

Anti- α 4 Integrin Antibody Blocks Monocyte/Macrophage Traffic to the Heart and Decreases Cardiac Pathology in a SIV Infection Model of AIDS

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Background—Cardiovascular disease (CVD), myocarditis and fibrosis are comorbidities of HIV⁺ individuals on durable antiretroviral therapy (ART). Although mechanisms for these vary, monocytes/macrophages are increasingly demonstrated to be key players.

Methods and Results—We directly blocked monocyte/macrophage traffic to the heart in an SIV model of AIDS using an anti-alpha-4 integrin antibody (natalizumab). Nineteen Rhesus macaques were SIVmac251 infected and CD8-lymphocyte depleted for the development of rapid AIDS. Ten animals received natalizumab once a week, for 3 weeks, and were sacrificed 1 week later. Six animals began treatment at the time of infection (early) and the remaining 4 began treatment 28 days post-infection (late), a time point we have previously established when significant cardiac inflammation occurs. Nine animals were untreated controls; of these, 3 were sacrificed early and 6 were sacrificed late. At necropsy, we found decreased SIV-associated cardiac pathology in late natalizumab-treated animals, compared to untreated controls. Early and late treatment resulted in significant reductions in numbers of CD163⁺ and CD68⁺ macrophages in cardiac tissues, compared to untreated controls, and a trend in decreasing numbers of newly recruited MAC387⁺ and BrdU⁺ (recruited) monocytes/macrophages. In late treated animals, decreased macrophage numbers in cardiac tissues correlated with decreased fibrosis. Early and late treatment resulted in decreased cardiomyocyte damage.

Conclusions—These data demonstrate a role for macrophages in the development of cardiac inflammation and fibrosis, and suggest that blocking monocyte/macrophage traffic to the heart can alleviate HIV- and SIV-associated myocarditis and fibrosis. They underscore the importance of targeting macrophage activation and traffic as an adjunctive therapy in HIV infection. (*J Am Heart Assoc.* 2015;4:e001932 doi: 10.1161/JAHA.115.001932)

Key Words: animal model • cardiomyopathy • fibrosis • HIV • myocarditis

Combination antiretroviral therapy (cART) has increased the life expectancy of HIV⁺ individuals, but comorbidities, including neurological,¹ renal,² bone,³ and cardiovascular (CV) disease (CVD)^{4–9} exist.^{10–12} HIV-associated CVD is a leading cause of HIV-associated mortality, where there is a 2-fold increase in the relative risk compared to noninfected, age-matched individuals.¹³ HIV-associated CVD, which includes atherosclerosis, dilated cardiomyopathy, myocarditis, and

myocardial infarction,^{7,14,15} likely has multiple etiologies, including toxic effects of cART,¹⁶ opportunistic infections,¹⁷ and chronic immune activation,¹⁸ but monocyte/macrophages are emerging as central players.

Dilated cardiomyopathy and myocarditis with HIV infection was evident in 40% to 50% of AIDS patients at necropsy in the pre-cART era.¹⁹ With effective cART in developed countries, incidence has decreased approximately 30%, suggesting that dilated cardiomyopathy and myocarditis with HIV and cART has declined.^{7,19,20} Despite this, magnetic resonance imaging and spectroscopy show that HIV⁺ individuals continue to have subclinical myocardial disease with myocardial fibrosis and alterations in cardiac function.^{21,22} Recent data comparing the rates of mortality in the pre- and post-cART era support these findings, where HIV⁺ individuals had a 6.3-fold increase in mortality resulting from cardiomyopathy and myocarditis in the post-cART era. Additionally, a recent study examining myocardial and microvascular inflammation showed that myocarditis is still present with HIV infection.²³ This suggests that HIV infection with effective cART can still lead to increased cardiac fibrosis and myocarditis.

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SIV-infected rhesus macaques are an excellent model to study the effects of SIV on cardiac inflammation and fibrosis. Previous work established that SIV-infected monkeys have dilated cardiomyopathy and myocarditis.^{24,25} Overall few SIV- or HIV-RNA or protein positive cells are found in cardiac tissues, similar to what is found in HIV-infected human cardiac tissues.^{26,27} Despite this, levels of SIV-RNA correlate with diastolic dysfunction, underscoring the role of lentiviral infection and cardiac dysfunction with AIDS.²⁷ We have previously demonstrated that SIV-infected, CD8-lymphocyte-depleted animals develop rapid and consistent AIDS with macrophage accumulation in the heart, cardiomyocyte damage, and fibrosis²⁸ and accumulation of CD163⁺ macrophages correlated with increased fibrosis.^{28,29} In these studies, bromodeoxyuridine (BrdU)-labeled monocyte/macrophages, which were labeled in the bone marrow and traffic to the heart, were increased late in infection. Overall, these observations support the hypothesis that macrophage activation and accumulation with SIV and HIV infection play a critical role in the development of cardiac pathology. To date, no studies have directly blocked monocyte/macrophage traffic to the heart in experimental infection with SIV.

