Technical Advance

Quantitative Analysis of Myocardial Inflammation by Flow Cytometry in Murine Autoimmune Myocarditis

Correlation with Cardiac Function

Marina Afanasyeva,* Dimitrios Georgakopoulos,[†]
Diego F. Belardi,[†] Amrish C. Ramsundar,*
Jobert G. Barin,* David A. Kass,[†] and
Noel R. Rose*[‡]

From the Departments of Pathology and Medicine,[†] and the
W. Harry Feinstone Department of Molecular Microbiology and
Immunology,[‡] the Johns Hopkins Medical Institutions,
Baltimore, Maryland*

Inflammation has been increasingly recognized as an important pathological component of heart failure. Existing methods of assessing myocardial infiltrate are labor-intensive and provide data that are difficult to quantify and not representative of the whole heart. As a result, little effort has been made to systematically assess the components of myocardial inflammation. We established an alternative method of quantitative assessment of myocardial inflammation by flow cytometry after enzymatic digestion of hearts to characterize the infiltrate and study the association between inflammation and cardiac function in murine experimental autoimmune myocarditis. The severity of acute myocarditis uniquely correlated with the proportion of neutrophils, but not T cells, B cells, or macrophages. Both acute and chronic phases were characterized by the presence of CD44^{high} (activated) T cells in the heart, whereas T cells trafficking through normal hearts exhibited CD44^{low} phenotype. During the chronic phase, the proportion of CD4⁺ T cells was associated with increased left-ventricular volumes and deterioration of systolic function, the hallmarks of dilated cardiomyopathy. We conclude that flow cytometry on uniformly digested mouse hearts provides sensitive and reproducible assessment of myocardial infiltrate and can be used to dissect out the specific role of individual immune components from the overall inflammatory response in the heart. (*Am J Pathol* 2004, 164:807–815)

Inflammation is an important component of cardiac pathology associated with a number of heart diseases, including myocarditis, dilated cardiomyopathy, myocardial infarction, and ischemia-reperfusion injury. However, the mechanisms underlying the progression of the inflammatory process in the heart and the development of cardiac dysfunction as a result of inflammation remain poorly understood. Animal models of experimentally induced myocarditis have been developed to gain insights into the pathogenesis of inflammatory heart disease.^{1–6}

Phenotypic characterization of the myocardial infiltrate has been traditionally assessed by immunohistochemical methods.^{7–9} Immunohistochemistry provides insights into the spatial distribution of the cellular infiltrate in the context of preserved cardiac morphology. However, the method is cumbersome for the purposes of quantifying cellular subsets and typically involves the assessment of one cellular marker at a time. Furthermore, the characterization of the infiltrate is limited to the chosen cross-section of the heart, which may not necessarily be representative of the whole myocardium because of the frequently patchy nature of myocarditis. These drawbacks necessitated the development of a more robust and quantitative method.

Flow cytometry represents a method of quantitative characterization of individual cells and provides a multiparametric assessment of cellular phenotypes by simultaneous cellular staining with multiple fluorochrome-labeled antibodies. It has been commonly applied for the analysis of peripheral blood, spleen, lymph nodes, and thymus, which was facilitated by the ease of individual cell isolation from these organs. The heart, however, pre-

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M.A. and D.G. contributed equally to this work.

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Address reprint requests to Noel R. Rose, M.D., Ph.D., Department of Pathology, Johns Hopkins School of Medicine, 720 Rutland Ave., Ross Bldg., Rm. 659, Baltimore, MD 21205. E-mail: nrrose@jhsph.edu.

sents a challenge in terms of cell separation. The myocardium represents a functional syncytium formed by anastomosing cardiomyocytes, which are supported and tethered by complex cardiac interstitium.^{10,11} These features of the myocardium make it difficult to achieve a uniform digestion with a satisfactory yield of infiltrating cells by simply placing myocardial tissue in a shaker with digestion solution, a method used for isolation of the inflammatory cells from other organs.^{12,13} These methodological difficulties have resulted in a limited use of flow cytometry in cardiac research. Meanwhile, *in vitro* studies of cardiac function have relied on cardiomyocyte isolation by means of retrograde perfusion of the coronary circulation with digestion solution.^{14,15} In this work, flow cytometry was used for the analysis of single cell suspensions obtained by cardiomyocyte isolation technique applied to individual murine hearts. Using this method, we assessed both the magnitude and phenotype of the cardiac infiltrate in normal and cardiac myosin (CM)-immunized mice. Finally, we combined flow cytometry with pressure-volume assessment^{16,17} to study the relationship between the nature of the inflammatory response and cardiac function in the chronic phase of experimental autoimmune myocarditis (EAM).

Materials and Methods

Mice

EAM was induced in 7- to 8-week-old male A/J mice and in 10- to 12-week-old female BALB/c mice obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained in The Johns Hopkins University School of Medicine conventional animal facility. The animal work was approved by the Animal Care and Use Committee of The Johns Hopkins University.

Induction and Gross Assessment of EAM

CM was purified according to the procedure of Shiverick and colleagues¹⁸ from pooled hearts of different mouse strains, 60% of which were from susceptible BALB/c and A/J mice. On days 0 and 7, BALB/c mice received subcutaneous injections of CM (200 μ g on day 0 and 250 μ g on day 7) and A/J mice received 100 nmol of peptide derived from the α -CM heavy chain¹⁹ [myh α (334-352) NH₂-DSAFDVLSTFAEEKAGVYK-COOH, synthesized by Macromolecular Resources, Colorado State University, Department of Biochemistry, Fort Collins, CO] emulsified in complete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO). When used in A/J mice, complete Freund's adjuvant was supplemented with 5 mg/ml of killed *Mycobacterium tuberculosis* strain H37Ra (Difco, Detroit, MI). On day 0, mice received an intraperitoneal injection of 500 ng of pertussis toxin (List Biological Laboratories, Campbell, CA). Hearts were scored grossly based on the extent of white discoloration by two independent investigators using the following classification: grade 0, no disease; grade 1, up to 10% of heart surface discolored; grade 2,

11 to 30%; grade 3, 31 to 50%; grade 4, 50 to 90%; and grade 5, more than 90%.

