Cardiovascular, Pulmonary and Renal Pathology

Bacterial Lipopolysaccharide Enhances Cardiac Dysfunction but Not Retroviral Replication in Murine AIDS

Roles of Macrophage Infiltration and Toll-Like Receptor 4 Expression

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Cardiovascular disease is an important complication of human immunodeficiency virus/acquired immune deficiency syndrome (AIDS), but the mechanism(s) involved are poorly understood. Although co-infecting pathogens have been implicated as an important factor in AIDS progression, no studies have investigated these interactions in cardiac tissue. We recently demonstrated that the murine AIDS model (LPBM5 retroviral infection) mimics human immunodeficiency virus-related cardiac dysfunction and pathology. We tested the hypothesis that subseptic lipopolysaccharide exposure (LPS) would enhance LPBM5 progression and exacerbate cardiovascular dysfunction during murine AIDS development. LPS (5 mg/kg, *Escherichia coli* 0111:B4) was administered at 1, 6, and 8 weeks during LPBM5 infection, and cardiac performance was evaluated at 10 weeks using noninvasive echocardiography. LPS alone had no significant effects, whereas it amplified abnormalities in cardiac structure and function observed in murine AIDS. Cardiac dysfunction was associated with selective increases in nonfocal infiltration of CD68⁺ cells and correlated with the extent of cardiac dysfunction. Retroviral progression and cardiac retroviral content remained unaltered, but cardiac toll-like receptor 4 was increased in retrovirus + LPS. We provide first-

time evidence of multipathogen enhancements to retrovirus-related cardiac complications and implicate innate immune responses, not co-pathogen-induced retroviral replication, as the primary mechanism in this setting. (*Am J Pathol* 2006, 168:727–736; DOI: 10.2353/ajpath.2006.050794)

Following the advent of highly active antiretroviral therapy regimens, acquired immune deficiency syndrome (AIDS)-free living and overall survival have significantly improved for patients infected with human immunodeficiency virus (HIV).^{1–4} However, a variety of chronic complications that are apparently not directly related to immunodeficiency or opportunistic infection have become evident in these patients. For example, early-onset cardiovascular disease is an increasingly significant cause of morbidity and mortality for HIV/AIDS patients, affecting as many as 70 to 80% of this population.^{2,5,6} A variety of mechanisms have been proposed to explain these phenomena, but the mechanisms by which cardiac injury and dysfunction occurs in HIV/AIDS and the direct role for HIV or related proteins in these events remain incompletely defined.^{7–9} Several investigators have recently suggested that local or systemic responses to the retroviral pathogen (eg, immune activation, cytokines, inflammatory response, oxidants, etc) may be putative mechanisms of cardiac pathogenesis rather than direct

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cardiac myocyte infection by HIV.^{8–11} The progression of HIV/AIDS and its secondary complications are highly variable among patients, suggesting that this transition may be highly context dependent.^{10–14} Co-infection with non-HIV pathogens has been postulated as a particularly important risk factor that predisposes HIV patients to more severe outcomes, and a broad spectrum of viral and bacterial pathogens have been identified as potential contributors to HIV disease progression, including cardiovascular complications.^{13,15–18} Enhanced retroviral induction as a result of co-pathogen-induced immune activation has been implicated by *in vitro* studies as the mechanism of this observed increase in toxicity.¹⁴ Several recent reports illustrate that gram-negative bacteria are also frequent concomitant pathogens to HIV infection and have been implicated in promoting HIV-1 pathogenesis through bacterial lipopolysaccharide (LPS).¹⁹ In addition to contributing to HIV-related progression and complications, several of these pathogens have individually been linked to cardiovascular disease alone.²⁰