Although no studies have directly blocked monocyte/macrophage traffic to the heart with SIV or HIV infection, data from experimental and clinical studies that diminished macrophage activation or accumulation in the heart support the notion that these cells are major players in cardiac pathogenesis. Thus, studies that blocked chemokine (C-C motif) ligand 5 (CCR5) using chemokine (C-C motif) ligand 5/regulated on activation normal T-cell expressed and secreted resulted in decreased monocyte/macrophage and T-lymphocyte accumulation,^{30,31} likely by indirect mechanisms. Similarly, blocking CCR5 with the anti-CCR5 antibody, maraviroc, resulted in reduced cardiac CD163 expression by macrophages and prevented diastolic dysfunction in SIV-infected monkeys.³² Similarly, studies blocking macrophage inhibitory factor showed decreased T-cell and macrophage migration and inhibition of onset of myocarditis in a rodent model of experimental autoimmune myocarditis.³³ Treatment with the statin, pravastatin, decreased macrophage numbers in abdominal aortic plaques of uninfected monkeys.³⁴ Another statin, atorvastatin, that did not reduce aortic inflammation in HIV⁺ infected individuals, did decrease the volume and high-risk features of noncalcified plaques.³⁵ In humans with coronary artery disease, angiotensin receptor blocker treatment resulted in decreased numbers of atherosclerotic lesions and significantly decreasing levels of soluble markers of inflammation C-reactive protein, interleukin-6 (IL-6), and monocyte chemoattractant protein 1.^{36,37}

In this study, we examined whether directly blocking leukocyte and monocyte/macrophage traffic to cardiac tissues with an anti- $\alpha 4$ integrin antibody, natalizumab,

decreased SIV-associated cardiac pathology (inflammation, fibrosis, and cardiomyocyte damage). Natalizumab is an anti- $\alpha 4$ antibody that binds to the $\alpha 4$ subunit of $\alpha 4\beta 1$ and $\alpha 4\beta 7$ integrins and blocks interactions between $\alpha 4$ and its ligands.³⁸ Natalizumab has been used effectively to treat multiple sclerosis³⁹ and Crohn's disease,⁴⁰ blocking accumulation of lymphocytes and monocytes/macrophages in the brain and gut, but not lymph nodes. In rodents, blocking $\alpha 4$ integrins reduced macrophage homing to atherosclerotic plaques.⁴¹ We have shown that natalizumab treatment in SIV-infected rhesus macaques with AIDS blocked monocyte/macrophage traffic to the central nervous system (CNS) and leukocytes to the gut, resulting in decreased numbers of SIV-RNA and SIV-p28-positive cells.⁴² Furthermore, natalizumab treatment of monkeys at the time of SIV infection resulted in undetectable SIV-RNA, -DNA and -p28 in the CNS and gut in the majority of animals, as well as the absence of leukocyte inflammation. In the current study, we examined whether natalizumab treatment decreased monocyte/macrophage accumulation in the heart and whether such treatment decreased cardiac fibrosis and myocyte damage.

Methods

Ethical Treatment of Animals

Treatment of animals in this study was in accord with the Guide for the Care and Use of Laboratory Animals, 8th edition. Animals were housed at the New England Regional Primate Center (NERPC; Southborough, MA), Tulane National Primate Research Center (TNPRC; Covington, LA), or BIOQUAL (Baltimore, MD). The NEPRC Protocol Number for this study is 04420 and the Animal Welfare Assurance Number is A3431-01. The TNPRC Number for this study is 3497 and the Animal Welfare Assurance Number is A4499-01. Animals were monitored daily for evidence of disease progression and changes in appetite or behavior, with clinical support administered under the direction of an attending veterinarian.

Animals, SIV Infection, and CD8 Lymphocyte Depletion

Nineteen rhesus macaques (*Macaca mulatta*) were infected with SIV mac251 (2 ng of SIV-p27) intravenously (kindly provided by Ronald Desrosiers, University of Miami; Table 1). All animals were CD8-lymphocyte depleted using cM-T807, a human anti-CD8 antibody, administered subcutaneously (10 mg/kg) on day 6 post-infection (pi) and intravenously (5 mg/kg) on days 8 and 12 pi, as previously described.¹ Ten animals (n=4 late natalizumab treated, n=6 untreated) were sacrificed at similar time points with progression to AIDS (49 to 65 days postinfection [dpi]). All CD8-lymphocyte-depleted

Table 1. Animals Used in This Study and Cardiac Pathology

Animal Groups	ID	Primate Center	Start of Natalizumab (dpi)	BrdU Administration (dpi)	Survival (dpi)	Cardiac Pathology		
						Inflammation	Fibrosis	Cardiomyocyte Degeneration
Early untreated n=3	A1	TNPRC	—	6, 20	22	NSF	NSF	NSF
	A2	TNPRC	—	6, 20	22	NSF	NSF	NSF
	A3	TNPRC	—	22	22	Mild	Mild	NSF
Early natalizumab n=6	A4	NERPC	0	6, 20	21	NSF	NSF	NSF
	A5	NERPC	0	6, 20	21	NSF	NSF	NSF
	A6	BIOQUAL	0	6, 20	21	NSF	NSF	NSF
	A7	BIOQUAL	0	6, 20	21	NSF	NSF	NSF
	A8	BIOQUAL	0	6, 20	21	Mild	Mild	Mild
	A9	BIOQUAL	0	6, 20	21	Mild	Mild	NSF
Late untreated without cardiac pathology n=3	A10	NERPC	—	49	56	NSF	NSF	NSF
	A11	NERPC	—	pre, 7, 20, 41, 54	56	NSF	NSF	NSF
	A12	NERPC	—	pre, 7, 20, 41, 54	55	Mild	Mild	NSF
Late untreated with cardiac pathology n=3	A13	TNPRC	—	pre, 7, 26, 55	56	Moderate	Moderate	Mild
	A14	TNPRC	—	pre, 7, 26, 55	65	Moderate	Moderate	Mild
	A15	NERPC	—	6, 20	60	Severe	Moderate	Moderate
Late natalizumab n=4	A16	NERPC	28	pre, 26, 47	49	Mild	NSF	NSF
	A17	NERPC	28	pre, 26, 47	49	Mild	NSF	NSF
	A18	NERPC	28	33, 47	49	Mild	Mild	NSF
	A19	NERPC	28	33, 47	49	Moderate	Mild	Mild