Cell Isolation

Mice were injected with 1000 U of heparin 10 minutes before cervical dislocation. The heart was rapidly removed, placed in a 37°C water bath, and the aorta was cannulated with a 22-gauge needle connected to a modified Langendorff preparation. The heart was perfused at a constant flow of 1.1 ml/minute at 37°C for 3 minutes with a Ca²⁺-free bicarbonate-based buffer containing (in mmol/L) 120 NaCl, 5.4 KCl, 1.2 NaH₂PO₄, 20 NaHCO₃, 5.6 glucose, 5 taurine, 1.6 MgCl₂, and 10 2,3-butanedione monoxime. Enzymatic digestion was initiated by perfusion with the above solution with the addition of collagenase type 2 and protease type XIV (0.895 mg/ml and 0.5 mg/ml respectively; Boehringer Mannheim, Indianapolis, IN) for an additional 7 minutes. All solutions were equilibrated with a 95% O₂-5% CO₂ mixture for 20 minutes before use. The heart was placed into a Petri dish containing chilled staining buffer (1% fetal calf serum, 0.05% sodium azide in phosphate-buffered saline) and manually dispersed into a single cell suspension using razor blades. Single cell suspensions were sequentially filtered through 40- μ m and 15- μ m cell strainers. All reagents were obtained from Sigma-Aldrich except where indicated.

Flow Cytometry

Cells from individual hearts were resuspended in 50 ml of staining buffer, centrifuged at 352 $\times$ g for 8 minutes, and split into approximately five samples per heart based on cell counting so that each sample contained $\sim 10^6$ live (trypan blue-negative) cells in 100 μ l of staining buffer. The mean and SD for live cell recoveries from individual mouse hearts were $(7.2 \pm 4.0) \times 10^6$ /heart. Cells were preblocked with anti-Fc γ receptor III/II monoclonal antibody (mAb) (clone 2.4G2) for 5 minutes and stained at +4°C for 30 minutes simultaneously with four colors using the following mAbs: fluorescein isothiocyanate-labeled anti-CD3 (clone 145-2C11), anti-CD44 (clone IM7), and Gr1 (clone RB6-8C5); phycoerythrin-labeled anti-CD4 (clone H129.19), anti-CD19 (clone 1D3), anti-MHC class II (clone NIMR-4), and anti-CD11b (clone M1/70); peridinin chlorophyll- α protein-labeled anti-CD45 (clone 30-F11); allophycocyanin-labeled anti-CD3 (clone 145-2C11), and anti-CD8 (clone 53-6.7). Cells were washed, resuspended in 450 μ l of staining buffer, and immediately analyzed. All reagents were from BD PharMingen (San Diego, CA), except for anti-MHC class II mAb, which was from Southern Biotechnology Associates, Inc. (Birmingham, AL). Data were acquired until 50,000 events were collected from the live gate using forward/side scatter plots. The live gate was set to exclude events with low forward scatter signals (Figure 1A). Cell fluorescence was measured with FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). Data analysis was performed using FCS Express2 (De Novo Software, Richmond Hill,

Figure 1. Flow cytometric assessment of heart-infiltrating leukocytes. **A:** Representative forward scatter/side scatter profile and a live cell gate. **B:** CD45/side scatter plot was used to obtain percentages of CD45⁺ leukocytes; cells were derived from the live cell gate shown in **A**. **C:** CD4/CD45 plot; cells were gated as shown in **A** to derive percentage of CD4⁺ T cells of total live cells. **D:** CD3/MHC class II plot was used to derive percentages of CD3⁺ T cells or MHC class II⁺ cells of CD45⁺ leukocytes. Cells were first gated as shown in **A** and then gated as shown in **B**. **E** and **F:** Histograms showing CD45⁺ leukocytes (the second peak) from unimmunized and immunized hearts, respectively; cells were gated as illustrated in **A**. Numbers are percentages of leukocytes of total live cells. Data in **A** to **D** and **F** are from day 30-immunized A/J mice and data in **E** are from an unimmunized A/J mouse. The **arrows** indicate the gating strategy.

Ontario, Canada). Heart cell isolation and flow cytometry were performed on 6 to 10 mice per day.

Assessment of Left Ventricular Function

Pressure-volume studies were performed in immunized BALB/c mice on days 58 to 61 after immunization and in age-matched unimmunized controls following a previously established protocol.¹⁷ Anesthesia was initiated with methoxyflurane followed by an intraperitoneal injection of urethane (800 to 1000 mg/kg), etomidate (20 to 30 mg/kg), and morphine (1 to 2 mg/kg). After placing the animal on a heating pad, a tracheotomy was performed, the trachea was cannulated with a smoothed 19-gauge needle, and ventilation was initiated with 100% O₂ at a tidal volume of 200 μ l and a rate of 140 breaths/minute. A custom designed ventilator provided constant flow at peak airway pressures of 5 to 10 cmH₂O. The left external jugular vein was exposed and cannulated using a 31-gauge needle. Volume expansion was achieved by infusion of 150 μ l of 12.5% human albumin. The left ventric-

ular apex was exposed via a subdiaphragmatic incision. An apical stab was made with a 26-gauge needle and a 1.4-F pressure-volume catheter (SPR-719; Millar, Houston, TX), containing four electrodes and a micromanometer, was placed to span the long axis of the left ventricle, such that the proximal electrode is just inside the endocardium and the distal electrode is just above or at the aortic valve. The catheter occupies a total volume of 0.76 μ l and ~20% of the cross-sectional area of the aortic valve at the widest part. Insertion of the pressure sensor beyond the valve confirms the absence of obstruction. The catheter was connected to a custom-designed signal conditioner, which provided a constant current of 30 μ A at a frequency of 20 kHz. Parallel conductance (offset) was estimated using the hypertonic saline dilution method. Measurements of pressure and volume were obtained at steady state and during the preload reduction by transiently occluding the inferior vena cava. After all measurements were made, the mouse was placed on its side, a limited lateral thoracotomy was performed and the descending aorta was dissected free from the spinal column just above the level of the diaphragm. For gain calibration, a flow probe (1RB; Transonic, Ithaca, NY) was placed around the aorta. All signals were digitized at 2 KHz and stored on disk for off-line analysis using custom-written software. The following indices were used: ejection fraction, cardiac output, end-systolic volume, end-diastolic volume (EDV), end-diastolic and end-systolic pressures, maximal rate of pressure development (dP/dt_{max}), preload-recruitable stroke work (PRSW),²⁰ preload-adjusted maximal power ($power_{max}/EDV$),²¹ diastolic stiffness coefficient (β),²² time constant of isovolumic pressure decay (τ_D),²³ and preload-adjusted peak filling rate (PFR/EDV).