Therefore, we tested the hypothesis that LPS enhances cardiac dysfunction through enhanced retroviral replication and immune activation in a murine AIDS (MAIDS) model. The LP-BM5 MAIDS model is a previously well-defined mouse model of retroviral infection that exhibits many characteristics similar to human HIV infection, including splenomegaly, lymphadenopathy, hypergammaglobulinemia, and B-cell hyperactivity at the early stages of retrovirus infection. Other similarities include aberrant and time-dependent cytokine release, changes in T-cell populations, and progression to severe immune deficiency at roughly 20 weeks after infection.^{21–23} Increased susceptibility to opportunistic infections and evidence of neurological abnormalities have also been documented.^{24,25} Furthermore, we have recently reported that this model recapitulates the major cardiovascular features already documented in HIV/AIDS patients, with initially detectable contractility deficits before overt immune compromise followed by more severe cardiomyopathy.^{26,27} Increased cardiac oxidative injury and left ventricular immune cell infiltration were also observed, similar to changes observed by others in human tissues.²⁶ We also corroborated our findings of reactive nitrogen species in this mouse model with a small set of human tissues, further supporting the relevancy of the animal preparation.^{26,27} Given our prior observations and the potential importance of combined pathogen effects with respect to retrovirus-related cardiovascular alterations, we investigated potential interactions among retroviral infection and a modest exposure to LPS. In the studies described herein, we tested the hypothesis that immunostimulation via low dose LPS (eg, at doses that did not induce cardiac toxicities alone) can modulate retrovirus-related cardiac deficiencies in this well-established model of the immunological and cardiac complications of retroviral infection.^{26,28} We used LPS as the immune activator rather than live bacteria to avoid confounding differences in pathogen growth in the immunocompromised hosts. Our investigative focus was on aspects of retroviral progression, leukocyte populations and trafficking to cardiac tissues. By using a specific pathogen-free mouse

colony, we had a unique opportunity to evaluate interactions between these two immune system challenges and to limit influences of other potential covariates commonly found in humans.

An additional component of our study was to address mechanisms by which such multipathogen interactions might develop *in vivo*. As key participants of the innate immune system, toll-like receptors (TLRs) are a newly identified class of receptors involved in the recognition and transmission of pathogenic stimuli. Of particular importance are TLR4 subtype receptors, which bind LPS and transduce rapid response to bacterial infection and elicit inflammatory and oxidative pathways in immune cells.²⁹ These receptors have also been detected on cardiac myocytes, and their up-regulation has been implicated in several human cardiac disease settings.³⁰ Furthermore, recent *in vitro* studies have shown that TLR4 activation is required for LPS-induced retroviral expression enhancement. We therefore tested the hypotheses that the TLR4 receptor system is involved in retrovirus-related cardiac dysfunction in the murine AIDS model.

Materials and Methods

LPBM5 Retroviral Infection in Mice

Active LPBM5 virus was prepared according to the methods of Watson³¹, as we have previously described.²⁶ Retrovirus-containing cell-free supernatant was collected from infected SCI/MuLV cells (AIDS Research and Reference Reagent Program, Bethesda, MD) and concentrated by centrifugation (Advanced Biotechnologies, Inc., Columbia, MA). Titers of esotropic MuLV were determined by the standard S'L plaque assay³² and by units of reverse transcriptase activity using a commercially available kit (Boehringer Mannheim, Mannheim, Germany).

Specific effort was made to procure and maintain pathogen-free female C57BL/6 mice (two specific barrier rooms at Harlan Laboratories [Indianapolis, IN]), which were housed in a sterile cage rack system with HEPA-filtered air circulation (approximately 50 air changes/hour; Allentown Caging Inc., Allentown, PA). After 1 to 2 weeks of acclimatization, 40 mice were divided into four treatment groups: control (CTRL), retrovirus infected alone (RTV), LPS treated alone (LPS), or retrovirus + LPS treated (RTV+LPS). LPBM5 retrovirus was dosed via a single intraperitoneal injection (100- μ l dose containing 200 reverse transcriptase units). Control animals received an identical injection of vehicle. At the time of injection, all mice were 6 weeks old and weighed 16 to 18 g. At 1, 6, and 8 weeks after LP-BM5 infection, animals in the LPS and the RTV+LPS treatment groups were administered a 5 mg/kg intraperitoneal dose of LPS (*Escherichia coli* 0111:B4; Sigma Chemical Co., St. Louis, MO). This dose is below the threshold required to induce a septic shock response in these mice.³³

Cardiac performance was assessed by echocardiography (see below) at 10 weeks of retroviral infection, allowing for sufficient time to clear LPS from the system ($t_{1/2}$ of LPS is 2 to 3 days). Immediately after echocardi-

ography, animals were sacrificed with an overdose of pentobarbital. Whole blood was collected from the descending abdominal aorta at the time of sacrifice, and complete blood chemistries were provided (Antech Diagnostics, Columbus, OH). Total cholesterol and triglyceride levels as well as aspartate aminotransferase, alanine aminotransferase, lipase, and amylase activities were also measured. Entire hearts were then rapidly isolated, rinsed in ice-cold physiological buffer, weighed, equatorially sectioned at the mitral valve, and fixed in 10% buffered formalin for later analysis.³⁴ Spleen weight was also measured as an index of retroviral progression, as documented by others.^{28,35}

