



# Myocarditis Associated with Combined Antiretroviral Therapy in CD8+ Cell-depleted Rhesus Macaques

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## Abstract

**Background:** Cardiovascular disease (CVD) is an emerging complication in HIV positive patients receiving highly active antiretroviral therapy (HAART). Although HAART is known to be associated with metabolic syndrome, a cluster of cardiovascular disease risk factors, the mechanism of HAART toxicity is unknown.

**Methods:** This retrospective study used tissues from eight CD8<sup>+</sup> cell-depleted adult rhesus macaques infected with SIMmac251. Four of the animals were treated for 28 days with Raltegravir and Tenofovir beginning 28 days postinfection. Four CD8<sup>+</sup> cell-depleted, uninfected animals served as additional controls. Histopathologic examination for inflammatory and degenerative lesions was performed on hematoxylin and eosin-stained sections of cardiac tissue. Viral replication in cardiac sections was evaluated by *in situ* hybridization for SIV RNA. The level of inflammation was assessed by immunohistochemistry, using antibodies to identify macrophages (CD68), B-lymphocytes (CD20), T-lymphocytes (CD8), interleukin-18, and caspase-3. The extent of myocardial fibrosis was evaluated by staining tissues for collagen deposition (Masson's trichrome stain).

**Results:** Multifocal mononuclear cell infiltrates were present in the heart tissue of animals that received antiretroviral therapy. No inflammatory lesions were present in untreated SIV+ controls. However, occasional small mononuclear infiltrates were seen in 2 of 4 uninfected controls. Degenerative changes in cardiomyocytes included loss of cross striations, hyalinization, and swelling. Rare atrophic and necrotic cardiomyocytes were seen only in tissue sections of treated animals. Myocardial fibrosis was not observed in either group. The inflammatory cells were positive for the macrophage marker Iba1 and negative for CD8 and CD20, suggesting the absence of lymphocytes in inflammatory foci. Expression of IL-18 was not observed in either treated or untreated controls, suggesting that the inflammation is not directly mediated by IL-18. In addition, caspase-3 expression was not seen in degenerating cardiomyocytes, indicating that the caspase-3 apoptotic pathway is not involved.

**Conclusions:** Macrophages are the predominant inflammatory cells involved in short-term antiretroviral therapy-associated myocarditis; however, the mechanisms mediating inflammation are unknown. This pilot assessment suggests that the process is IL-18 and caspase-3 independent. Further studies are needed to elucidate the mechanism of myocardial damage.

Supported by NIH grants NS050041, NS048831, MH61232, and RR00168.

## Methods

### *In situ* hybridization (ISH) for SIV

Productively infected cells in formalin-fixed, paraffin-embedded sections of left ventricles were localized by ISH for SIV RNA, as described elsewhere. Tissue sections were deparaffinized in xylene and rehydrated in graded ethanol to diethyl pyrocarbonate (Sigma Chemical Co., St. Louis, MO) treated water. Endogenous alkaline phosphatase activity was blocked with levamisole (Sigma). Tissue sections were hybridized with HCl (Sigma), digested with proteinase K (Roche Diagnostics Corp., Indianapolis, IN), acetylated in acetic anhydride (Sigma), and hybridized overnight at 50°C with a digoxigenin-labeled antisense riboprobe which spans the entire genome of the SIMmac239 molecular clone of SIVmac251 (Lofstrand Labs, Gaithersburg, MD). The following day, tissue sections were washed extensively and bound probe was detected by immunohistochemistry (IHC), using alkaline phosphatase-conjugated sheep anti-digoxigenin F(ab) fragments (Roche) and the chromogen nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP, Roche). Sections were counterstained with nuclear fast red (Vector Labs, Burlingame, CA). Sections of heart from a rhesus macaque infected with SIMmac251 served as both positive control (when incubated with SIV antisense probe) and negative control (when reacted with SIV sense probe). Additional negative controls included sections of heart from uninfected macaques reacted with SIV antisense probe.