Statistical Analyses

The Mann-Whitney *U*-test was used to compare gross severity scores and Student's *t*-test was used for other comparisons. Associations between cell populations and hemodynamic indices were assessed using linear regression. The data were analyzed using SigmaStat 3.01 (SPSS Inc., Chicago, IL) software. *P* values of ≤ 0.05 were considered statistically significant.

Results

Flow Cytometric Analysis of Cardiac Infiltrate in A/J Mice

We first applied flow cytometry to assess cardiac inflammation in CM peptide-immunized A/J mice, which are highly susceptible to the induction of severe EAM.^{6,24} For the analysis, only the events with relatively high forward scatter were selected using forward/side scatter plots to exclude cell debris, red blood cells, and cells in advanced apoptosis as demonstrated by the live cell gate in Figure 1A. CD45, a panleukocyte marker, was used to estimate percentages of heart-infiltrating leukocytes (Figure 1; B, E, and F). The live cell gate was used to estimate

Figure 2. Severity of myocarditis in A/J and BALB/c mice. **A** and **B**: Gross scores in A/J (**A**) and BALB/c (**B**) mice. **C** and **D**: Flow cytometry-derived percentages of infiltrating leukocytes (CD45⁺ cells) of total live cells in the heart in A/J (**C**) and BALB/c (**D**) mice. **E** and **F**: Relationship between gross scores and percentages of leukocytes in the heart in A/J (**E**) and BALB/c (**F**) mice. *P* values were calculated using the Mann-Whitney *U* (gross scores) or the Student's *t* (leukocytes)-test. Day 0 represents unimmunized mice. The gross scores and flow cytometry data are from the same mice and each triangle or dot represents an individual mouse. In **E** and **F** the data are pooled from different time points after immunization.

percentages of leukocytes or other subsets of infiltrating cells of total live cells (Figure 1, B and C, respectively). To estimate proportions of cell subsets out of all leukocytes, events from the live cell gate were further gated on CD45⁺ population as illustrated by the arrows in Figure 1; A, B, and D.

Using CD45 as a marker, we found that immunized A/J mice had significantly higher percentages of heart-infiltrating leukocytes compared to the unimmunized controls

(Figure 2C). Flow cytometry discriminated among mice with the same gross score (Figure 2E), providing a more sensitive assessment of inflammation severity. We also examined the individual subsets of the total pool of CD45⁺ infiltrating cells: total T cells (CD3⁺), helper T cells (CD4⁺), cytotoxic T cells (CD8⁺), B cells (CD19⁺), macrophages (CD11b⁺Gr1^{low}), and neutrophils (CD11b⁺Gr1^{high}). Percentages of all inflammatory cell subsets of total live cells increased with disease, with a

Table 1. Cellular Composition of the Heart Infiltrate in Unimmunized and Immunized A/J and BALB/c Mice

Cell populations	A/J		BALB/c				
	Day 0	Day 30	Day 0	Day 15	Days 22 to 24	Days 30 to 34	Days 58 to 61
CD45	12.1 ± 1.7	34.6 ± 13.8*	11.3 ± 1.9	17.2 ± 6.4	17.3 ± 6.4	11.8 ± 5.2 [†]	9.3 ± 4.0
CD3	0.8 ± 0.7	7.2 ± 3.6*	0.6 ± 0.4	1.8 ± 0.9*	1.4 ± 0.8	1.6 ± 1.5	1.2 ± 0.7
CD3/CD45	6.8 ± 3.5	32.5 ± 4.5*	5.6 ± 0.9	10.6 ± 2.4*	7.3 ± 2.3	12.9 ± 5.6* [†]	12.7 ± 2.8*
CD4	0.2 ± 0.01	4.2 ± 3.0	0.4 ± 0.1	0.9 ± 0.4*	0.6 ± 0.5	0.6 ± 0.6	0.5 ± 0.2
CD4/CD45	2.5 ± 1.3	10.1 ± 7.0	3.4 ± 1.2	5.1 ± 1.9	3.3 ± 1.9	4.8 ± 2.4	5.1 ± 1.9
CD8	0.1 ± 0.09	1.4 ± 0.5*	0.1 ± 0.01	0.3 ± 0.2	0.4 ± 0.3	0.3 ± 0.3	0.3 ± 0.1*
CD8/CD45	1.3 ± 1.2	4.3 ± 2.5	0.9 ± 0.05	1.8 ± 0.4*	2.1 ± 1.0*	2.4 ± 1.7*	3.2 ± 1.7*
CD4:CD8 ratio	1.3 ± 0.01	2.5 ± 1.8	3.5 ± 1.1	2.9 ± 1.1	1.7 ± 0.9*	1.8 ± 0.6*	1.5 ± 0.3*
CD19	0.2 ± 0.07	1.3 ± 1.1	0.4 ± 0.2	1.6 ± 1.6	0.7 ± 0.6	0.6 ± 0.3	0.5 ± 0.6
CD19/CD45	3.0 ± 1.1	5.8 ± 2.8	3.6 ± 1.9	8.1 ± 6.2	4.6 ± 4.0	5.9 ± 2.8	5.1 ± 6.4
CD11b ⁺	8.6 ± 1.8	14.1 ± 10.3	7.6 ± 0.9	9.6 ± 3.8	14.8 ± 4.6*	8.8 ± 3.7	4.9 ± 2.1*
CD11b/CD45 [†]	59.2 ± 5.9	44.4 ± 15.8	67.5 ± 5.3	55.7 ± 7.0*	76.8 ± 6.5*	74.6 ± 5.0* [†]	53.5 ± 12.0*
Gr1	0.01 ± 0.01	2.1 ± 2.3	0.1 ± 0.03	1.1 ± 0.7*	1.0 ± 1.0	0.4 ± 0.4	0.9 ± 1.3
Gr1/CD45	0.2 ± 0.2	14.2 ± 10.7	0.8 ± 0.2	6.5 ± 2.4*	5.2 ± 3.8*	3.0 ± 1.5*	7.5 ± 6.8
MHC II	2.0 ± 1.8	11.4 ± 3.4*	ND	ND	ND	ND	ND
MHC II/CD45	22.7 ± 7.8	57.3 ± 8.0*	ND	ND	ND	ND	ND