Nineteen animals were used in this study, housed at either the New England Regional Primate Center (NERPC), Tulane National Primate Research Center (TNPRC), or BIOQUAL, as indicated. Six animals began natalizumab treatment at the time of infection at 0 days postinfection (dpi) and were sacrificed at 21 dpi. Three early untreated controls were sacrificed at 22 dpi. Four late natalizumab-treated animals began treatment at 28 dpi and were sacrificed at 49 dpi. Three animals each for late untreated controls without cardiac pathology and with cardiac pathology were sacrificed at 56 to 65 dpi. Pathology was assessed based on the degree of inflammation, fibrosis, and cardiomyocyte degeneration. To investigate whether blocking monocyte/macrophage traffic to the heart decreased SIV-associated cardiac pathology, 10 randomly chosen, $\times 200$ fields of view were chosen and analyzed blindly by a veterinary pathologist. Sections of cardiac tissue were scored based on the degree of change as having no significant findings (NSF), mild, moderate, or severe inflammation, fibrosis, and cardiomyocyte degeneration. BrdU indicates bromodeoxyuridine.

animals had high viral load at peak viremia that remained elevated and was not different between early and late treated animals and controls. Six early natalizumab-treated animals were sacrificed at 21 dpi and 3 untreated controls were sacrificed at 22 dpi. Sections of left ventricular myocardium (hereafter referred to as cardiac tissue) were analyzed by a board-certified veterinary pathologist (A.D.M.), and scored based on degree of inflammation, fibrosis, and cardiomyocyte degeneration. Sections were scored as either no significant findings (NSF), mild, moderate, or severe, based on degree of change in cardiac tissues, as previously described.²⁸

Anti- $\alpha 4$ Integrin (Natalizumab) and BrdU Administration

The anti- $\alpha 4$ integrin monoclonal antibody (natalizumab) was provided by Biogen Idec (Cambridge, MA) in a sterile concentrated solution. Natalizumab has specificity for the

$\alpha 4$ subunit of $\alpha 4\beta 1$ and $\alpha 4\beta 7$ integrins expressed on surfaces of all leukocytes, except neutrophils.⁴³ Natalizumab was administered weekly for 3 weeks beginning on the day of infection (0 dpi; n=6) or on 28 dpi (n=4), as previously described.⁴² This treatment regimen maintains high levels of natalizumab in serum of rhesus macaques during treatment.⁴⁴ To study monocyte/macrophage traffic to the heart, animals were administered BrdU (60 mg/kg) at indicated time points (Table 1), as previously described.⁴²

Immunohistochemistry

Numbers of macrophages and T lymphocytes present in formalin-fixed, paraffin-embedded tissues were determined by immunohistochemistry and cell counting. Cardiac tissues were stained with antibodies against CD163 (1:250; AbD Serotec, Kidlington, UK), CD68 (1:200; Dako, Carpinteria, CA), MAC387 (1:100; Dako) macrophages, and CD3⁺ T lympho-

cytes (1:300; Dako). The number of macrophages that traffic to cardiac tissues was determined using a mouse monoclonal BrdU antibody (1:50), as previously described.²⁸ Twenty random, nonoverlapping, $\times 200$ microscopic fields of view were taken for each animal and the number of positive cells/mm² calculated for each. Data are represented as the average number of positive cells/mm² from the 20 random fields.

Masson's Trichrome Stain

Percent of collagen per tissue area used as a marker of fibrosis²⁹ was measured using a modified Massons Trichrome Stain kit (Newcomer Supply, Middleton, WI), according to the manufacturer's recommendation. Tissue sections were imaged using a Zeiss Axio Imager M1 microscope (Zeiss, Oberkochen, Germany) using Plan-Apochromat $\times 20/0.8$ Korr objectives, as previously described.^{28,45} The area of red and blue dyes corresponding to cytoplasm and collagen, respectively, were measured to determine the percentage of total tissue area.

Statistical Analysis

Statistical analyses were conducted using Prism software (version 6.0; GraphPad Software Inc., La Jolla, CA). *P* values were calculated using the nonparametric Mann–Whitney *t* test with significance accepted at $P < 0.05$ when comparing early and late natalizumab-treated animals to early and late untreated controls. ANOVA was used to compare late natalizumab-treated animals to late untreated animals with and without cardiac pathology. If the ANOVA was significant ($P < 0.05$), then a post-hoc nonparametric Mann–Whitney *t* test was performed. To determine whether changes in numbers of macrophages in cardiac tissues correlates with changes in fibrosis, a nonparametric Spearman rank correlation was used where $P < 0.05$ was significant.

Results

Natalizumab Treatment Decreases the Frequency and Severity of Pathology in SIV-Infected, CD8-Lymphocyte-Depleted Rhesus Macaque Cardiac Tissues

The relative degree of pathology in cardiac tissues was assessed based on levels of inflammation, fibrosis, and cardiomyocyte degeneration. Normal sections were scored as having no significant findings (NSF). When present, inflammation, fibrosis, and cardiomyocyte degeneration were scored as mild, moderate, or severe. We found no significant changes in the pathology of cardiac tissues in early natalizumab-treated animals, compared to untreated controls.

Of 3 SIV-infected untreated animals sacrificed early, 21 dpi ($n=3$), 2 had no significant findings with regard to inflammation or fibrosis. The remaining SIV-infected untreated controls, sacrificed at 21 dpi, had mild inflammation and mild fibrosis. In the early natalizumab-treated group ($n=6$), sacrificed at 21 dpi, 4 animals had no significant findings in regard to inflammation and fibrosis and 2 had mild inflammation and fibrosis (Table 1).

