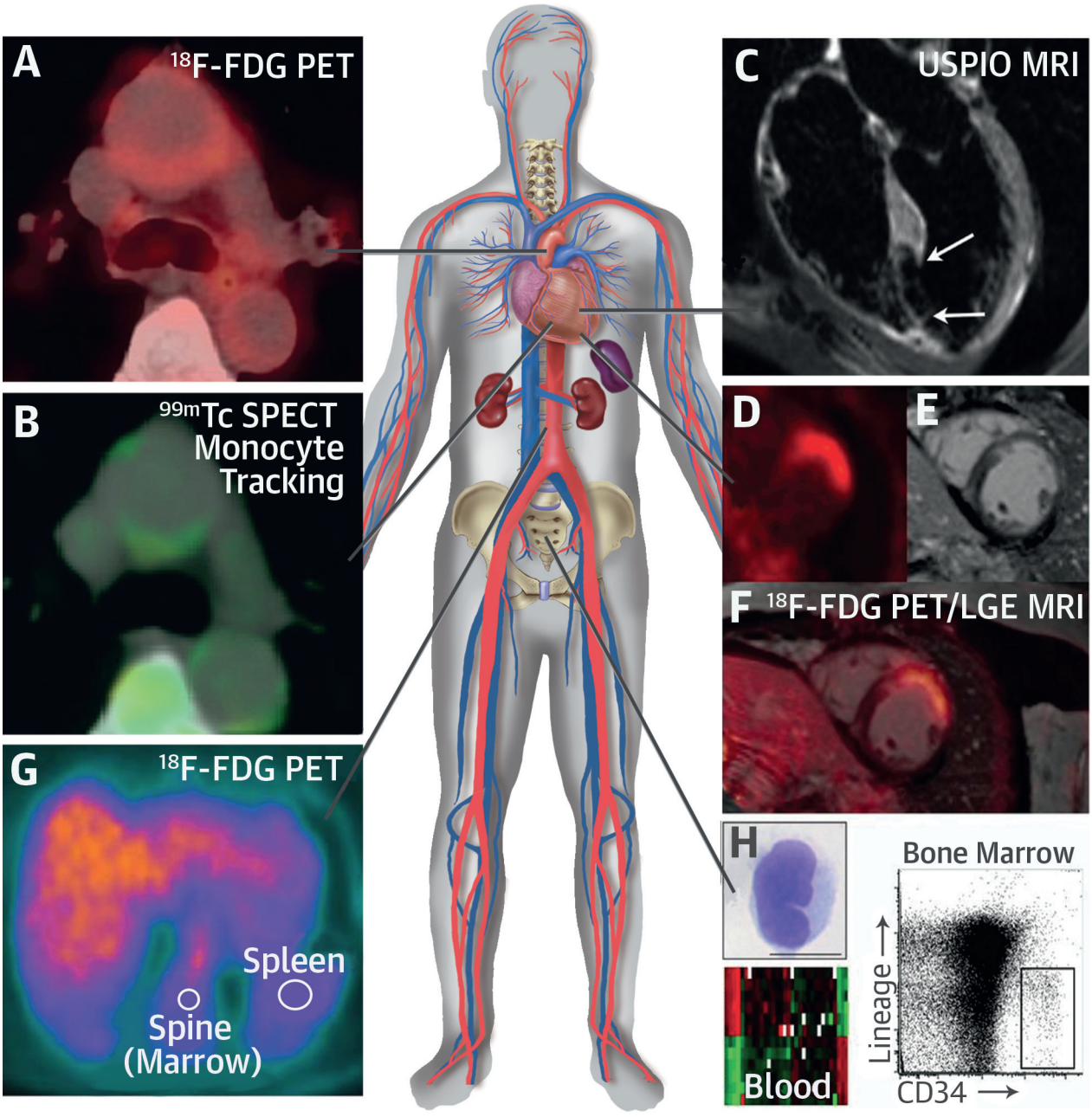
CENTRAL ILLUSTRATION **Imaging Systemic Inflammatory Networks in Ischemic Heart Disease**



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Integrated clinical datasets, including whole-body imaging, and immunological assays, such as flow cytometry of blood and bone marrow, could provide insight into the systemic immune response after acute myocardial infarction (AMI). **(A)** ^{18}F-FDG PET-CT in a patient with atherosclerosis (17). **(B)** Infiltration of ^{99m}Tc-labeled leukocytes into ^{18}F-FDG-rich aortic lesions in the same patient (17). **(C)** MRI in a patient with AMI after injection of USPIO, **arrows** indicate infarct (57). **(D)** ^{18}F-FDG PET, **(E)** LGE MRI, and **(F)** fused image in a patient with AMI (Schwaiger, unpublished data, 2014). **(G)** Uptake of ^{18}F-FDG in the bone marrow and spleen of a patient with AMI (71). **(H)** Measurements obtained in the blood and bone marrow include the number and phenotype of monocytes and their progenitors. A monocyte, a PCR array of monocytes, and a flow cytometry plot of progenitor cells are shown. FDG = fluorodeoxyglucose; LGE = late gadolinium enhancement; MRI = magnetic resonance imaging; PCR = polymerase chain reaction; PET = positron emission tomography; SPECT = single photon emission computed tomography; ^{99m}Tc = technetium-99m; USPIO = ultrasmall superparamagnetic iron oxide nanoparticles.