### Immunohistochemistry for CD68, Cleaved Caspase-3 (CC3), CD20, and CD8

Cells expressing CD68, CD8, CC3 and CD20 were localized in sections of myocardium by IHC, using the respective antibodies (DAKO Corp.) and a commercial kit (ABC Elite, Vector Laboratories). Formalin-fixed, paraffin-embedded sections of brain were deparaffinized in xylene and rehydrated through graded ethanol to distilled water. Endogenous peroxidase activity was blocked by incubation in 3% H<sub>2</sub>O<sub>2</sub>, and antigen retrieval was accomplished by microwave sections for 20 minutes in citrate buffer (DAKO). Sections were then incubated for 30 minutes at room temperature with antibodies and reacted sequentially with biotinylated secondary antibody and horseradish peroxidase-conjugated avidin DH. Antigen-antibody complex formation was localized by development of the chromogenic substrate 3, 3'-diaminobenzidine (DAKO). Tissue sections were counterstained in Mayer's hematoxylin (Sigma-Aldrich), cleared, and cover-slipped with permanent mounting medium.

### RNA Isolation and Real Time RT-PCR

Heart tissue (left ventricle) collected during necropsy and stored at -80°C was homogenized in Trizol reagent (Invitrogen) using silica beads and a bead beater. Chloroform was added to the homogenate and the aqueous phase was collected after centrifugation. An equal volume of 70% ethanol was added to the aqueous phase and the total RNA was purified using the RNeasy kit (Qiagen). RNA quantification was carried out using the Ribogreen kit (Invitrogen). Real time RT-PCR was carried out in an ABI 7700 thermal cycler using one step RT-PCR master mix, gene specific primers, and FAM-labeled TaqMan probes. *In vitro* transcription of the SIMmac251 gag gene, TNF-alpha, and RPL13A were carried out using their respective plasmid constructs. Based on the molecular mass of the transcripts, copy numbers were determined and the standards for real time PCR were generated by serial dilution of known copy numbers of the transcripts. RPL13A was used as internal control and the copy numbers of SIVmac251 gag and TNF-alpha were determined by using 250 ng of total RNA.

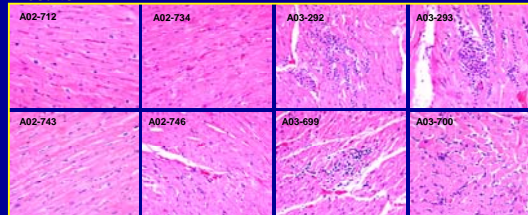
### Statistical analyses

A two-tailed, nonparametric Mann-Whitney test was used to compare the virus burden measured by real time RT-PCR between control animals and animals that received CART. Significant differences were assumed for probability values of  $p < 0.05$ .

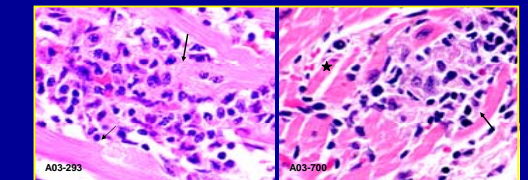
TABLE 1. Rhesus Macaque cohort

Control	CART
A02-712	A03-292
A02-734	A03-293
A02-743	A03-699
A02-746	A03-700

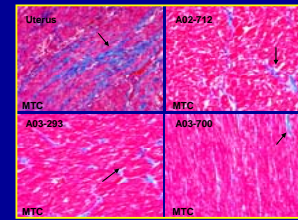
## Results



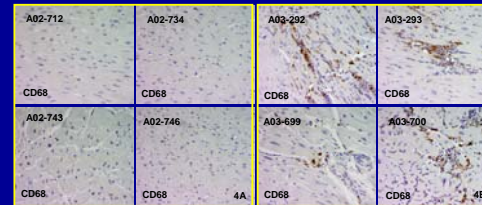
**Figure 1.** Mononuclear cell infiltrates in myocardial sections of animals that received CART. H&E stained tissue sections of left ventricle from untreated control animals (Panel 1A) show no significant lesions. In contrast, the myocardium of animals that received CART show multifocal mononuclear inflammatory cell infiltrates compressing myocardial fibers (Panel 1B). Note the degeneration and necrosis of cardiomyocytes. All sections are at 200X magnification.