* and [†], *P* < 0.05, Student's *t*-test, compared to the control group for the same strain (*) or to A/J mice for the same time point ([†]). Data represent percent of either total live cells or of CD45⁺ leukocytes. Data are mean ± SD. ND, not determined.

[†]These numbers represent percentages of CD11b⁺Gr1⁺ macrophages. Day 0 group represents unimmunized age-matched controls.

particularly pronounced increase in CD3⁺ T cells ($P = 0.007$, Table 1) and a relative decrease in the percentage of CD45⁺ population, which presumably includes mainly endothelial cells along with other types of resident cells. The proportion of these populations among total infiltrating leukocytes also changed on immunization, including increases in proportions of T lymphocytes ($P < 0.001$) and neutrophils and a moderate decrease in the proportion of macrophages. The latter population, however, still represented the largest fraction of total leukocytes. We also found that a greater percentage of infiltrating cells expressed MHC class II in EAM compared to the unimmunized control ($P < 0.001$, Table 1).

Flow Cytometric Assessment of the Progression of EAM in BALB/c Mice

Once the method had been established in a highly susceptible A/J mouse, we studied myocardial inflammation in BALB/c mice, which are moderately susceptible but commonly used because of the greater availability of genetically modified strains.^{25–27} Unimmunized BALB/c mice did not differ from unimmunized A/J mice in terms of the presence of different leukocytic subtypes in the heart (Table 1). In accord with our previous observations,²⁵ BALB/c mice developed mild myocarditis undetectable by gross examination of the hearts at early time points (Figure 2B). During peak inflammation in the heart (approximately day 21 after immunization), these mice typically develop disease with a 50% prevalence based on light microscopic examination with a median histological score among diseased animals of 1 (on a scale from 0 to 5). More than half of the immunized mice had increased percentages of total leukocytes (>15%) on days 15 and 22 to 24, but this change did not reach statistical significance compared to the unimmunized mice (Table 1 and Figure 2D). BALB/c mice had significantly less severe myocardial inflammation on days 30 to 34 compared to the same time point in A/J mice, as assessed by CD45⁺ population, and significantly lower gross scores ($P = 0.002$). At this stage of disease, BALB/c mice also had significantly lower proportions of CD3⁺ T cells and significantly higher proportions of CD11b⁺Gr1^{low} macrophages out of CD45⁺ leukocytes (Table 1).

CM immunization changed the quality of the infiltrating cells in BALB/c mice with significantly greater proportions of T cells and neutrophils ($P = 0.001$ and $P < 0.001$, respectively, on day 15) (Table 1). The proportion of CD11b⁺Gr1^{low} macrophages significantly decreased by day 15 ($P = 0.03$) but then increased during the acute phase and dropped again during the chronic phase. Interestingly, the chronic phase of disease (days 58 to 61) was characterized by very few total CD45⁺-infiltrating cells in the heart with the majority of mice having fewer CD45⁺ cells than the unimmunized controls (Figure 2D), while the severity of gross pathology increased (Figure 2B). Overall, BALB/c mice exhibited no association between gross scores and percentages of infiltrating leukocytes (Figure 2F). Importantly, on days 58 to 61 the quality of the infiltrate was different from that of the unim-

Figure 3. Increased activation state of heart-infiltrating T cells in EAM. Flow cytometric events were gated on live CD45⁺CD3⁺ cells. **A:** Percentages of cells expressing high levels of CD44 of total CD3⁺ T cells. Data are from BALB/c mice and shown as mean $\pm$ SD, $n \geq 5$ per group. *, $P < 0.05$ compared to unimmunized controls (day 0), Student's *t*-test. Day 0 corresponds to unimmunized age-matched BALB/c control mice. **B:** Overlay histogram shows CD44 expression on CD3⁺ T cells from a representative unimmunized mouse (thin line, shaded) and an immunized mouse on day 23 after immunization (thick line, unshaded).

mized mice and was characterized by an increased proportion of T cells. At all time points, the proportion of CD4⁺ T cells remained greater than that of CD8⁺ T cells. However, the CD4/CD8 T-cell ratio decreased with disease progression and at later time points was significantly lower compared to that in unimmunized controls (Table 1). At all time points of EAM, CD3⁺ T cells expressed higher levels of CD44, exhibiting a more activated state compared to unimmunized mice (Figure 3).

Percentages of CD4⁺ T cells, CD8⁺ T cells, macrophages (data not shown), and neutrophils (Figure 4A), but not CD19⁺ B cells, of total live cells significantly correlated with the severity of inflammation, as assessed by percentages of infiltrating CD45⁺ leukocytes, on days 22 to 24 after immunization. However, only the proportion of neutrophils, but not any other cell type, of leukocytes had significant positive association with severity in both BALB/c and A/J mice (Figure 4; B, C, E, and F).

