Noninvasive Contrast-Enhanced Ultrasound Molecular Imaging Detects Myocardial Inflammatory Response in Autoimmune Myocarditis

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Background—Cardiac tests for diagnosing myocarditis lack sensitivity or specificity. We hypothesized that contrast-enhanced ultrasound molecular imaging could detect myocardial inflammation and the recruitment of specific cellular subsets of the inflammatory response in murine myocarditis.

Methods and Results—Microbubbles (MB) bearing antibodies targeting lymphocyte CD4 (MB_CD4), endothelial P-selectin (MB_Psel), or isotype control antibody (MB_Iso) and MB with a negative electric charge for targeting of leukocytes (MB_Lc) were prepared. Attachment of MB_CD4 was validated in vitro using murine spleen CD4+ T cells. Twenty-eight mice were studied after the induction of autoimmune myocarditis by immunization with α-myosin-peptide; 20 mice served as controls. Contrast-enhanced ultrasound molecular imaging of the heart was performed. Left ventricular function was assessed by conventional and deformation echocardiography, and myocarditis severity graded on histology. Animals were grouped into no myocarditis, moderate myocarditis, and severe myocarditis. In vitro, attachment of MB_CD4 to CD4+ T cells was significantly greater than of MB_Iso. Of the left ventricular ejection fraction or strain and strain rate readouts, only longitudinal strain was significantly different from control animals in severe myocarditis. In contrast, contrast-enhanced ultrasound molecular imaging showed increased signals for all targeted MB versus MB_Iso both in moderate and severe myocarditis, and MB_CD4 signal correlated with CD4+ T-lymphocyte infiltration in the myocardium.

Conclusions—Contrast-enhanced ultrasound molecular imaging can detect endothelial inflammation and leukocyte infiltration in myocarditis in the absence of a detectable decline in left ventricular performance by functional imaging. In particular, imaging of CD4+ T cells involved in autoimmune responses could be helpful in diagnosing myocarditis. (Circ Cardiovasc Imaging. 2016;9:e004720. DOI: 10.1161/CIRCIMAGING.116.004720.)

Key Words: cardiomyopathy ◼ contrast media ◼ echocardiography ◼ leukocyte ◼ myocarditis

M yocarditis is characterized by an inflammatory infiltration of the myocardium with myocyte degeneration and necrosis in the absence of ischemia.1 Infectious agents, systemic diseases, drugs, and toxins cause myocarditis.2 Myocarditis has been found in 1% to 9% of autopsies and in a higher proportion (5% to 12%) in young individuals with sudden unexplained cardiac death.3,4 This is in contrast to the perceived low frequency of a diagnosis of myocarditis in patients. The clinical presentation of myocarditis is variable, and routine cardiac tests either lack sensitivity (ECG) or specificity (troponins) for diagnosing myocarditis. Endomyocardial biopsy is the gold standard for securing the diagnosis of a suspected myocarditis.5 However, endomyocardial biopsy is invasive, susceptible to sampling error and suffers from low sensitivity and a high interobserver variability in interpretation.6 Thus, there is a need for noninvasive imaging methods for the diagnosis and characterization of myocarditis. Echocardiography, radionuclide imaging, and cardiac magnetic resonance imaging have all been used for the detection of myocarditis in clinical and preclinical studies. Echocardiographic features of myocarditis including left ventricular (LV) dilatation, segmental wall motion abnormalities, increased LV sphericity, and transient increases in LV wall thickness7–9 are limited in specificity and hence diagnostic accuracy. For nuclear imaging, Indium-111 radiolabeled antimyosin antibodies have been used for the detection of myocardial necrosis in murine models of myocarditis and in clinical studies.10–11 Whereas Indium-111 antimyosin antibody