Murine Echocardiography

Mice were placed under light anesthesia with halothane inhalation (0.5 to 1% halothane United States Pharmacopeia in a mixture of 95% O₂ and 5% CO₂), as we have previously described.³⁶ Two-dimensional and M-mode echocardiographic images were recorded and analyzed by a Sonos 5500 echocardiogram and a 15-MHz ultrasonic probe (Agilent Technologies). Two-dimensional transverse LV imaging was used to position the probe just distal to the mitral valve leaflets, and M-mode images were then captured. Three loops of M-mode data were captured from each animal at approximately 5-minute intervals and stored on digital disk until analysis. Each of these captured image loops provided 7 to 12 heart cycles; data were averaged from at least five cycles per loop. LV systolic (LVIDs) and diastolic (LVIDd) internal dimensions were measured according to the American Society for Echocardiography leading-edge technique by a blinded investigator.³⁷ These parameters allowed the determination of LV fractional shortening (%FS), a measure of systolic function, by the equation: %FS = [(LVIDd – LVIDs)/LVIDd] × 100%.

Ascending aortic flow velocity was determined using the continuous Doppler wave mode. Peak aortic flow velocity and velocity-time integral were determined for at least five beats per loop for each animal. At sacrifice, aortic outflow tract (aortic root) was isolated, and cross-sectional area was measured via light microscopy with area-calibrated digital image analysis (Image-Pro Plus; Media Cybernetics, Silver Spring, MD). Stroke volume (SV) was calculated by velocity-time integral × aortic root cross-sectional area.³⁶ Cardiac output (CO) was calculated by SV × heart rate.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis of LPBM5 and TLR4 Expression

Total RNA was isolated from frozen cardiac and splenic tissue (Trizol; Gibco BRL). An absorbance reading at 260 nm was used to quantify the amount of RNA in each sample. The integrity of the RNA was verified by fractionating the RNA on a formaldehyde agarose gel. Total RNA

(2 μg) was reverse transcribed to cDNA (cDNA cycle kit; Invitrogen) in 20 μl of reverse transcription reaction mix at 42°C (60 minutes). The reaction was stopped by heat inactivation at 95°C (2 minutes) and chilled on ice. A 5-μl portion of the resulting cDNA was amplified using primers specific for the p12 region in the *gag* gene in the LP-BM5 genome,³⁸ a region spanning 317 to 726 bp of the murine *TLR4* gene,³⁰ and β -actin.³⁹ The RTV primers included sense primer, 5'-CCT TTATCGACACTTCCCTT-3', and antisense primer, 5'-CCGCTCTTCTTAAGTGGTC-3'. The TLR4 primers for the murine gene included sense primer, 5'-GCTTACACCACCTCTCAAAGTTGAT-3', and antisense primer, 5'-ATTACCTCTTAGAGTCAGTTCATGG-3'. Similarly, β -actin primers were sense primer, 5'-ATG-GATGACGATATCGCT-3', and antisense primer, 5'-ATGAGGTAGTCTGCTAGGT-3'. PCR reactions were performed under the same conditions, which included an initial denaturation at 95°C (5 minutes), followed by 30 cycles each of denaturation (95°C/1 minute), annealing (55°C/1 minute), and extension (72°C/1 minute), followed by a final extension (72°C/10 minutes). The PCR products were separated and visualized on 2% agarose ethidium bromide-stained gel. Band intensity was assessed using imaging software (Labworks 4.0; Media Cybernetics, Silver Spring, MD). RTV expression was normalized to β -actin expression in each tissue. Cardiac RTV expression was further expressed as a percentage of splenic RTV expression.