Overall, natalizumab treatment decreased cardiac pathology in late treated animals ($n=4$), compared to late untreated controls ($n=6$; Table 1). Three of 4 late treated animals had no significant findings with respect to cardiomyocyte degeneration, and 2 of 4 had no significant findings with regard to fibrosis. SIV-infected, untreated late control animals with cardiac pathology ($n=3$) had an increased severity, compared to late treated SIV-infected animals (Table 1). Two of the late controls with pathology had moderate inflammation and 1 had mild inflammation. All 3 late controls with pathology had moderate fibrosis, whereas 2 had mild and 1 had moderate cardiomyocyte degeneration. Compared to the late controls with pathology, the severity of pathology in SIV-infected, late natalizumab-treated animals ($n=4$) was diminished (Table 1). Three late treated animals had mild inflammation, whereas the remaining had moderate inflammation. Two late treated animals had no inflammation and 2 had only mild inflammation. Three late treated animals had no significant findings with regard to cardiomyocyte degeneration and 1 had mild cardiomyocyte degeneration (Table 1).

Natalizumab Treatment Decreases the Number of Macrophages Present in Cardiac Tissue in Early and Late Treated Animals

In animals that began natalizumab treatment early at 0 dpi, there was a significant decrease in the number of CD163⁺ and CD68⁺ macrophages in heart tissues, compared to untreated controls sacrificed at the same time point (Figure 1). There was a significant 3.35- and 3.74-fold decrease in the numbers of CD163⁺ and CD68⁺ macrophages, respectively, in cardiac tissue in early natalizumab-treated animals, compared to controls (Figure 1B; Table 2; nonparametric Mann–Whitney *t* test, $P < 0.05$). Though not significant, there were decreased numbers of newly infiltrating MAC387⁺ macrophages and CD3 T-lymphocytes in early treated animals (Figure 1B; Table 2).

Natalizumab treatment beginning on 28 dpi (late) resulted in a significant decrease in numbers of CD163⁺ and CD68⁺ macrophages, when compared to all SIV-infected untreated late control animals (Figure 1). We found a 3.53- and 1.19-fold decrease in numbers of CD163⁺ and CD68⁺ macrophages, respectively, in cardiac tissues of late natalizumab-treated tissues, compared to controls (Table 2; nonparametric Mann–