Association between Inflammation and Cardiac Dysfunction

We examined the association between phenotypes of infiltrating cells and cardiac function using flow cytometry and pressure-volume relations¹⁶ on the same BALB/c mice during the chronic phase of EAM (days 58 to 61). This time point was chosen because it was associated with greater functional abnormalities compared to earlier time points (our unpublished observations). The chronic phase was characterized by increased heart weights, increases in left-ventricular volumes, and deterioration of both systolic (reduced ejection fraction, dP/dt_{max} , and PRSW) and diastolic (increased β and prolonged τ_D) performance (Table 2).

The percentage of CD45⁺ leukocytes did not correlate with any hemodynamic indices, but the proportion of CD4⁺ T cells of leukocytes was significantly associated with increased EDV and reduced systolic function assessed by ejection fraction, PWR_{max}/EDV , and PRSW (Figure 5; A to D). The range of cardiac performance during the chronic phase is shown in Figure 5, E and F, and represents hearts with the lowest and highest pro-

Figure 4. Severity of acute inflammation is associated with the proportion of neutrophils. Percentages of leukocytes (CD45⁺ cells) of total live cells correlate with percentages of neutrophils of total live cells in BALB/c mice (A) and with the proportion of neutrophils of leukocytes in BALB/c mice (B) and in A/J mice (C). D: Side scatter/CD45 dot plot of heart cells represents severe myocarditis in A/J mice. The rectangular and oval gates indicate granulocytes and lymphocytes, respectively. These gates were obtained by backgating Gr1⁺ and CD3⁺ populations. Representative CD45/Gr1 dot plots from A/J mice with mild (E) or severe (F) myocarditis. Neutrophils are CD45⁺Gr1^{high} cells (upper right quadrants). Numbers are percentages of neutrophils of total live cells. Data were obtained using the live cell gate from individual hearts on days 22 to 24 in BALB/c mice and on day 30 after immunization in A/J mice. R^2 represents a regression coefficient. P values indicate the significance of the slope of the regression line (the strength of the linear association).

portions of CD4⁺ T cells. The proportion of CD4⁺ T cells, however, did not correlate with diastolic indices τ_D or β .

Among other inflammatory subsets, proportion of CD8⁺ T cells tended to be inversely associated with indices of systolic function, but these associations did not reach statistical significance. Proportions of other cells including neutrophils, B cells, or macrophages did not correlate with any hemodynamic parameters. Similar to the proportion of CD4⁺ T cells, gross scores were asso-

ciated with increased EDV, reduced ejection fraction, and reduced PRSW, but not τ_D or β (data not shown).

Discussion

In this report, we describe a technique of flow cytometric assessment of cardiac inflammation after enzymatic digestion of hearts by delivery of digestion solution through

Table 2. Cardiac Function in Chronic EAM

Index	Day 0	Days 58 to 61	<i>P</i> value
HW (mg)	96.7 ± 4.5	129.9 ± 14.8	<0.001
HW/BW (mg × g ⁻¹)	4.3 ± 0.5	5.8 ± 0.6	<0.001
ESV (μL)	13.1 ± 3.7	33.4 ± 30.8	0.04
EDV (μL)	35.5 ± 3.4	54.4 ± 30.4	0.16
EF (%)	63.4 ± 8.3	45.2 ± 15.2	0.03
CO (mL × min ⁻¹)	13.1 ± 1.3	12.5 ± 3.2	0.67
dP/dt _{max} (mmHg × s ⁻¹)	14,587.2 ± 1363.3	10,824.4 ± 2,569.5	0.01
PWR _{max} /EDV (mmHg × s ⁻¹ × 100)	31.5 ± 7.2	22.1 ± 8.4	0.06
PRSW (mmHg)	76.3 ± 8.3	63.9 ± 6.1	0.02
β (mmHg × mg × μL ⁻¹ × 100 ⁻¹)	0.05 ± 0.01	0.17 ± 0.1	0.02
τ_D (ms)	6.8 ± 0.3	8.9 ± 1.7	0.01

HW, heart weight; HW/BW, heart weight-to-body weight ratio; ESV, end-systolic volume; EDV, end-diastolic volume; EF, ejection fraction; CO, cardiac output; dP/dt_{max}, maximal rate of pressure development; PWR_{max}/EDV, preload-adjusted maximal power; PRSW, preload-recruitable stroke work (stroke work – EDV relations); β , diastolic stiffness index; τ_D , isovolumic relaxation time constant. P values were calculated using Student's *t* test. Day 0 group represents unimmunized age-matched control BALB/c mice.

Figure 5. Proportion of CD4⁺ T cells is associated with cardiac dysfunction during the chronic phase of EAM. The proportions of CD4⁺ T cells of leukocytes are associated with increased EDV (**A**), reduced ejection fraction (EF) (**B**), reduced preload-adjusted maximal power (PWR_{max}/EDV) (**C**), and reduced PRSW (**D**). Each dot represents an individual mouse. Pressure-volume loops and a flow cytometric plot are from the same mouse with the lowest (2.8%) (**E**) and highest (8.7%) proportion of CD4⁺ T cells of leukocytes (**F**). R^2 represents the regression coefficient. P values indicate the significance of the slope of the regression line (the strength of the linear association).

coronary circulation. There has been a limited number of previous studies of hematopoietic cell isolation from the heart.^{28–30} The authors digested minced hearts for 1 to 2 hours in collagenase solution and reported the necessity to pool hearts to obtain satisfactory cellular yields.^{28,30} Huber and colleagues³¹ were able to perform flow cytometric assessment of infiltrates of individual hearts collected on day 7 after coxsackievirus B3 infection using an enzymatic digestion of minced hearts. The authors, however, did not report their cellular yields. Their method can potentially be used in a setting of a high degree of infiltration, as it is the case during the acute phase of coxsackieviral myocarditis. The method reported in this study also provides an opportunity to study cellular populations in healthy hearts and in hearts with low-grade myocarditis. This was possible because of high yields of cells that were achieved because of homogeneous and relatively fast (7 minutes) digestion. Shorter exposure time to the digestion solution may also yield healthier cells, which is important for *in vitro* functional studies, such as T-cell proliferation, cytokine production, cytotoxicity assays, and so forth. Furthermore, this method has been used to obtain viable cardiomyocytes^{14,15} and can potentially be used for flow cytometric assessment of their phenotypes along with physiological studies of cardiomyocyte function.

