ORIGINAL RESEARCH

Number of Circulating CD73-Expressing Lymphocytes Correlates With Survival After Cardiac Arrest

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***Background*—**Patients resuscitated from cardiac arrest (CA) have highly variable neurological, circulatory, and systemic ischemia-reperfusion injuries. After the initial hypoxic-ischemic insult, a cascade of immune and inflammatory responses develops and is often fatal. The role of the immune response in pathophysiological characteristics and recovery is not well understood. We studied immune cell activity and its association with outcomes in a cohort of CA survivors.

***Methods and Results*—**After informed consent, we collected blood samples at intervals over a week after resuscitation from CA. We examined the expression of CD39 and CD73 (alias 5′-nucleotidase), production of tumor necrosis factor-α, generation of reactive oxygen species, and secretion of vascular endothelial growth factor by circulating myeloid and lymphoid cells, in comparison to cells obtained from control subjects before coronary artery bypass grafting surgery. The number of circulating total and CD73-expressing lymphocytes correlated with survival after CA. Incubation of immune cells, obtained from post-CA subjects, with AMP, a substrate for CD73, resulted in inhibition of tumor necrosis factor-α production and generation of reactive oxygen species. This effect was blocked by adenosine 5′-(α, β-methylene) diphosphate, a specific inhibitor of CD73 and ZM 241385, an A2 adenosine receptor antagonist. We also found that AMP-dependent activation of CD73 induces production of vascular endothelial growth factor.

***Conclusions*—**CD73-expressing lymphocytes mediate cellular protection from inflammation after CA through inhibition of proinflammatory activation of myeloid cells and promotion of vascular endothelial growth factor secretion. The contribution of CD73 lymphocytes in the regulation of acute inflammation and tissue injury after CA warrants further study. **(*J Am Heart Assoc*. 2019;8:e010874. DOI: 10.1161/JAHA.118.010874.)**

Key Words: cardiac arrest • CD73 • inflammation • lymphocytes

Global ischemia during cardiac arrest (CA), the subsequent trauma of cardiopulmonary resuscitation, and a cascade of secondary injurious processes result in heterogeneous injury to the brain and other tissues. Despite improved cardiopulmonary resuscitation techniques and refinement of postresuscitation care,[1–3] outcomes remain poor.[4] The initial ischemic neurological damage after CA is followed by a post-CA syndrome,[5] which may be severe enough to cause death by neurological injury, circulatory collapse, or multiorgan system failure. In some patients, post-CA syndrome may resolve with cognitive dysfunction or a good outcome. Post-CA syndrome is characterized by a systemic inflammatory response,[6–8] and several studies have demonstrated an association between increased levels of circulating proinflammatory cytokines and poor outcome,[9,10] suggesting a possible role for anti-inflammatory and immunomodulatory therapies.[11] However, these studies have focused on mechanisms that promote inflammation without taking into account protective mechanisms also mediated by the immune response.

Brain ischemia is associated with rapid accumulation of immune cells, including neutrophils, monocytes, and lymphocytes.[12,13] Myeloid cells play an essential role in clearance of apoptotic cells and toxic cell debris after injury,[14] contributing to efficient tissue repair,[15] but excessive activation of myeloid

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Clinical Perspective

What Is New?

- Little is known about the cellular and humoral immune response after cardiac arrest.
- This study demonstrates an association between the number of circulating CD73-expressing lymphocytes during postresuscitation cardiac arrest care and shows how CD73-positive–mediated production of adenosine may be necessary to modulating the activation status of neutrophils.

What Are the Clinical Implications?