Histology and Immunohistochemistry

After formalin fixation (48 hours), heart and spleen tissues were embedded in paraffin for histological studies (cross-sectional orientations were used). Tissue sections (5 μm) were mounted onto microscope slides and prepared for histological and immunohistochemical analyses, as we have previously described.³⁴ Cardiac and splenic cross-sections were stained using hematoxylin and eosin and Masson's trichrome for routine morphological and histological assessments. Cardiac tissues from each treatment group were assessed for evidence of specific leukocyte infiltrates assessed in the complete blood chemistry profile. Histological stains for mast cells (Astra Blue stain; Sigma) and eosinophils (Vital Red stain; Sigma) and immunohistochemical probes for neutrophils (anti-myeloperoxidase antibody; 1:2000 dilution; Neomarkers) and monocytes/macrophages (anti-CD68⁺; 1:400; Neomarkers) were used. In additional studies, cardiac myocyte prevalence of TLR4 was assessed in left ventricular cross-sections (anti-TLR4; raised against the carboxy terminus of murine TLR4; 1:400 dilution; Santa Cruz Biotechnology). Isotypic and preadsorbed staining controls demonstrated antibody specificity. NOS2-stained tissues were assessed for NOS2-positive cell bodies (monocytes/macrophages) as well. Diaminobenzidine (0.06% [w/v]; DAKO, Carpinteria, CA) was used to provide visualization of immunoreactivity, with methyl green counterstaining.

Digital Image Analysis

Digital images were acquired using a Polaroid DMC camera and Olympus microscope (model BX40) and transferred to Image Pro Plus software (Media Cybernetics) for both area and intensity analyses, as we have previously described.⁴⁰ All images were captured using identical light and software settings. For morphological studies, cross-sectional images of whole spleen and heart stained with H&E were captured at $\times 4$ magnification, and circumferential traces were used to calculate tissue areas. All other images were captured at $\times 400$ and then segmented to eliminate background and nuclear counterstain from analysis. Cardiac residence of specific immune infiltrates was quantitated using a digitized cell counting approach. Tissue areas were calibrated, and positive cells were segmented and gated based on size and then counted and normalized as a function of left ventricular area. In parallel studies, immunoreactivity for cardiac TLR4 was determined by measuring optical density of diaminobenzidine signal in each tissue, giving a quantitative measure of relative staining intensities, as we have previously described.⁴⁰ More than 200 cardiac images were analyzed, and intraobserver and interobserver variability for these automated procedures were each $<2\%$.

Statistical Analysis

All data presented herein represent 6 to 12 observations per group. All statistical analyses were performed using Sigma Stat statistical software (Jandel Scientific, San Rafael, CA). Analyses of variance were used for statistical comparisons among groups with Student-Newman-Keuls for post hoc analysis. Spearman's nonparametric correlation analysis was used to define significant associations. A total of 30 to 35 data points were used for each analysis (controls, RTV alone, LPS alone, and RTV in combination with LPS), providing a statistical power of greater than 0.95 at $r = 0.5$ and $\alpha = 0.05$. $P < 0.05$ was considered statistically significant.

Results

Murine AIDS Studies

Cardiac Structure and Function after Retrovirus, LPS, or Their Combination

Shown in Figure 1 are changes in total heart weight and systolic and diastolic dimensions from mice treated with retrovirus, LPS, or their combination. Representative photomicrographs from control- and RTV+LPS-treated animals illustrate the significant LV chamber remodeling induced by combination treatment (Figure 1, top panel). Neither retrovirus nor LPS alone significantly altered LV dimensions (LVIDs: CTRL, 1.88 ± 0.04 mm; RTV, 1.96 ± 0.04 mm; LPS, 1.78 ± 0.7 mm; and LVIDd: CTRL, 3.66 ± 0.95 mm; RTV, 3.49 ± 0.05 mm; LPS, 3.45 ± 0.07 mm), but their combination caused a significant increase in

Figure 1. Development of cardiac left ventricular hypertrophy in murine AIDS and dilation in animals infected with RTV in conjunction with LPS. **Top panel:** Representative images (magnification, $\times 25$) of equatorial cardiac cross-sections of control mouse and RTV-infected mouse in combination with LPS at 10 weeks. **Middle panel:** Average heart weights (normalized to body weight) at 10 weeks after RTV infection. **Bottom panels:** Average interior cardiac dimensions (systolic and diastolic) at 10 weeks after RTV. * $P < 0.05$ compared with control. † $P < 0.05$ compared with RTV alone.

luminal LV dimensions at both systole (LVIDs, 2.33 ± 0.03 mm) and diastole (LVIDd RTV+LPS, 3.91 ± 0.1 mm) (Figure 1, bottom panels). Hearts from RTV-treated mice

Figure 2. Cardiac alterations during murine AIDS and RTV infection in combination with LPS. **Top panels:** Average data for heart rate (HR) and fractional shortening (FS%) at 10 weeks after RTV infection. **Bottom panel:** Average data for CO and SV at 10 weeks after RTV infection. * $P < 0.05$ compared with control. † $P < 0.05$ compared with RTV alone.

developed a hypertrophic response with both RTV alone (CTRL heart weight, 5.83 ± 0.02 versus RTV, 7.33 ± 0.27 mg/g body weight) and in combination with LPS (LPS alone, 5.76 ± 0.51 g/mg; LPS+RTV, 6.87 ± 0.32 mg/g) (Figure 1, middle panel). Total body weights were not different among groups, and no animals in the study developed cachexia during the 10-week study; thus, the increase in heart weight normalized to body weight was apparently not related to changes in body mass.