See Editorial by Porter
See Clinical Perspective
Ultrasound Molecular Imaging of Myocarditis

imaging has a high sensitivity and negative predictive value, its clinical use is hampered by limited tracer availability, radiation exposure, and delayed imaging to prevent blood pool signal. Cardiac magnetic resonance imaging allows detection of myocardial edema or irreversible tissue damage inflicted by the inflammatory infiltrate, but sensitivity is variable and may be reduced in the absence of tissue necrosis. Animal models of both virus-induced and autoimmune myocarditis suggest that heart-specific autoimmune inflammatory responses are important in the development of chronic cardiomyopathy, and that CD4+ T cells are instrumental in driving the transition to cardiomyopathy.15–17 Recently, fluorine-19 cardiac magnetic resonance has been used for detecting the inflammatory infiltrate in murine autoimmune myocarditis. Thus, with the exception of fluorine-19 cardiac magnetic resonance, a common weakness of the aforementioned techniques is that they detect tissue injury (edema, necrosis), which may be absent in lymphocytic myocarditis or which can also be because of, for example, myocardial ischemia, rather than the inflammatory infiltrate. In the past years, microbubble (MB) ultrasound contrast agents have been modified for the purpose of molecular imaging. Either modifications in the shell properties or the conjugation of specific ligands to the MB shell surface have been used to promote adhesion of MB to disease-specific intravascular events. The feasibility of this strategy for molecular imaging in the myocardium has been proven in vivo. In this study, we hypothesized that contrast-enhanced ultrasound (CEU) molecular imaging can be used to detect (1) the inflammatory cell recruitment and endothelial inflammatory activation and (2) the recruitment of CD4+ T cells in a murine model of myocarditis.

Methods

Study Design and Animal Model

All experiments were performed in accordance with Swiss Legislation and were approved by the Animal Care Committee of Basel. A total of 52 female BALB/c mice were used. In 4 mice, specific targeting of CD4 with targeted MB was validated in vitro. In 34 mice, experimental autoimmune myocarditis (EAM) was induced by intraperitoneal injection of 400-ng pertussis toxin (List Biological Laboratories) followed by washing with PBS. The culture plates were mounted on a microscope (Olympus BX51W). A suspension of either MBIso or MBCD4 at a concentration of 3×10^6 mL^−1 was drawn through the flow chamber at a shear rate of 2 dynes/cm². After 5 minutes of continuous flow, the number of attached MB was counted in 20 optical fields (×20 magnification, total area 2.94 mm²). Experiments were performed in triplicate.

Specific attachment of MBCD4 to CD4+ lymphocytes was assessed on cell-based adhesion assays. Single-cell suspensions of murine spleen cells from 4 mice were generated using a cell strainer. CD4+ T cells were separated from the cell suspension by depletion of magnetically labeled nontarget cells using a cocktail of biotin-conjugated monoclonal antibodies and antibiotin monoclonal antibodies conjugated to MicroBeads (DiaMaTr + Cell Isolation Kit II, Miltenyi Biotec). Both the separated nontarget cells and the CD4+ cells were resuspended at 2×10⁶ cells per 100 μL. 1×10⁶ leukocytes (CD4+ or CD4−) were then incubated with 1×10⁵ MB (MBiso or MBCD4) for 10 minutes followed by washing with PBS. Microscopy at ×20 magnification (Zeiss LSM 710) was used to assess MB attachment to the cells with the investigator (E.E.) blinded to all image information. The results are presented as the average number of MB attached per cell.

Animal Instrumentation

On day 21 after immunization, the mice were anesthetized with 5% isoflurane, which was subsequently reduced to 2% and adapted to stabilize the heart rate between 350 and 450 bpm. The animal core temperature was maintained at 37°C. The chest and neck were depilated. For MB injections, the right internal jugular vein was cannulated and the animals were transferred onto a temperature controlled imaging stage (Vevo Imaging Station).

Echocardiography

High-frequency (40 MHz, MS 550D transducer) ultrasound imaging (Vevo 2100, VisualSonics Inc) was performed for the assessment of cardiac structure and function. M-Mode images of the LV at the height of the papillary muscles were used to measure anterior and posterior wall thickness, LV end-diastolic diameter, and LV end-systolic diameter. LV ejection fraction and LV mass were derived from these measurements. E-wave and A-wave velocities were measured using pulsed Doppler from a modified apical 4-chamber view. Parasternal long- and short-axis B-mode images were acquired at a frame rate of >200 frames per second. Longitudinal, radial, and circumferential global peak strain values were derived from the B-mode images using a speckle-tracking algorithm (VevoStrain, VisualSonics). Cine-loops with 2 cardiac cycles not distorted by...
respiratory movements were selected, and the endocardial and epicardial borders were traced on end-diastolic frames. After automated tracing, manual adjustments were made as necessary for good tracking throughout the cardiac cycles. The strain measurements were then averaged over the 2 cardiac cycles without the use of smoothing filters. All echocardiographic images were acquired and analyzed by the same investigator (L.X.) who was blinded to all other experimental data.