Reproducibility and sensitivity of the method was demonstrated in unimmunized mice, which had similar percentages of total leukocytes with comparable proportions of different inflammatory subsets. Using flow cytometry,

we confirmed our previous observations that A/J mice develop more severe acute and subacute myocarditis compared to BALB/c mice and our data corroborate earlier findings that the inflammatory infiltrate in acute EAM is composed predominantly of macrophages, has more CD4⁺ than CD8⁺ T cells, and few B220⁺ B cells.^{7,8,32} The flow cytometric method, however, provided us with the ability to precisely quantify these differences.

One of the important findings in this report is that the severity of acute myocardial inflammation is strongly associated with neutrophil accumulation in the heart. Simultaneous staining for CD11b and Gr1 gave us an opportunity to distinguish between macrophages and neutrophils. Previous studies of EAM using immunohistochemistry used CD11b (or Mac1) as a marker of macrophages and failed to make this distinction.⁸ Recent *in vitro* studies have demonstrated the ability of neutrophils to cause free radical-mediated injury of cardiomyocytes and impair their ability to shorten.^{33,34} These observations combined with our findings warrant further investigation on the contribution of neutrophils to the pathogenesis of autoimmune myocarditis.

We have previously shown that eosinophils accumulate in the myocardium in severe EAM.²⁴ In this report, we did not address whether eosinophils correlate with either overall severity of disease or cardiac dysfunction. Such assessment should be performed in the future, because eosinophils have the ability to produce local tissue damage by releasing the cytotoxic contents of their cytoplasm.

mic granules.^{35,36} Unlike neutrophils, eosinophils represent a population of cells that are Gr1^{low}.³⁷ Their flow cytometric analysis, however, is less straightforward compared to other hematopoietic cells discussed in this report. Eosinophils fall into the same granulocyte region as neutrophils on side scatter/CD45 plot in Figure 4D, but exhibit higher side scatter signal. The distinction based on the side and forward scatter properties is enhanced by aldehyde fixation.³⁸ In this report, the cells were not fixed to minimize the length of the procedure given the fact that all of the steps were performed on the same day.

Notably, the percentages of CD4⁺ and CD8⁺ T cells in the heart infiltrate did not add up to the percentages of CD3⁺ T cells. This was a consistent finding in both unimmunized and immunized mice, as well as in A/J and BALB/c mice. CD4⁺ and CD8⁺ T cells could account for approximately half of CD3⁺ population; these results were confirmed by using two different types of anti-CD3 mAb. Future experiments should address the nature of this CD3⁺CD4[−]CD8[−] population. Double-negative T cells have been shown to exist outside thymus in adult mice; they have been ascribed a regulatory role.³⁹ CD3⁺CD4[−]CD8[−] population may also represent a subset of double-negative NKT cells.⁴⁰ Finally these cells may be $\gamma\delta$ T cells, which have been shown to accumulate in the myocardium during fulminant myocarditis in humans and represent between 5% and 20%, and in some cases up to 50%, of the acute inflammatory infiltrate in coxsackievirus B3-induced myocarditis in mice.^{41–43}

Flow cytometry, unlike immunohistochemistry, is useful in assessing changes in the intensity of expression of surface markers and therefore can be used to quantify their up-regulation or down-regulation. We demonstrated that heart-infiltrating T cells in immunized mice had higher surface expression of CD44 compared to those of unimmunized mice, suggesting a more aggressive nature of T cells in the inflamed hearts because CD44 is known to be up-regulated on activated and memory, but not naïve, T cells. Furthermore, it may prove to be a useful marker of potentially pathogenic T cells or of a previous inflammation. In this regard it is noteworthy that T cells on days 58 to 61 after immunization still expressed increased levels of CD44.

To our knowledge, this is the first report of a comprehensive characterization of the inflammatory infiltrate during both the acute and chronic phases of EAM in mice. We found that by days 58 to 61 after immunization very few CD45⁺ cells were left in the heart; however, the changes in the proportions of individual inflammatory cells remained. Despite the reduction in percentages of infiltrating leukocytes, the chronic phase was associated with increased gross pathology. These higher gross scores may reflect cardiac remodeling, such as fibrosis, which is typical of late stages of EAM, and therefore these scores may not represent myocardial inflammation per se.

During the chronic phase, percentages of total infiltrating leukocytes did not correlate with cardiac dysfunction, but proportions of CD4⁺ T cells in the heart infiltrate significantly correlated with systolic dysfunction and large left-ventricular volumes, major hallmarks of dilated cardiomyopathy. The importance of CD4⁺ T cells in dis-

ease initiation in EAM was demonstrated by T-cell depletion in A/J mice and adoptive transfer studies from C.B-17 mice with myocarditis to SCID mice.⁴⁴ The role of CD8⁺ T cells in EAM is less well defined. It has been demonstrated that CD8 deficiency in B10.Br mice leads to exacerbation of CM-induced myocarditis.⁸ However, depletion of CD8⁺ T cells with a mAb reduced the severity of EAM in A.SW mice.^{32,45} The ability of CD8⁺ T cells to induce myocarditis was demonstrated in mice transgenically expressing an ovalbumin peptide in the heart under cardiac-specific promoter.⁴⁶ These mice developed severe myocarditis on the transfer of CD8⁺ T cells from transgenic mice that expressed TCR specific for the ovalbumin peptide.