In vivo echocardiogram cardiac performance indicators are shown in Figure 2. Heart rate was maintained throughout the studies at physiological rates by using light halothane anesthesia, and no differences were observed among treatment groups, as we have published previously.²⁶ Similar to our previous report, LPBM5 retrovirus alone caused significant reductions in fractional shortening (CTRL, $51 \pm 0.58\%$; RTV, $44.2 \pm 0.8\%$), cardiac output (CTRL, 17.2 ± 0.64 ml/min; RTV, 14 ± 0.6 ml/min), and stroke volume (CTRL, 35.2 ± 1.5 μ l; RTV, 31.2 ± 0.7 μ l).²⁶ LPS alone caused a slight increase in stroke volume (41.7 ± 0.7 μ l) when administered alone but had no other demonstrable effects on cardiac performance (FS%, 49 ± 1.13 ; CO, 19.6 ± 0.47 ml/min, NS). In contrast, LPS exacerbated the cardiac contractility deficits observed in RTV-treated mice, because RTV+LPS treatment caused a significantly lower stroke volume (26.7 ± 1.2 μ l), cardiac output (11.8 ± 0.4 ml/min), and LV shortening fraction ($40.4 \pm 0.9\%$) relative to RTV treatment alone.

Influence of LPS on Retroviral Progression in Vivo

Given the capacity of LPS to modulate RTV-induced cardiac functional and structural changes, we tested the hypothesis that LPS treatment can promote retroviral progression in spleen and cardiac tissue. Splenomegaly is consistently observed in the murine AIDS model and is used as a marker of retroviral progression and immune dysfunction.³¹ LPBM5 retrovirus caused significant in-

Figure 3. Splenomegaly and increased viral load in cardiac and splenic tissue during the progression of murine AIDS. **A:** Representative images of retroviral RT-PCR products from cardiac (C) and splenic (S) tissue in mice infected with LPBM5 and in combination with LPS. **B: Right panel,** average data for total spleen weight in control versus retrovirus infected animals at sacrifice. Data are expressed as milligrams per gram of body weight ($n = 6-12$). * $P < 0.05$ compared with control. **Left panel,** RTV expression was normalized to β -actin expression in each tissue. Total cardiac viral load at 10 weeks after RTV infection (further expressed as a percentage of splenic RTV expression) with RTV alone and in combination with LPS. Data are represented as means $\pm$ SEM ($n = 3-6$).

creases in spleen weight compared with CTRL and LPS alone treatment groups (Figure 3), but this effect was not further augmented in the RTV+LPS group.

LPBM5 infectivity is facilitated by the gag, pol, and env genes.⁴¹ The p12 region in the gag gene has been shown to be unique to the LPBM5 virus and important to infectivity.⁴² Thus, we selected this region to develop a primer set specific to LPBM5. A representative agarose gel after separation of RT-PCR products is shown in Figure 3 (top panel). The 229-bp RT-PCR product, specific for the p12gag region of LPBM5, was confirmed by a restriction enzyme analysis with *Sma*I (data not shown). RTV-treated mice yielded a positive PCR product for LPBM5 in splenic and cardiac tissue. This signal was absent in CTRL and LPS alone treatment groups, with equivalent β -actin signal. RTV+LPS-treated mice had comparable levels of LPBM5 signal (cardiac to splenic %, $69 \pm 15\%$) to RTV alone ($50 \pm 7.4\%$); LPS apparently did not augment retroviral load in splenic or cardiac tissue, despite dramatic alterations in cardiac functional changes.