**CEU Molecular Imaging and Assessment of Myocardial Perfusion**
Contrast ultrasound imaging (Sequoia Acuson C512; Siemens Medical Systems) was performed with a high-frequency linear-array probe (15L8) held in place by a railed gantry system. The LV was imaged in short axis at the papillary muscle level. ECG-gated triggering was used to acquire end-systolic frames. For the detection of the contrast agent, power modulation and pulse inversion imaging at a frequency of 7 MHz and a dynamic range of 50 dB was used. The gain settings were adjusted to levels just below visible speckle and held constant. MBlc, MBiso, MBPSel, and MB CD4 (5x10⁶ MB per injection) were injected in each mouse in random order. Ultrasound imaging was paused from injection until 8 minutes later when imaging was resumed at a mechanical index of 0.87. The first acquired image frame was used to derive the total amount of MB present within the LV myocardium. The MB in the ultrasound beam were then destroyed with several (>10) image frames. Several image frames at a long pulsing interval (every 9 heartbeats) were subsequently acquired to measure signal attributable to freely circulating MB. After log-linear conversion using known dynamic range lookup tables, frames representing freely circulating MB were digitally subtracted from the first image to derive signal from attached MB. Contrast intensity was measured from a region of interest encompassing the whole LV myocardium. The selection of the region of interest was guided by fundamental frequency anatomic images of the LV acquired at 14 MHz. In 7 additional mice with myocarditis, imaging was performed 10 minutes after blocking of CD4 by intravenous injection of antimouse CD4 mAb (H129.19, BD Bioscience; 5 µg). After CEU molecular imaging, microvascular perfusion was assessed using constant infusions of nontargeted MB at an infusion rate of 3x10⁶ MB per minute. After a high-energy destruction impulse (5 frames, mechanical index of 1.9), end-systolic short-axis image frames of the LV were acquired over several heartbeats (>10) at a low mechanical index of 0.2. On the resulting destruction–replenishment sequences, the average signal intensity of a region of interest placed over the septal, anterior, and lateral wall was fitted to the monoexponential function \(y = A(1 - e^{-\beta t})\), where \(y\) is the signal intensity at a given time point, \(A\) is the rate of increase of the curve, which reflects myocardial blood flow velocity, and \(A\) is the plateau intensity, which is proportional to myocardial blood volume. Myocardial blood flow was estimated by the product of \(A\) and \(\beta\). For each animal, 2 to 3 destruction–replenishment curves were averaged.²⁶ CEU imaging data were analyzed blinded to all other experimental data (D.S.).

**Histology and Immunohistochemistry**
For the assessment of myocarditis severity, hematoxylin and eosiin–stained short-axis cross-sections of the hearts were analyzed by a pathologist blinded for all other experimental data. The cross-section of the LV was divided into 4 quadrants by using (1) the papillary muscles and (2) an ink mark on the posterior interventricular sulcus as landmarks. Each of the 4 quadrants was then individually scored on a scale from 0 to 4 with 0 representing no inflammatory infiltrates, 1 small foci of leukocytes between cells, 2 larger foci of >100 leukocytes, 3>10% of the quadrant cross section involved, and 4>50% of the quadrant cross section involved. In addition, an overall score for the entire cross section using the same criteria was given and used for the classification of animals.²³ For immunohistochemistry, snap-frozen heart sections were stained with antibodies to CD4 (MCA4635T, AbD Serotec). A fluorescent secondary antibody (A-11006 Alexa 488, Invitrogen) was used to visualize CD4, and the cells/mm² of tissue staining positive for CD4 were quantified.

**Statistical Analysis**
Data were analyzed on GraphPad Prism (version 7). Data are expressed as medians and 25th–75th percentiles unless stated otherwise. Comparisons between MB agents in the flow-chamber experiments were made using a Mann–Whitney rank sum test. Attachment of MB to CD4+ versus CD4− cells and MB signals within and between animal groups were compared using a Kruskal–Wallis ANOVA with Dunn post hoc test. Correlations between MB signals and CD4+ cells in tissue were assessed using Spearman correlation. A P value of <0.05 (2 sided) was considered statistically significant.