in metabolically-active cells and can be harnessed to image inflammation in cardiovascular organs, as immune cells, particularly macrophages, internalize ^{18}F isotope-derivatized glucose. Vascular ^{18}F-FDG imaging in patients with atherosclerosis robustly correlates with macrophage density as measured by histology (56). It is increasingly used to monitor inflammation in vascular beds as a function of therapy in patients, particularly in the aorta and carotid arteries (60–64). ^{18}F-FDG imaging in the heart, that is, in coronaries or the acutely-infarcted myocardium, is more complex, as distressed myocytes rely on glycolysis and avidly ingest the agent. Myocyte ^{18}F-FDG uptake can be attenuated with meals low in carbohydrates on the day before imaging, which is routinely done for cardiac PET imaging in patients with sarcoidosis. In mice with AMI, the majority of infarct ^{18}F-FDG signals derive from immune cells (65). In patients, however, macrophage-enriched ischemic tissue is more heterogeneously distributed than in mice after coronary ligation. Islands of surviving myocytes interspersed with inflamed necrotic areas may compound the interpretation of ^{18}F-FDG data. Ongoing work should clarify these questions, and hybrid PET/CMR will amplify our understanding of signal sources by comparing simultaneously acquired ^{18}F-FDG with USPIO data. In addition, a number of promising immune imaging approaches have been applied to ischemic heart disease, including imaging integrins expressed by leukocytes and stromal cells during angiogenesis (66), imaging leukocyte receptors (67,68), calcium imaging with sodium fluoride (69), and fluorine-19 perfluorocarbon CMR (70).

WHAT IS ON THE HORIZON FOR CLINICAL IMAGING IN ISCHEMIC HEART DISEASE?

Several immune-targeting imaging agents are in the pipeline, including protease reporters, PET isotope-labeled peptides, nanoparticles, and minibodies. These will be more specific than currently-available agents and will increase our ability to measure the abundance of leukocytes and their subsets, adhesion molecules that recruit leukocytes into tissue, and targets related to leukocyte function in cardiovascular tissues. Recently published clinical studies show that even currently-approved tools can provide insight into the pathophysiology of ischemic heart disease. For instance, a SPECT/CT study in patients with atherosclerosis (17) tracked autologous peripheral blood mononuclear cells to atherosclerotic plaques that were also high in ^{18}F-FDG PET signal, implying that leukocytes migrate to inflamed atherosclerotic lesions in patients. Several small ^{18}F-FDG PET/CT studies in patients with AMI reported increased PET signals in ischemic myocardial regions in association with higher ^{18}F-FDG uptake in remote nonculprit atherosclerotic plaque, as well as in hematopoietic organs (39,71–73), highlighting the systemic inflammation that follows organ ischemia, as previously described in pre-clinical models. A retrospective trial in patients with atherosclerosis reported that increased splenic ^{18}F-FDG predicts higher cardiovascular event rates (73). These trials exemplify the opportunities generated by whole-body imaging, including the ability to sample more than 1 organ system. A next step could include characterizing inflammatory networks, for instance, by integrating PET data in multiple target tissues (bone marrow, spleen, heart, plaque) with MRI (USPIO uptake in infarct, functional data on left ventricular wall motion) and studies of inflammatory chemokines and circulating cells in blood during imaging (**Central Illustration**). In addition, targeted imaging of pathways that confer risk and interfere with the immune system, such as sympathetic nervous signaling (74), may allow us to simultaneously assess information transfer between involved organ systems.

Imaging's recent evolution toward quantitative reporting on leukocytes and molecules will most assuredly accelerate the expansion of our knowledge regarding the immune system's role in ischemic heart disease. Directing imaging toward this task requires a wide range of expertise spanning hematology, cellular immunology, clinical cardiology, isotope physics, optical physics, and imaging hardware development. Because no one person unifies this knowledge, multidisciplinary teams are necessary to advance the field. The authors of this paper formed a collaboration (75) to focus on deciphering systemic inflammatory networks in ischemic heart disease. Our goals include the adoption of imaging for basic discovery, clinical translation, and testing of immunomodulatory interventions in AMI.

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REPRINT REQUESTS AND CORRESPONDENCE: Dr. Matthias Nahrendorf, Center for Systems Biology, Massachusetts General Hospital, Harvard Medical School, Simches Research Building, 185 Cambridge Street, Boston, Massachusetts 02114. E-mail: mnahrendorf@partners.org.

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