Circulating Leukocytes after Retrovirus, LPS, or Their Combination

Shown in Figure 4 are circulating blood leukocyte counts for the three treatment groups at 10 weeks. RTV alone caused a significant reduction in circulating monocytes (CTRL, 106.7 ± 52.2 count/ μ l versus RTV, 10.5 ± 5.5 count/ μ l whole blood), with neutrophils, total lymphocytes, and eosinophils remaining unchanged. LPS alone caused no significant changes on any measured cell type, whereas the combined treatment caused a reduction in monocytes equivalent to RTV alone (RTV+LPS, 39.9 ± 11.7 count/ μ l) plus a marked eosinophilia (CTRL, 12.6 ± 6.9 count/ μ l; RTV, 5.7 ± 2.6 count/ μ l; LPS, $13.2 \pm$

Figure 4. Circulating monocyte levels are significantly diminished during murine AIDS, whereas circulating eosinophils are significantly enhanced with RTV infection in combination with LPS. Total number of monocytes, neutrophils, lymphocytes, and eosinophils per microliter of whole blood from control and RTV-infected mice. * $P < 0.05$ compared with control. † $P < 0.05$ compared with RTV alone.

6.7 count/ μ L; and RTV+LPS, 138.6 ± 21.1 count/ μ L) with no alterations in total lymphocytes of neutrophils.

Cardiac Leukocyte Infiltration

In situ measures were used to assess the cardiac residence of multiple immune cell infiltrates by immunohistochemistry (see Materials and Methods). In our preliminary examinations, inflammatory lesion sites were not observed in any of the treatment groups (eg, no focal accumulations of cells); thus, the following measures assessed the interstitial presence of immune cells throughout the myocardium. Cardiac presence of CD68⁺ infiltrates was significantly elevated in RTV-treated mice (CTRL, 14.8 ± 2.1 cells/mm² versus RTV, 21.0 ± 1.4 cells/mm²), whereas cell counts for LPS-treated mice (16.4 ± 5.6 cells/mm²) were not different from control. This cellular infiltration was further elevated in RTV+LPS-treated mice (27.0 ± 3.6 cells/mm², $P < 0.05$ versus RTV alone; Figure 5). The extent of myocardial infiltration of CD68⁺ cells was inversely correlated to cardiac performance parameters in these same mice: LV fractional shortening, stroke volume, and cardiac output each yielded significant negative correlations to CD68⁺ cell densities in the same hearts. Nominal cardiac presence of neutrophils, mast cells, and eosinophils were detected, with no significant increases in any treatment group studied (data not shown).

Additional immunohistochemical studies revealed the presence of NOS2-positive mononuclear cells, which were consistent with the general distribution of CD68⁺ infiltrates. Using automated cell counting methods identical for the studies described above, we found that NOS2-positive infiltrates were significantly elevated in hearts from RTV mice at 10 weeks of retroviral infection, relative to control, and this was further enhanced in conjunction with RTV+LPS (NOS2-positive cells, 14.8 ± 2.1 , $21.0 \pm 3.0^*$, 16.5 ± 2.6 , $27.0 \pm 2.8^{*†}$ cells/mm² for CTRL, RTV, LPS, RTV+LPS, respectively; * $P < 0.05$ compared with control; † $P < 0.05$ compared with RTV alone). The

Figure 5. Increased cardiac presence of CD68⁺ infiltrates during murine AIDS. Total number of infiltrating immune cells per square millimeter of cardiac tissue was assessed using digital imaging approaches. * $P < 0.05$ compared with control. † $P < 0.05$ compared with RTV alone. Cardiac function related to cardiac CD68⁺ cells. ●, control; ▲, LPS alone; ■, RTV alone; ◆, RTV and LPS. * $P < 0.05$.

relative increases and densities paralleled those observed for CD68⁺ cells over the same time course, consistent with these cells being of apparent monocyte/macrophage lineage. The NOS2 prevalence was primarily confined to immune cells within cardiac interstitium, rather than exclusive expression in cardiac myocytes. This observation is potentially important and would have been overlooked if we had used more traditional techniques using homogenates and Western blotting.

TLR4 Expression and Prevalence after Retrovirus, LPS, or Their Combination

Shown in Figure 6A (top panel) is a representative gel for TLR4 and β -actin expression in cardiac tissue by RT-PCR. Band intensities for the 410-bp TLR4 PCR product are expressed as a percentage of β -actin expression, as illustrated in Figure 6A (bottom panel). Cardiac mRNA expression of TLR4 was greatly enhanced by LPS stimulation of retroviral disease. This mRNA expression was translated into increased cardiac TLR4 protein levels as determined by immunohistochemistry (Figure 6B), such that mRNA expression was significantly correlated to protein content by immunohistochemistry ($r = 0.5$, $P < 0.05$). Integrated optical density analysis for TLR4 protein was reflective of cardiac myocytes themselves, not infiltrative immune cells, because LPS stimulated expression of TLR4 in cardiac myocytes during RTV disease.