**Results**

**In Vitro Assessment of MB–CD4 Interaction**
The specific targeting of MBlc and MBPSel has been validated previously, whereas this has not been the case for MB CD4.²³ Therefore, we performed flow chamber and in vitro cell-based assays to characterize MB CD4 interactions with CD4. In a parallel plate flow-chamber system coated with CD4 protein, attachment of MB CD4 was significantly greater than for MB iso at a shear stress encountered in the myocardial microcirculation (Figure 1A). In cell-based assays, MB iso attached to CD4+ lymphocytes. Attachment to CD4− cells was 6-fold less, and similar to the attachment of MB iso to both CD4+ and CD4− cells (Figure 1B–1D).

**Baseline Characteristics and Echocardiographic Parameters**
Myocarditis developed in 21 of the injected mice (n=7 severity degree 1, n=7 severity degree 2, n=1 severity degree 3, and n=6...
severity degree 4; Table I in the Data Supplement), whereas n=13 of the injected mice did not develop myocarditis (Figure 2). For all subsequent analyses, mice with severity degrees 3 and 4 were grouped together as severe myocarditis, severity degrees 1 and 2 as moderate myocarditis, whereas injected mice that did not develop myocarditis were grouped together with sham-injected mice as no myocarditis. In animals with myocarditis, the inflammatory infiltrate consisted primarily of mononuclear cells, including macrophages and lymphocytes.

Mice with severe myocarditis had lower body weight than both the mice with no and moderate myocarditis (Table). Both the heart weight determined postmortem and the LV mass determined by echocardiography were higher in mice with severe myocarditis compared with the 2 other groups. E-wave velocity was lower in mice with severe myocarditis compared with the other groups. For all other echocardiographic parameters, there were no significant differences between the groups with a trend toward reduced values in animals with severe myocarditis. Of note, both $\beta$ as an estimate of myocardial blood flow velocity and $A^*\beta$ as an estimate of myocardial blood flow were not significantly different between the groups, albeit with large differences between individual animals.

LV Strain Parameters
Speckle-tracking–based strain measurements have been reported to be more sensitive for the detection of global changes in LV function when compared with conventional echocardiography.25 We, therefore, assessed global peak strain and peak strain rate on LV long- and short-axis images. In mice with severe myocarditis, radial and circumferential strain showed a trend toward lower values, and longitudinal strain was significantly reduced. However, in mice with moderate myocarditis, strain and strain rate values were identical to the ones in mice without myocarditis (Figure 3).

Ultrasound Molecular Imaging
Figure 4 shows data for ultrasound molecular imaging in all the 3 groups of mice, and Figure 5 represents examples of color-coded images for all 4 MB in an animal without, with moderate and with severe myocarditis. Signal enhancement was similarly low for all MB in mice without myocarditis. In contrast, in mice with myocarditis, the signals from MB_{CD4}, MB_{PSel} and MB_{Lc} were all significantly higher than the signal from MB_{iso}. Importantly, this was not just the case in mice with severe myocarditis but also in mice with moderate myocarditis, as well as when comparing mice without myocarditis to mice with myocarditis (Figure 1 in the Data Supplement). In mice with severe myocarditis, an increase in signal from MB_{iso} could be observed, which was, however, not significant when compared with the corresponding signals in the 2 other groups. Whereas the absolute signal intensities for MB_{CD4}, MB_{PSel} and MB_{Lc} increased in mice with severe myocarditis compared with mice with moderate myocarditis, the ratios for the targeted versus the control MB remained constant at a 2- to 3-fold increase because of nonsignificantly elevated signal from MB_{iso}. When examining the performance of ultrasound molecular imaging for diagnosing myocarditis using receiver-operating-characteristic curves, all 3 targeted MB performed better than ejection fraction, longitudinal strain, and MB_{iso} (Figure 2 in the Data Supplement). In addition to in vitro validation, we performed in vivo experiments to confirm the specific attachment of MB_{CD4} to CD4+ T cells. After injection of a blocking antibody in a dosage sufficient to saturate CD4 on CD4+ T cells. After injection of a blocking antibody in a dosage sufficient to saturate CD4 on CD4+ T cells in circulation, signal for MB_{CD4} was abolished in mice with myocarditis (Figure 6).