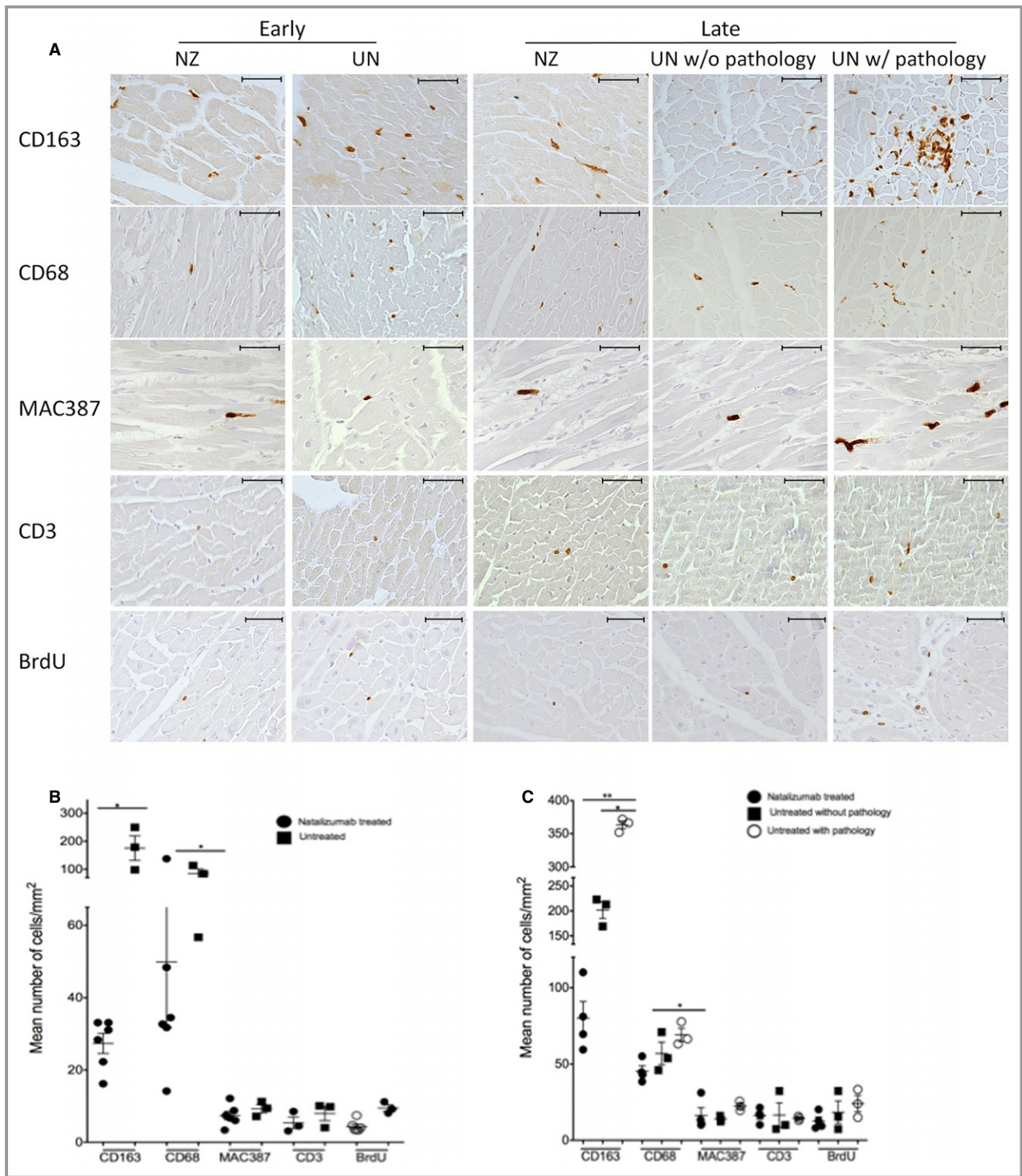


Figure 1. Natalizumab treatment decreases the number of macrophages in cardiac tissues in SIV-infected, CD8-lymphocyte-depleted rhesus macaques. A, Sections of left ventricular tissues from early and late natalizumab-treated animals and matched controls were immunohistochemically stained with antibodies recognizing CD163⁺, CD68⁺, and MAC387⁺ macrophages and CD3⁺ T lymphocytes. B and C, Twenty random, nonoverlapping, ×200 fields of view were taken for each animal and the average number of cells/mm² calculated. In both early and late natalizumab-treated animals, there was a decrease in the numbers of CD163⁺ and CD68⁺ macrophages when compared to controls, with no differences in T lymphocytes detected. Statistical analysis between early natalizumab-treated animals and controls was done using a nonparametric Mann–Whitney *t* test. For late natalizumab-treated animals and untreated controls with and without cardiac pathology, an ANOVA was performed first, and, if significant, a post-hoc nonparametric Mann–Whitney *t* test was performed (**P*<0.05; ***P*<0.01). Scale bar=50 μm, ×400 magnification. Error bars represent the average number of positive cells/mm²±SEM. BrdU indicates bromodeoxyuridine; NZ, natalizumab treated; UN, untreated.

Table 2. Numbers of Macrophages and T Lymphocytes in Natalizumab-Treated Animals and Controls

Immune Markers	Early				Late			
	Untreated (n=3)	NZ (n=6)	P Value	Fold Decrease	Untreated (n=6)	NZ (n=4)	P Value	Fold Decrease
CD163	158.84 (±55.78)	47.36 (±18.77)	*	3.35	282.45 (±36.97)	80.06 (±10.95)	**	3.53
CD68	84.34 (±16.67)	22.54 (±5.09)	*	3.74	63.01 (±4.71)	52.87 (±10.83)	*	1.19
MAC387	9.33 (±1.17)	7.43 (±3.19)	ns	—	18.25 (±2.11)	15.91 (±7.46)	ns	—
CD3	8.05 (±2.13)	5.13 (±3.02)	ns	—	15.55 (±3.60)	12.27 (±5.42)	ns	—
BrdU	9.31 (±1.57)	4.39 (±0.64)	ns	—	21.19 (±5.85)	16.42 (±3.19)	ns	—

Numbers represent the mean number of positive cells (cells/mm²)±SEM, in parentheses. All animals were SIV-infected and CD8-lymphocyte depleted, with 10 of the animals receiving natalizumab. Twenty random, nonoverlapping, ×200 fields of view were counted for each animal and the average number of positive cells/mm² calculated. *P* values were calculated by comparing the mean number of positive cells for the indicated groups using the nonparametric Mann–Whitney *t* test (**P*<0.05; ***P*<0.01). Fold change was calculated for the numbers of cells where there was a significant difference between the indicated groups. Early natalizumab-treated animals began treatment at the time of infection, 0 days postinfection (dpi). Late natalizumab-treated animals began treatment 28 dpi. All treated animals were treated weekly for 3 weeks with a dose of 30 mg/kg of α-VLA-4. BrdU indicates bromodeoxyuridine; ns, no significance; NZ, natalizumab treated.

Whitney *t* test, *P*<0.05; *P*<0.01). We next examined whether the numbers of macrophages in late natalizumab-treated animals differed between late untreated control animals with and without cardiac pathology.

Late treated animals had decreased numbers of CD163⁺ macrophages, compared to untreated late controls without cardiac pathology (Figure 1A). There was a 2.51-fold decrease in the number of CD163⁺ macrophages in cardiac tissue in late treated animals, compared to controls without cardiac pathology (Figure 1B; Table 3; nonparametric Mann–Whitney *t* test, *P*<0.05). There were no differences in numbers of CD68⁺, MAC387⁺ macrophages and CD3 T lymphocytes in cardiac tissues of late natalizumab-treated animals and untreated controls without pathology (Table 3).

Significant reductions in the number of CD163⁺ and CD68⁺ macrophages were found in late treated animals, compared to late untreated controls with cardiac pathology (Figure 1A).

There was a 4.53- and 1.59-fold decrease in numbers of CD163⁺ and CD68⁺ macrophages present in cardiac tissues, compared to late natalizumab treated animals (Figure 1C; Table 3; nonparametric Mann–Whitney *t* test, *P*<0.05, *P*<0.01). Similar to early treated animals, late treated animals had a trend of decreased numbers of newly infiltrating macrophages expressing MAC387 and CD3 T lymphocytes, compared to late untreated animals with cardiac pathology (Table 3).

Natalizumab Treatment Blocks Traffic of Macrophages to the Cardiac Tissues

BrdU experiments were used to further determine whether natalizumab treatment blocks traffic of monocyte/macrophages to the heart. Previously, we have shown that the majority of BrdU⁺ macrophages in the heart are MAC387⁺ macrophages.²⁸ In early natalizumab-treated animals, there

Table 3. Numbers of Macrophages and T Lymphocytes in Late Natalizumab-Treated Animals and Controls Without and With Cardiac Pathology

Immune Markers	Late						
	Untreated w/o Pathology n=3	Untreated w/Pathology n=3	NZ n=4	P Value NZ vs w/o	P Value NZ vs w/	Fold Decrease NZ vs w/o	Fold Decrease NZ vs w/
CD163	195.33 (±16.37)	363.23 (±15.87)	80.06 (±10.95)	*	**	2.51	4.53
CD68	56.96 (±7.37)	84.13 (±4.38)	52.87 (±10.83)	ns	*	—	1.59
MAC387	14.01 (±1.09)	22.51 (±1.77)	15.91 (±7.46)	ns	ns	—	—
CD3	13.17 (±3.76)	16.63 (±7.92)	12.27 (±5.42)	ns	ns	—	—
BrdU	19.86 (±10.08)	22.51 (±5.23)	16.17 (±3.19)	ns	ns	—	—

Numbers represent the mean number of positive cells (cells/mm²)±SEM, in parentheses. All animals were SIV-infected and CD8-lymphocyte depleted. Twenty random, nonoverlapping, ×200 fields of view were counted for each animal and the average number of positive cells/mm² calculated. ANOVA was used to compare late natalizumab-treated animals to late untreated animals with and without cardiac pathology. If the ANOVA was significant (*P*<0.05), then post-hoc Mann–Whitney *t* tests were performed. BrdU indicates bromodeoxyuridine; ns, no significance; NZ, natalizumab treated; w/, untreated with cardiac pathology; w/o, untreated without cardiac pathology.