Discussion

Although concurrent pathogen presentation is a well-appreciated phenomenon in retrovirus-induced immunodeficiencies, few studies have investigated mechanistic consequences, especially as they relate to cardiovascular complications. Similar to our previous report, we found that LPBM5 retroviral infection alone in pathogen-free C57BL/6 mice induced detectable ventricular dysfunction.²⁶ Interestingly, despite the fact that the LPBM5 retroviral preparation is different from HIV per se, this murine model recapitulates nearly all major complications of the HIV/AIDS condition, including overt immunodeficiency and increased opportunistic infections, neurocognitive deficits, increased tumor incidence.²⁸ Based on our observations, cardiac dysfunction can be included in this list.²⁶ Because the retroviral pathogen is substantially different from HIV, an immune system response to retrovirus rather than retrovirus per se may be the operable mechanism for many of these parenchymal organ abnormalities.^{26,43}

As expected, the LPS exposure we used in this mouse strain had no significant effects on cardiovascular status alone. In contrast, LPS substantially enhanced retrovirus-related cardiac structural and functional alterations. In these studies, we chose to use LPS rather than a live gram-negative pathogen to avoid confounding issues of bacterial growth differences among treatment groups. Because previous *in vitro* studies have shown that various pathogen exposures, including LPS, can enhance HIV replication rate and infectivity, we investigated content of

Figure 6. Increased cardiac TLR4 expression and protein prevalence during RTV infection in combination with LPS. **A: Top panel**, representative image of cardiac TLR4 expression during murine AIDS. **Bottom panel**, average data from total cardiac TLR4 expression (expressed as percentage of β -actin expression). * $P < 0.05$ compared with control. † $P < 0.05$ compared with RTV alone. **B: Top panels**, representative images of TLR4 prevalence in left ventricular myocardium of control and RTV infected mice in combination with LPS (magnification, $\times 400$). **Bottom panel**, relative prevalence of TLR4 staining was determined by digital imaging. Average data from control, RTV alone, LPS alone, and RTV and LPS in combination; means $\pm$ SEM depicted. * $P < 0.05$ compared with control.

retroviral RNA in spleen and cardiac homogenates by RT-PCR.^{13,17,19} Retrovirus was readily detectable in the LPBM5-treated animals; however, there was no signifi-

cant enhancement in the RTV+LPS group. Total spleen weight was also not enhanced in the combination treatment, suggesting that the LPS dosing did not alter retroviral pathogenesis or cardiac abundance of retrovirus *in vivo*. These findings suggest that cardiac content of retrovirus per se is not closely linked to organ dysfunction *in vivo*, a concept supported by previous publications.⁹ Isolated cellular experiments demonstrate that HIV and other retroviruses can apparently infect cardiac myocytes, but whether this phenomenon occurs substantially *in vivo* and plays a causative role in commonly observed cardiomyopathies remains to be resolved.^{9,44}

Our measurements of circulating leukocytes demonstrated that LPBM5 infection alone caused a reduction in circulating monocytes, with no change in total lymphocytes, neutrophils, or eosinophils at 10 weeks. LPS alone caused no significant effects, but the combination caused marked increases in eosinophilia.¹⁹ Although previous studies have also shown that eosinophilia is a common occurrence in HIV/AIDS patients, the significance and mechanisms of this "allergen-like" response are unclear. Eosinophilic myocarditis has been previously documented in HIV case reports, but we did not observe significant presence of this cell type in cardiac cross-sections, and total cell counts were not associated with any index of cardiac performance in the mouse model (see below).^{45–47}