![Figure 2](http://circimaging.ahajournals.org/)

**Figure 2.** Hematoxylin and eosin–stained histological sections of the myocardium in a control mouse (A and D), a mouse with moderate myocarditis (B and E), and a mouse with severe myocarditis (C and F). The dashed lines (B) denote 2 inflammatory foci that covered $\approx$8% of the left ventricular area, and the arrowhead (E) denotes a focus of $>100$ inflammatory cells, (F) shows extensive inflammatory infiltration in a mouse with severe myocarditis.
Immunohistochemistry was used to quantify CD4+ T cells in myocardial tissue to assess whether signal from MB<sub>iso</sub> tracks with the degree of CD4+ T cell infiltration. MB<sub>iso</sub> signal correlated, albeit modestly, with CD4+ cells within the myocardium on histology (Figure 7), this was not the case for MB<sub>iso</sub> in the same animals.

**Discussion**

Myocarditis and the ensuing inflammatory autoimmune phenomena can lead to chronic dilated cardiomyopathy and heart failure. Currently available noninvasive imaging methods detect the functional or structural consequences of infection or inflammation, such as tissue necrosis or edema, and are limited in diagnostic accuracy. We, therefore, investigated whether CEU molecular imaging can detect the inflammatory process in myocarditis, and whether the specific detection of cellular subsets of the inflammatory infiltrate is possible. Our results indicate that (1) CEU ultrasound molecular imaging detects myocardial inflammatory infiltration and endothelial cell activation independent of functional consequences in autoimmune myocarditis and (2) we show for the first time that CEU molecular imaging allows for the specific detection of CD4+ T cells, which contribute to the pathogenesis of myocarditis and dilated cardiomyopathy.

**CEU Molecular Imaging Detects Myocarditis Independent of Functional Impairment**

Despite advances in noninvasive imaging, myocarditis continues to represent a challenging diagnosis. Conventional echocardiography can detect structural and functional alterations in fulminant myocarditis, but it is limited in sensitivity and specificity in lower-grade acute myocarditis, which has a higher likelihood of progression to dilated cardiomyopathy. In our study, LV mass was increased and LV function was tended to be decreased in the group with severe myocarditis. However, despite the use of high-resolution ultrasound and speckle-tracking analysis, there were no differences in LV size and function between the group without and the group with moderate myocarditis. Direct assessment of the inflammatory process responsible not only for acute myocarditis but also for the progression to dilated cardiomyopathy may improve the diagnostic performance of cardiac ultrasound. Our study shows that CEU molecular imaging with MB targeted to leukocytes and to P-selectin yields increased signal not only in the group with severe myocarditis but also in the group with moderate myocarditis, suggesting that the direct detection of the inflammatory infiltrate, rather than its consequences may result in better sensitivity. In addition to relatively nonspecific targeting of activated leukocytes using negatively charged MB<sub>iso</sub>.
We used antibody targeting of the endothelial cell adhesion molecule P-selectin. P-selectin is expressed on the surface of the vascular endothelium on inflammatory stimulation. In myocarditis, P-selectin plays an important role by mediating the recruitment of pathogenic T cells into myocardial tissue. Our results are in line with earlier reports that show that CEU molecular imaging can detect cardiac transplant rejection by targeting activated leukocytes or intercellular adhesion molecule-1. However, whereas the inflammatory process in transplant rejection shares similarities with myocarditis, an important difference that increases the diagnostic challenges is the low grade and focal nature of the inflammatory infiltrate in moderate myocarditis (Figure 2B and 2E). Also, the finding that CEU molecular signal for both leukocyte-targeted and P-selectin–targeted MB was greater in severe compared with moderate myocarditis indicates that various degrees of disease activity can be discerned. With regard to the clinical translation of this method for detecting myocarditis, leukocyte-targeted MB perform as well as MB targeted to P-selectin with the advantage of a greater ease of preparation of the contrast agent.