We found that CD4⁺ T cells predominated over CD8⁺ T cells in the myocardial infiltrate not only during the acute phase in both A/J and BALB/c mice, but also during the chronic phase of myocarditis in BALB/c mice. Unlike EAM, coxsackievirus B3-induced myocarditis in BALB/c mice is characterized by the predominance of CD8⁺ over CD4⁺ T-cell population in the myocardium during the acute phase of disease.⁴² The contribution of different inflammatory cells in disease progression at later time points and their role in cardiac dysfunction was not previously studied. Our findings show that the proportion of CD4⁺ T cells in chronic EAM represents a marker of progression to dilated cardiomyopathy, but future studies are needed to determine whether CD4⁺ T cells play a causal role in the deterioration of cardiac function. Similarly, the pathogenic potential of other cell types, including those that correlate with disease severity during the acute phase, should be assessed in terms of their ability to produce local damage and cardiac dysfunction. In this regard, it should be mentioned that a pathogenically important cell type may appear in the myocardium before the onset of cardiac dysfunction and therefore the existing association may be missed by examining individual time points. Finally, further analysis of CD4⁺ T-cell subsets, including those of Th1 and Th2 phenotypes, will provide insights into their relative contribution to cardiac injury.

In summary, we successfully applied flow cytometry to study cardiac inflammation in a mouse model of EAM and demonstrated the feasibility of using invasive hemodynamic studies and subsequent cardiac digestion and flow cytometry on the same mouse to study the effects of inflammation on cardiac function. We conclude that flow cytometry on digested hearts represents a reproducible, simple, and a relatively fast method of assessing both cellular frequencies and phenotypes and its application should facilitate our understanding of the role of inflammation in heart disease.

References

1. Wolfgarm LJ, Beisel KW, Herskowitz A, Rose NR: Variations in the susceptibility to coxsackievirus B3-induced myocarditis among different strains of mice. *J Immunol* 1986, 136:1846–1852
2. Hill SL, Afanasyeva M, Rose NR: Autoimmune myocarditis. The Molecular Pathology of Autoimmune Diseases. Edited by CA Bona, AN Theofilopoulos. Ann Arbor, Taylor & Francis, 2002, pp 951–964