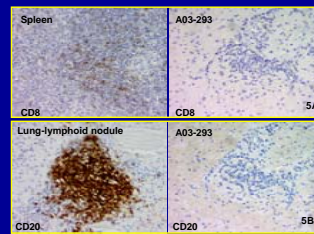
**Figure 2.** Myocardial degeneration and necrosis. Inflammatory lesions contain degenerating cardiomyocytes as shown in A03-293 (arrows). In addition, lesions contain atrophied myocardial fibers (asterisks) and mononuclear cells within cardiomyocytes (arrow), as shown in A03-700. Sections are at 600X magnification.



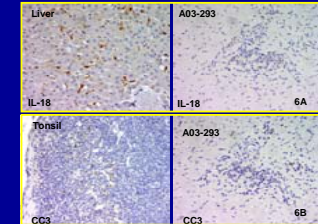
**Figure 3.** Myocardial fibrosis if not evident in either animals that received CART or controls. Masson's trichrome staining was performed to localize collagen fibers (blue stain, indicated by arrow) within the myocardial sections of both control animals and animals that received CART. Significant fibrosis was not apparent in any animals from either group. A section of uterus with fibrosis served as a positive control. All sections are at 200X magnification.



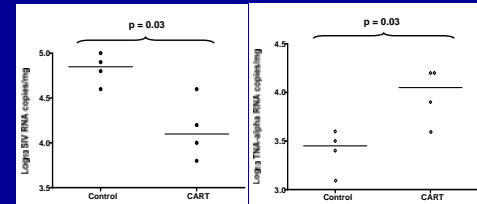
**Figure 4.** Activated macrophages are localized in myocardium from monkeys that received CART but not controls. IHC for CD68, an antigen expressed on activated macrophages. Panel 4B shows large numbers of CD68+ macrophages within inflammatory infiltrates in myocardial sections from animals that received CART. In contrast, CD68 expressing cells are absent from myocardial sections from control animals (Panel 4A). DAB (brown) chromogen with hematoxylin counterstain. All sections are at 200X magnification.



**Figure 5.** Myocardial infiltrates are not composed of T or B lymphocytes. Panel 5A, CD8 expressing T lymphocytes are not localized within myocardial lesions. A section of spleen containing CD8+ T lymphocytes (brown DAB chromogen) served as positive control. Panel 5B, myocardial lesions did not contain CD20-expressing B lymphocytes. Large numbers of DAB (brown) chromogen-stained CD20 positive B-lymphocytes are shown in a lymphoid nodule within a section of lung tissue, which served as positive control. All sections are at 200X magnification.



**Figure 6.** IL-18 and Cleaved Caspase 3 (CC3) are not expressed by cardiomyocytes within lesions. Panel 6A, small numbers of mononuclear cells expressing the pro-inflammatory cytokine IL-18 were localized within myocardial lesions; however, IL-18 expressing cardiomyocytes were not seen in the tissues from either group. A section of liver containing IL-18 expressing Kupfer cells (brown chromogen) served as a positive control. Panel 6B, CC3 is not expressed by cardiomyocytes in myocardial sections from either group. CC3 expression localized by DAB (brown) chromogen in the lymphocytes of a section of tonsil served as positive control. All sections are at 200X magnification.



**Figure 7.** Quantification of virus burden in myocardial tissue by real time RT-PCR. Tissue virus burdens were significantly lower in the left ventricles of animals that received CART as compared to untreated controls. Horizontal bars represent median values for each data set.

**Figure 8.** Enhanced expression of TNF-alpha in the myocardium of animals that received CART. Real-time RT-PCR with TNF-alpha specific TaqMan probe revealed significantly greater levels of TNF-alpha expression in the myocardium of animals that received CART as compared to control animals. Horizontal bars represent median values for each data set.

## Conclusions

1. Macrophages are the predominant inflammatory cells involved in myocarditis associated with short-term antiretroviral therapy.
2. IL-18 may not be a key cytokine involved in the inflammatory process.
3. Caspase-3 mediated apoptosis of cardiomyocytes is not evident; however, apoptosis mediated by other caspases and caspase-independent pathways was not investigated.
4. The quantity of virion-associated RNA was significantly lower in myocardial tissue from animals that received CART as compared to controls, suggesting that CART was successful in reducing SIV burden.
5. Enhanced expression of TNF-alpha in the myocardium of animals that received CART indicates that the inflammation is mediated through TNF-alpha.