**CEU Molecular Imaging Assesses the Infiltration With Pathogenic CD4+ Lymphocytes**

In animal models of both viral and autoimmune myocarditis, CD4+ T cells represent a relatively small subset of leukocytes infiltrating the myocardium. However, development of myocarditis on transfer of appropriately activated CD4+ T cells in host animals suggests a decisive role of CD4+ T cells. In addition, the number of CD4+ T cells within tissue correlates with cardiac functional impairment in the chronic stage of EAM. Thus, the specific detection of CD4+ T cells could not only be used to characterize the inflammatory infiltrate in myocarditis but could also be of value in predicting late functional consequences of myocarditis. We, therefore, developed and validated a novel, CD4+-targeted MB using a monoclonal antibody. Flow-chamber assays and in vitro experiments showed that this contrast agent attaches specifically to CD4+ T cells, and that attachment occurs also under flow. In vivo, CEU molecular imaging showed increased signals both in moderate and in severe myocarditis, and the signal correlated with CD4+ T cells present in tissue. Abolishment of signal by blocking of CD4+ (Figure 6) indicates selective targeting similar to in vitro observations. A previous study used imaging of CD8+ T-cell granzyme B activity to assess immune-mediated myocarditis triggered by injection of cytotoxic T cells with fluorescence reflectance imaging. However, whereas fluorescence imaging is limited to small-animal models, CEU molecular imaging as used in our study does not have limited penetration and, therefore, could also be feasible in humans. In addition, molecular imaging of specific cellular subsets of...
the inflammatory immune response in myocarditis may help further elucidating the mechanisms of immune-mediated myocardial disease and be of use in the assessment of treatment effects in the future.

There are several limitations of this study that deserve attention. We studied a model of EAM, rather than virus-induced disease. However, EAM shares many pathophysiological features with virus-induced myocarditis, and it has been used extensively to study the autoimmune processes caused by initial viral myocyte infection.36 Regarding CEU molecular imaging, differences in myocardial perfusion between the groups could potentially influence the results. Although there was a high variability in myocardial perfusion in mice with severe myocarditis, and localized perfusion abnormalities in areas with tissue necrosis cannot be excluded in this animal group, we did not find significant differences between the groups, and perfusion was identical in mice without and mice with moderate myocarditis. In addition, the low signals for control MB in both the groups with myocarditis argue against an influence of myocardial flow on CEU molecular imaging results. Also, although moderate myocarditis could be detected independent of functional defects, ultrasound molecular imaging may not be sensitive enough to diagnose the early stages of myocarditis when only a few inflammatory foci or minimal myocyte necrosis are present. Finally, the limited spatial resolution of CEU at 7 MHz precluded a regional assessment of disease activity, which would be desirable for the guidance of endomyocardial biopsies in patients.

In summary, the results of our study indicate that CEU molecular imaging can be used to detect the inflammatory process in myocarditis and discriminate disease severity independent of cardiac function. Importantly, specific molecular imaging of a leukocyte subset that is driving the autoimmune process in myocarditis is possible. As a consequence, CEU molecular imaging can potentially be useful in diagnosing myocarditis. Future studies will have to address whether imaging of the acute inflammatory response and its components also allows a risk assessment about the future development of dilated cardiomyopathy.

**Sources of Funding**

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**Disclosures**

None.

**References**


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SUPPLEMENTAL MATERIAL
**Supplementary table.** Global and regional histology scores in animals with moderate (grade 1 and 2) and severe (grade 3 and 4) myocarditis.

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Supplementary Figure 1. Comparison of conventional echocardiography, strain measurements and molecular imaging between mice without and with myocarditis. Radial (A), circumferential (B) and longitudinal strain (C), as well as ejection fraction (D) did not show differences between the groups. In contrast, molecular imaging (E) showed significantly elevated signals in animals with myocarditis for microbubbles targeted to CD4, P-selectin and to leukocytes (**p<0.01 vs MBiso. (**p<0.001 vs MBiso).
Supplementary Figure 2. Receiver–operating characteristic (ROC) curves for diagnosing myocarditis (n=20 mice without myocarditis, n=21 mice with myocarditis). Area under the curve (95% confidence interval) was 0.55 (0.35 – 0.7) for ejection fraction, 0.60 (0.41 – 0.78) for longitudinal strain, 0.57 (0.39 – 0.75) for MB_Ctr, 0.86 (0.74 – 0.98) for MB_CD4, 0.90 (0.81 – 0.99) for MB_PSel, and 0.82 (0.68 – 0.95) for MB_Lc.