P*<0.05; *P*<0.01.

were few BrdU⁺ cells (4.38 ± 0.64 cells/mm²) and a trend of decreasing numbers of BrdU⁺ cells, compared to untreated controls (9.31 ± 1.57 cells/mm²; Figure 1A and 1B; Table 2). Animals that began natalizumab treatment at 28 dpi had decreased numbers of BrdU⁺ cells (16.42 ± 3.19 cells/mm²), compared to untreated animals with cardiac pathology (22.51 ± 5.23 cells/mm²; Figure 1A and 1C; Table 3). The number of BrdU⁺ cells in late natalizumab-treated animals did not differ, when compared to untreated animals without cardiac pathology (Figure 1A and 1C; Table 3).

Decreased Fibrosis in Cardiac Tissues of Natalizumab-Treated Animals Correlates With Significant Decreases in Macrophage Numbers

Using a modified Masson's trichrome stain, the percent collagen per total tissue area in cardiac tissues of natalizumab-treated animals and untreated controls was quantified (Figure 2A and 2B). Compared to controls, both early and late natalizumab-treated animals had decreased amounts of collagen (Figure 2C). In early natalizumab-treated animals, the average percent collagen per total tissue area was $5.58 \pm 2.56\%$, compared to $9.6 \pm 2.06\%$ for untreated controls, a significant 1.72-fold decrease in percent collagen (Figure 2C, left; $P < 0.05$, nonparametric Mann–Whitney *t* test).

Animals that began natalizumab treatment at 28 dpi (late) had a significantly higher average percentage of collagen per tissue area in the left ventricle ($8.66 \pm 2.31\%$), compared to animals that began treatment early (5.58 ± 1.47 ; Figure 2C, right; $P < 0.05$, nonparametric Mann–Whitney *t* test). Late natalizumab-treated animals had no significant differences in the percent of collagen per total tissue area, compared to untreated animals without cardiac pathology ($8.66 \pm 2.31\%$ versus $10.33 \pm 1.84\%$). However, when compared to untreated animals with cardiac pathology, there was a significant decrease in the percentage of collagen per tissue area. Whereas late natalizumab-treated animals had an average percentage of collagen of $8.66 \pm 2.31\%$, untreated animals with cardiac pathology had an average of $19.91 \pm 1.85\%$, a significant 2.29-fold decrease in the average percent collagen per total tissue area (Figure 2C, right; $P < 0.05$, nonparametric Mann–Whitney *t* test).

We next examined whether there was a correlation between decreased fibrosis in natalizumab-treated animals and changes in macrophage numbers in cardiac tissues if significant differences in macrophage numbers were found between groups. There was a correlation between increased fibrosis and increased numbers of CD163⁺ ($r = 0.9$; $P < 0.05$) and CD68⁺ ($r = 0.86$; $P < 0.05$) macrophages in untreated controls sacrificed at 21 dpi, compared to early natal-

izumab-treated animals (Figure 2D). A correlation also existed in late natalizumab-treated animals, compared to all late untreated controls for CD163⁺ ($r = 0.85$; $P < 0.05$), CD68⁺ ($r = 0.55$; $P < 0.05$) macrophages and fibrosis (Figure 2E).

Discussion

Chronic inflammation persists within HIV-infected individuals despite effective cART and decreased plasma viral load to undetectable levels.^{46–48} With chronic inflammation, there are increased comorbidities, compared to the general non-HIV-infected population.^{49–51} In particular, there is an increased incidence of CVD⁴ where monocytes/macrophages are increasingly considered to play a role.^{52,53} Previously, we have shown that SIV-infected, CD8-lymphocyte-depleted monkeys have increased numbers of macrophages (CD163⁺, CD68⁺, and MAC387⁺) in cardiac tissues that positively correlate increased fibrosis.²⁸ In this study, we examined whether an anti- $\alpha 4$ integrin antibody, natalizumab, diminishes leukocyte and monocyte/macrophage traffic to the heart resulting in decreased fibrosis.

Natalizumab blocks the interaction between $\alpha 4$ integrin and its ligand, vascular cell adhesion molecule 1 (VCAM-1).³⁸ VCAM-1 is expressed on endothelial cells of the arterial lumen with atherosclerosis.⁵⁴ Studies in mice showed that inhibiting the interaction between $\alpha 4$ and VCAM-1 decreased macrophage recruitment to atherosclerotic plaques.⁴¹ Previously, we have shown that there is a higher level of macrophage traffic to the heart later in SIV infection (>21 dpi) and that there are few macrophages present in untreated animals sacrificed at 21 dpi.²⁸ In the current study, we found that natalizumab treatment beginning at 0 dpi resulted in decreased numbers of CD163⁺ and CD68⁺ macrophages, compared to uninfected controls, but cardiac pathology in early infection is minimal and most of the pathology occurred in the later stages of infection.

When compared to untreated controls with cardiac pathology, late treated animals had significant decreases in the number of CD163⁺ and CD68⁺ macrophages. In fact, the numbers of macrophages in late natalizumab-treated animals were similar to untreated animals without cardiac pathology. Additionally, we found a correlation between decreased macrophage numbers in natalizumab-treated animals with decreased cardiac fibrosis. Overall, these data show that blocking monocyte/macrophage traffic to the heart alleviates HIV- and SIV-associated cardiac pathology that resulted in reduced inflammation, fibrosis, and cardiomyocyte degeneration.