3. Gauntt C, Huber S: Coxsackievirus experimental heart diseases. *Front Biosci* 2003, 8:E23–E35
4. Fairweather D, Kaya Z, Shellam GR, Lawson CM, Rose NR: From infection to autoimmunity. *J Autoimmun* 2001, 16:175–186
5. Kawai C: From myocarditis to cardiomyopathy: mechanisms of inflammation and cell death: learning from the past for the future. *Circulation* 1999, 99:1091–1100
6. Neu N, Rose NR, Beisel KW, Herskowitz A, Gurri-Glass G, Craig SW: Cardiac myosin induces myocarditis in genetically predisposed mice. *J Immunol* 1987, 139:3630–3636
7. Wang Y, Afanasyeva M, Hill SL, Rose NR: Characterization of murine autoimmune myocarditis induced by self and foreign cardiac myosin. *Autoimmunity* 1999, 31:151–162
8. Penninger JM, Neu N, Timms E, Wallace VA, Koh DR, Kishihara K, Pummerer C, Mak TW: The induction of experimental autoimmune myocarditis in mice lacking CD4 or CD8 molecules. *J Exp Med* 1993, 178:1837–1842
9. Hirasawa M, Deguchi H, Ukimura A, Kitaura Y: Immunologic interaction between infiltrating eosinophils and T lymphocytes in murine spontaneous eosinophilic myocarditis. *Int Arch Allergy Immunol* 2003, 130:73–81
10. Sonnenblick EH: Correlation of myocardial ultrastructure and function. *Circulation* 1968, 38:29–44
11. Weber KT: Cardiac interstitium in health and disease: the fibrillar collagen network. *J Am Coll Cardiol* 1989, 13:1637–1652
12. Wasowska B, Baldwin WM, Howell DN, Sanfilippo F: The effects of donor-specific blood transfusion enhancement of rat renal allografts on cytotoxic activity and phenotypes of peripheral blood lymphocytes, splenocytes, and graft-infiltrating cells. *Transplantation* 1991, 51:451–459
13. Gavett SH, Chen X, Finkelman F, Wills-Karp M: Depletion of murine CD4+ T lymphocytes prevents antigen-induced airway hyperreactivity and pulmonary eosinophilia. *Am J Respir Cell Mol Biol* 1994, 10:587–593
14. Zhou YY, Wang SQ, Zhu WZ, Chruscinski A, Kobilka BK, Ziman B, Wang S, Lakatta EG, Cheng H, Xiao RP: Culture and adenoviral infection of adult mouse cardiac myocytes: methods for cellular genetic physiology. *Am J Physiol* 2000, 279:H429–H436
15. Fiset C, Clark RB, Larsen TS, Giles WR: A rapidly activating sustained K+ current modulates repolarization and excitation-contraction coupling in adult mouse ventricle. *J Physiol* 1997, 504:557–563
16. Georgakopoulos D, Mitzner WA, Chen CH, Byrne BJ, Millar HD, Hare JM, Kass DA: In vivo murine left ventricular pressure-volume relations by miniaturized conductance micromanometry. *Am J Physiol* 1998, 274:H1416–H1422
17. Georgakopoulos D, Kass D: Minimal force-frequency modulation of inotropy and relaxation of in situ murine heart. *J Physiol* 2001, 534:535–545
18. Shiverick KL, Thomas LL, Alpert NR: Purification of cardiac myosin. Application to hypertrophied myocardium. *Biochim Biophys Acta* 1975, 393:124–133
19. Donermeyer DL, Beisel KW, Allen PM, Smith SC: Myocarditis-inducing epitope of myosin binds constitutively and stably to I-Ak on antigen-presenting cells in the heart. *J Exp Med* 1995, 182:1291–1300
20. Glower DD, Spratt JA, Snow ND, Kabas JS, Davis JW, Olsen CO, Tyson GS, Sabiston Jr DC, Rankin JS: Linearity of the Frank-Starling relationship in the intact heart: the concept of preload recruitable stroke work. *Circulation* 1985, 71:994–1009
21. Kass DA, Beyar R: Evaluation of contractile state by maximal ventricular power divided by the square of end-diastolic volume. *Circulation* 1991, 84:1698–1708
22. Gaasch WH, Battle WE, Oboler AA, Banas Jr JS, Levine HJ: Left ventricular stress and compliance in man. With special reference to normalized ventricular function curves. *Circulation* 1972, 45:746–762
23. Raff GL, Glantz SA: Volume loading slows left ventricular isovolumic relaxation rate. Evidence of load-dependent relaxation in the intact dog heart. *Circ Res* 1981, 48:813–824
24. Afanasyeva M, Wang Y, Kaya Z, Park S, Zilliox MJ, Schofield BH, Hill SL, Rose NR: Experimental autoimmune myocarditis in A/J mice is an interleukin-4-dependent disease with a Th2 phenotype. *Am J Pathol* 2001, 159:193–203
25. Afanasyeva M, Wang Y, Kaya Z, Stafford EA, Dohmen KM, Sadighi Akha AA, Rose NR: Interleukin-12 receptor/STAT4 signaling is required for the development of autoimmune myocarditis in mice by an interferon-gamma-independent pathway. *Circulation* 2001, 104:3145–3151
26. Eriksson U, Kurrer MO, Sonderegger I, Iezzi G, Tafuri A, Hunziker L, Suzuki S, Bachmaier K, Bingisser RM, Penninger JM, Kopf M: Activation of dendritic cells through the interleukin 1 receptor 1 is critical for the induction of autoimmune myocarditis. *J Exp Med* 2003, 197:323–331
27. Afanasyeva M, Rose NR: Immune mediators in inflammatory heart disease: insights from a mouse model. *Eur Heart J Suppl* 2002, 4:I31–I36
28. Austyn JM, Hankins DF, Larsen CP, Morris PJ, Rao AS, Roake JA: Isolation and characterization of dendritic cells from mouse heart and kidney. *J Immunol* 1994, 152:2401–2410
29. Stinn JL, Taylor MK, Becker G, Nagano H, Hasegawa S, Furakawa Y, Shimizu K, Libby P, Mitchell RN: Interferon-gamma-secreting T-cell populations in rejecting murine cardiac allografts: assessment by flow cytometry. *Am J Pathol* 1998, 153:1383–1392
30. Furukawa Y, Mandelbrot DA, Libby P, Sharpe AH, Mitchell RN: Association of B7-1 co-stimulation with the development of graft arterial disease. Studies using mice lacking B7-1, B7-2, or B7-1/B7-2. *Am J Pathol* 2000, 157:473–484
31. Huber SA, Mortensen A, Moulton G: Modulation of cytokine expression by CD4+ T cells during coxsackievirus B3 infections of BALB/c mice initiated by cells expressing the gamma delta + T-cell receptor. *J Virol* 1996, 70:3039–3044
32. Pummerer C, Berger P, Fruhwirth M, Ofner C, Neu N: Cellular infiltrate, major histocompatibility antigen expression and immunopathogenic mechanisms in cardiac myosin-induced myocarditis. *Lab Invest* 1991, 65:538–547
33. Poon BY, Ward CA, Giles WR, Kubes P: Emigrated neutrophils regulate ventricular contractility via alpha4 integrin. *Circ Res* 1999, 84:1245–1251
34. Poon BY, Ward CA, Cooper CB, Giles WR, Burns AR, Kubes P: Alpha(4)-integrin mediates neutrophil-induced free radical injury to cardiac myocytes. *J Cell Biol* 2001, 152:857–866
35. Tepper RI, Coffman RL, Leder P: An eosinophil-dependent mechanism for the antitumor effect of interleukin-4. *Science* 1992, 257:548–551
36. Patella V, de Crescenzo G, Marino I, Genovese A, Adt M, Gleich GJ, Marone G: Eosinophil granule proteins are selective activators of human heart mast cells. *J Immunol* 1996, 157:1219–1225
37. Ramalingam T, Rajan B, Lee J, Rajan TV: Kinetics of cellular responses to intraperitoneal *Brugia pahangi* infections in normal and immunodeficient mice. *Infect Immun* 2003, 71:4361–4367
38. Shapiro HM: *Practical Flow Cytometry*. Hoboken, John Wiley & Sons, 2003, p 486
39. Cauley LS, Cauley KA, Shub F, Huston G, Swain SL: Transferable anergy: superantigen treatment induces CD4+ T cell tolerance that is reversible and requires CD4–CD8– cells and interferon gamma. *J Exp Med* 1997, 186:71–81
40. Azuma T, Takahashi T, Kunisato A, Kitamura T, Hirai H: Human CD4+ CD25+ regulatory T cells suppress NKT cell functions. *Cancer Res* 2003, 63:4516–4520
41. Eck M, Greiner A, Kandolf R, Schmausser B, Marx A, Muller-Hermelink HK: Active fulminant myocarditis characterized by T-lymphocytes expressing the gamma-delta T-cell receptor: a new disease entity? *Am J Surg Pathol* 1997, 21:1109–1112
42. Huber SA: Coxsackievirus-induced myocarditis is dependent on distinct immunopathogenic responses in different strains of mice. *Lab Invest* 1997, 76:691–701
43. Huber SA, Sartini D, Exley M: Vgamma4(+) T cells promote autoimmune CD8(+) cytolytic T-lymphocyte activation in coxsackievirus B3-induced myocarditis in mice: role for CD4(+) Th1 cells. *J Virol* 2002, 76:10785–10790
44. Smith SC, Allen PM: Myosin-induced acute myocarditis is a T cell-mediated disease. *J Immunol* 1991, 147:2141–2147
45. Neu N, Pummerer C, Rieker T, Berger P: T cells in cardiac myosin-induced myocarditis. *Clin Immunol Immunopathol* 1993, 68:107–110
46. Grabie N, Delfs MW, Westrich JR, Love VA, Stavrakis G, Ahmad F, Seidman CE, Seidman JG, Lichtman AH: IL-12 is required for differentiation of pathogenic CD8+ T cell effectors that cause myocarditis. *J Clin Invest* 2003, 111:671–680