In parallel to blood measurements, we investigated the presence of macrophages, neutrophils, mast cells, and eosinophils in cardiac cross sections using *in situ* techniques. LPBM5 alone caused significant enhancement of CD68⁺ (monocyte/macrophage) cells in cardiac cross-sections, and these were found to be widely distributed throughout cardiac tissues. We found no significant evidence of classical focal lesions in this model in any treatment group, suggesting that this typically used endpoint for evidence of myocarditis may not serve as an adequate marker of myocyte/immune cell interaction in this setting and at these time points. Although LPS alone caused no significant changes in cardiac CD68⁺ cells (shown in Figure 5) or other leukocytes (data not shown), the treatment combination caused further increases in cardiac CD68⁺ abundance relative to retrovirus alone. We found that these cells were positive for NOS2, but surrounding myocardium was not. In addition, we found that the extent of macrophage presence in cardiac tissue correlated with the degree of LV dysfunction observed in the same animals (Figure 5). These findings are consistent with a cell-specific monocyte/macrophage induction of inflammatory responses, and widely distributed infiltration of these activated cells may play an important role in cardiac parenchymal injury and dysfunction.⁴⁸ Many previous reports have investigated NOS2 induction as a potential contributor to various forms of cardiac failure, and thus far, the findings have been inconsistent. Our *in situ* approach illustrated a strong NOS2 prevalence only in the CD68⁺ cell type, suggesting that homogenates and Western blotting techniques may not offer adequate sensitivity to detect such changes. Recent investigations have demonstrated an enhanced presence of CD68⁺ cells in brain regions from HIV dementia autopsy cases

and in human HIV cardiac tissues.^{49,50} Our findings in this murine model recapitulate these human findings and further argue the relevance of the animal preparation and the potential importance of immune cell interactions with cardiac myocytes in RTV-related cardiac complications.

A number of autopsy studies (wherein a few tissues have been specifically described) have suggested an increased prevalence of focal immune cell lesions in hearts from HIV⁺ patients.^{6,51–55} This observation of focal infiltrates is often pathologically described as "inflammation" (although it is more specifically evidence of infiltration). One issue of such studies and observations is that these autopsy studies, investigating cardiac pathology in AIDS patients, by definition occur late in the disease progression and in many cases at the time of fulminant myocarditis. In our MAIDS mouse studies, we observed that cardiac dysfunction (apparent at 5 weeks) preceded development of overt immune deficiency (20 weeks). We therefore focused on early changes in immune regulation and the more subtle interactions that may occur with long-term residence of immune cells in the cardiac interstitium, which have been largely overlooked in other studies. Our data suggest that immune cell interactions in cardiac muscle may be an important participant in cardiac complications but that focal lesions may be a later event.

Several very recent reports have suggested that innate immunity pathways, more specifically the TLR family, may play a critical role in many forms of pathogen-induced cardiovascular disease states.⁵⁶ Thus far, the TLR4 receptor has been shown to mediate much of the host response to gram-negative bacterial infections *in vivo*, and these processes are upstream from several inflammatory cascades and transcriptional controllers already implicated in cardiomyopathies (eg, nuclear factor- κ B, tumor necrosis factor- α , and others).⁵⁷ In HIV/AIDS, activation of the TLR4 pathway has been suggested as important for HIV replication enhancement by coexisting pathogens. In the mouse model, neither retrovirus alone nor LPS alone had significant impact on cardiac TLR4 expression or protein content, whereas substantial increases in expression were observed with combined treatment. Increases in TLR4 mRNA were paralleled by increased protein prevalence in which the distribution was consistent with cardiac myocyte localization rather than infiltrating immune cells. These observations provide first-time evidence that the activation of the cardiac innate immunity pathways may contribute to cardiac complications during retroviral infection and not solely through the enhancement of retroviral replication. Innate immunity pathways (in particular TLR4) may play an important role in a multipathogen setting commonly observed in humans and warrant further investigation.

In summary, a multipathogen setting is commonly observed in HIV-related cardiomyopathy cases, but few studies have addressed important interactions with respect to retroviral pathogenesis versus organ dysfunction. Here, we have demonstrated that a modest exposure of LPS (eg, at doses that did not yield significant *in vivo* effects when administered alone) amplified abnormalities in cardiac structure and function observed in a

murine AIDS model. The observed cardiac dysfunction was associated with selective increases in nonfocal infiltration of CD68⁺ cells; these cells were found to be NOS2 positive and correlated with the extent of cardiac dysfunction. This amplification interaction was not associated with alterations in retroviral progression or cardiac retroviral content, but an important increase in TLR4 was observed in the combination treatment group only. Co-existing pathogens may be an under-appreciated and critically important modulator of cardiac status in HIV/AIDS patients. These studies provide first-time evidence that multipathogen exposures may represent an important contributor to retrovirus-related cardiac complications and implicate innate immunity responses in this setting.

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