Though not significant, we found a trend of decreasing numbers of MAC387⁺ macrophages in the left ventricle of late treated animals, compared to untreated animals with cardiac

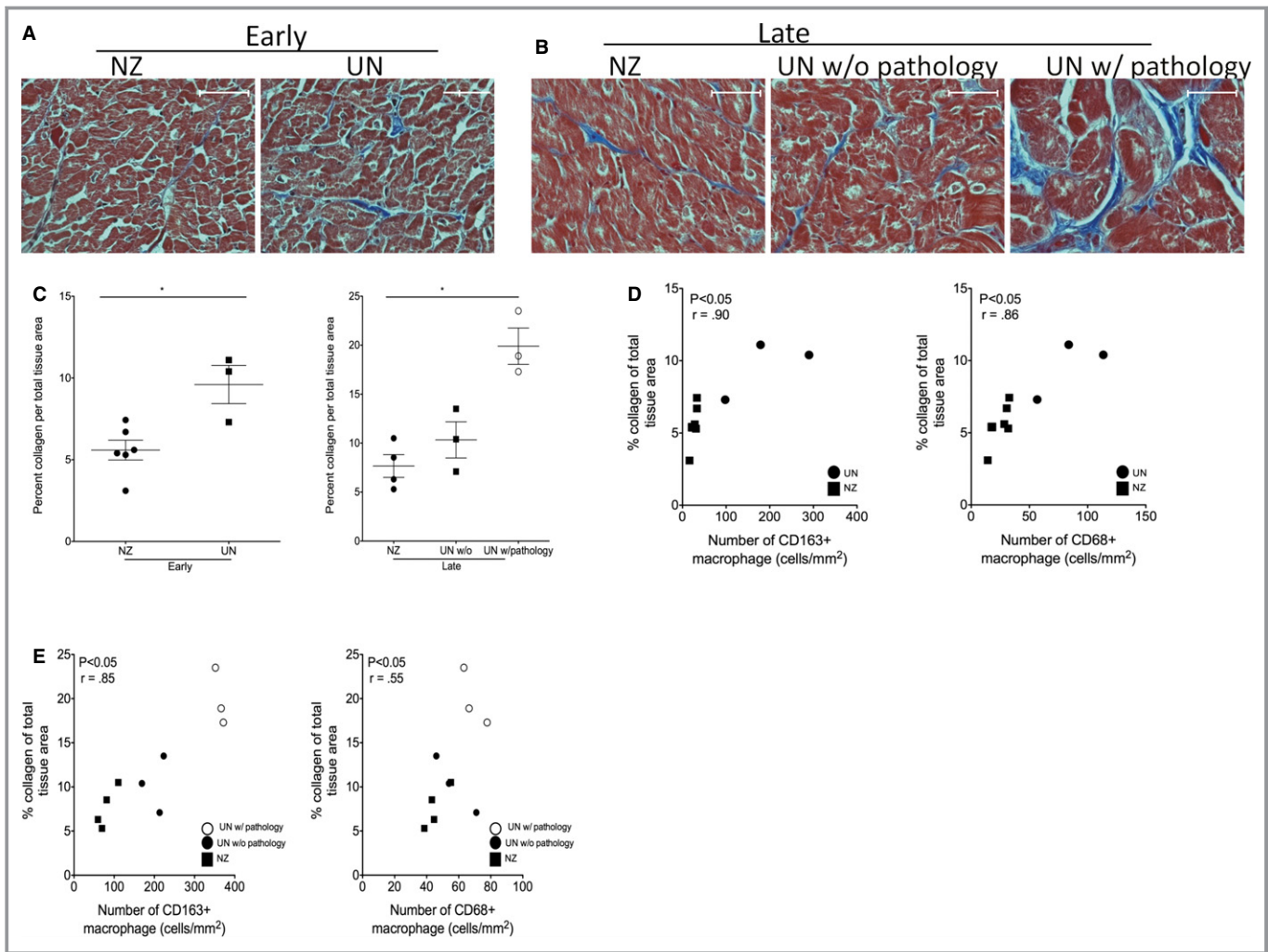


Figure 2. Natalizumab treatment decreases fibrosis in the left ventricle of SIV-infected, CD8-lymphocyte-depleted rhesus macaques. A and B, Modified Masson's trichrome stain was used to compare the percent collagen per total tissue area, a marker of fibrosis, in the left ventricle of early and late natalizumab-treated animals compared to untreated controls. C, Natalizumab treatment resulted in decreased fibrosis in cardiac tissues, compared to controls, regardless of when treatment began. In animals that began natalizumab treatment at 0 days postinfection (dpi), there was a significant decrease in the amount of collagen per tissue area ($5.58 \pm 2.56\%$), compared to untreated controls ($9.6 \pm 2.06\%$; nonparametric Mann–Whitney t test, $P < 0.05$). Animals that began natalizumab treated at 28 dpi also showed a significant decrease in the amount of collagen per total tissue area ($8.66 \pm 2.31\%$), when compared to untreated controls with cardiac pathology ($19.91 \pm 1.85\%$). There was no difference in the percent collagen per total tissue area in late natalizumab-treated animals, compared to untreated controls without cardiac pathology. Spearman rank test was used to determine whether there was a correlation between decreases in fibrosis in natalizumab-treated animals and numbers of macrophages. D, In early natalizumab-treated animals (closed square), there was a correlation between the decrease in numbers of CD163⁺ and CD68⁺ macrophages and decreases in fibrosis, when compared to untreated controls (closed circle). E, In late natalizumab-treated animals (closed square), there was a correlation between decreases in CD163⁺ and CD68⁺ macrophages, compared to untreated controls with (open circle) and untreated controls without pathology (closed circle). r = spearman coefficient, $P < 0.05$. Statistical analysis between early natalizumab-treated animals and controls was done using a nonparametric Mann–Whitney t test. For late natalizumab-treated animals and untreated controls with and without cardiac pathology, an ANOVA was performed first, and, if significant, a post-hoc nonparametric Mann–Whitney t test was performed ($*P < 0.05$). Scale bar = 50 μ m, $\times 400$ magnification. Error bars represent the average number of positive cells/mm² \pm SEM. NZ indicates natalizumab treated; UN, untreated.

pathology. The finding of a decrease in newly recruited macrophages is supported by our observation of fewer BrdU-labeled macrophages (that traffic from the bone marrow), suggesting that macrophage traffic to the heart results in increased fibrosis. Whereas previous research showed that

natalizumab decreased traffic of CD3 T lymphocytes and MAC387⁺ to the brain and gut,⁴² in the current study, we did not find significant differences in the number of CD3 T lymphocytes or MAC387⁺ macrophages in cardiac tissues of SIV-infected animals with or without natalizumab treatment. This possibly

suggests that CD3⁺ T lymphocytes and MAC387⁺ macrophages use different integrins to traffic to the heart than to the brain or gut; however, we have previously shown that MAC387⁺ macrophages and not CD3⁺ T lymphocytes correlate with increased fibrosis in cardiac tissues.²⁸ Our lack of finding a statistically significant reduction in the number MAC387⁺ macrophages may be owing to the relatively few numbers of those cells in cardiac tissues.

Previously, we have shown that the rate of monocyte/macrophage traffic to the heart is increased later in infection (after 48 dpi), as opposed to early infection.²⁸ Using BrdU labeling, we found a trend of decreased traffic of monocytes/macrophages to the heart in late natalizumab-treated animals, compared to untreated animals that developed cardiac pathology. Late natalizumab-treated animals had a similar rate of traffic of newly released monocytes/macrophages to the heart as untreated animals without cardiac pathology. This provides evidence that potentially blocking traffic of monocyte/macrophages later during SIV infection can alleviate SIV-associated cardiac pathology.

Although cART can decrease HIV to nondetectable levels in plasma, it does not necessarily target monocyte/macrophages that play a role in the development of cardiac pathology and CVD.³² Chronic immune activation with HIV infection is posited to play a role in HIV-associated cardiovascular pathology. Previous studies show that HIV-infected individuals have increased inflammation in the ascending aorta that correlates with levels of sCD163 in plasma.⁵⁵ Increased inflammation in the aorta is also been linked to high-risk noncalcified plaques that are prone to rupture.⁸ 18-Fluorodeoxyglucose positron emission tomography imaging studies have demonstrated that such plaque areas are comprised of areas with accumulation of macrophages.^{8,56,57} Whereas macrophage accumulation in the aorta and cardiac plaques are critical in HIV-associated cardiac disease, it is not surprising that also there is increased macrophage inflammation in cardiac tissues at the same time. Unpublished data from our laboratory, using matched cardiac tissues (left ventricle) and aorta from HIV⁻ and HIV⁺ individuals, shows that, with HIV infection, there is an increased macrophage accumulation inflammation in ventricular tissues and the aorta. Moreover, increased macrophage inflammation in cardiac tissues correlates with increased fibrosis, macrophage accumulation in the aorta, and increased aortic intima-media thickness. To date, there are few therapies that target macrophages specifically or indirectly to diminish HIV-associated CV pathology. Emergent data underscore the importance of such therapy strategies.

Therapeutic agents that have been successful in the treatment noncalcified cardiac plaque in HIV⁺ individuals include 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (statins), which, fortuitously, have anti-inflammatory

effects on macrophages.^{58,59} Statin therapy in conjunction with cART reduced serum levels of inflammatory markers, including IL-6, IL-8, and tumor necrosis factor alpha, more so than cART alone.⁶⁰ Statins have similarly been used in monkeys, where they decreased the macrophage content in plaques in the abdominal aorta.³⁴ A recent study in HIV-infected individuals showed that statins significantly decreased the volume on noncalcified plaques, but whether statin use in this study directly affected monocyte and/or macrophage activation and traffic was not studied.³⁵ In rodent models of experimental autoimmune myocarditis, rosuvastatin-reduced numbers in macrophages, T lymphocytes, and multinucleated giant cells in the heart resulted in decreased numbers of apoptotic cardiomyocytes. The effects of statins on myocarditis with HIV infection have not been examined.⁶¹

Other studies found that maraviroc treatment decreased chemotaxis of monocyte/macrophages, *in vitro*,⁶² but in clinical studies with advanced HIV, it did not affect the development of immune reconstitution inflammatory syndrome.⁶³ Maraviroc is used primarily to inhibit viral replication of R5-tropic HIV by blocking interactions between the virus and CCR5 on host cells.^{64,65} Studies using maraviroc in SIV-infected monkeys demonstrated fewer CD163⁺ macrophages in the heart, but this could have been owing to a decreased CD163 expression (activation) on macrophages already present in the heart and not a decrease in inflammatory cells. All together, these experiments add further evidence to the role that monocyte/macrophages play in cardiac pathology with HIV and SIV infection, and suggest that therapies blocking monocyte/macrophage traffic to the heart could diminish HIV-associated cardiac pathology.

In this study, we showed that directly blocking monocyte/macrophage traffic to cardiac tissues with natalizumab successfully decreased the numbers of macrophages present in tissues. Studies examining whether blocking traffic to vessels result in decreased high-risk vascular plaques with HIV infection are warranted. Our data suggest that studies examining the efficacy of blocking monocyte/macrophage traffic, or directly targeting monocyte/macrophage activation as an adjunctive therapy with cART, should be examined with an aim to decrease HIV-associated cardiac pathology.

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Disclosures

None.

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