- Lymphocytes and lymphocyte subpopulations are novel prognosticators.
- In addition, CD73-mediated generation of adenosine may be a crucial pathway to downregulate inflammation and could be manipulated to improve survival after cardiac arrest.

cells worsens tissue damage. After entering damaged tissues, neutrophils and monocytes amplify cerebral inflammation and aggravate postischemic brain damage through mechanisms like generation of reactive oxygen species (ROS), production of cytotoxic tumor necrosis factor-α (TNF-α), and secretion of chemokines that further promote migration and accumulation of proinflammatory immune cells.[16] Immature neutrophils are characterized by lower phagocytic activity and higher basal intracellular TNF-α/interleukin-10 ratio.[17] The number of immature neutrophils is increased after CA and is associated with increased mortality and worse neurological outcomes.[18] Many studies have shown that neutrophils contribute to the tissue damage induced by ischemia and reperfusion in subjects with cardiovascular and cerebrovascular events.[19–23] It has also been shown that the neutrophil/lymphocyte ratio is increased after CA and associated with higher mortality and unfavorable neurological outcomes.[24,25] Recently, Villois et al[26] performed a retrospective analysis of data on subjects admitted to the intensive care unit after CA and demonstrated that up to 40% of subjects with CA have lymphopenia. Survivors have a higher number of lymphocytes compared with nonsurvivors, and lymphopenia was an independent predictor of poor outcome in subjects with out-of-hospital CA.[26] Although the mechanism of lymphopenia after CA is not known, it has been shown that stimulation of lymphocyte β_2-adrenergic receptors inhibited egress of lymphocytes from lymph nodes and rapidly produced lymphopenia in mice.[27] Lymphocytes are highly mobile cells, continually recirculating between the blood and the tissues via the lymph.[28] The estimated residence time of lymphocytes in the blood is ≈30 minutes.[29] It is, therefore, plausible that activation of sympathetic nervous system after CA resulted in retention of lymphocytes within lymph nodes. Interestingly, prior use of corticosteroid therapy was identified as a predictor of lymphopenia after CA.[26]

Endotoxin induces proinflammatory activation of myeloid cells. An increased level of endotoxin in plasma was detected in 46% of subjects with CA.[5] Endotoxin mediates its action via activation of toll-like receptors (TLRs). The expression levels of TLR2 and TLR4 were upregulated on monocytes during first 24 hours after CA, followed by relative downregulation of both receptors at later time points.[30] It has been shown that deficiency or functional blockade of TLR2 is associated with increased survival in a mouse model of CA/cardiopulmonary resuscitation.[31] TLR4 contributes to acute kidney injury after CA and promotion of accumulation and proinflammatory activation of neutrophils and monocytes after experimental cerebral ischemia.[32–34]

Adenosine is a potent endogenous anti-inflammatory and immunosuppressive purine nucleoside.[35] Adenosine mediates inhibition of proinflammatory cytokine production from myeloid cells[36,37] and limits lymphocyte activation.[38,39] Adenosine is produced from ATP by two anti-inflammatory ectonucleotidases, CD39 and CD73. It has been previously shown that CD39 and CD73 are expressed on a subpopulation of lymphocytes. These lymphocytes produce adenosine and inhibit immune/inflammatory responses.[40,41] Adenosine, acting via A2 adenosine receptors, inhibited production of TNF-α,[36,42] interferon-γ,[38,43] and ROS,[44] factors contributing to brain inflammation.[16,45] We and others have previously shown that adenosine induces vascular endothelial growth factor (VEGF) production in a variety of cell types, including myeloid cells.[46,47] Using an animal model, it has also been shown that VEGF is upregulated in the brain after CA.[48] VEGF increased the neuroprotective efficacy of bone marrow mesenchymal stem cells in a rat model of CA-induced global cerebral ischemia.[49] It is unclear, however, if CD73-expressing lymphocytes can protect against injury induced by global ischemia/reperfusion.

The goal of the current study was to characterize subpopulations of circulating lymphocytes after CA and determine the association between number of cells expressing CD39 and CD73 at different time points after resuscitation (return of spontaneous circulation [ROSC]) and outcome (survivors versus nonsurvivors). We also examined the role of CD73 lymphocytes, determined the effects of CD73 and adenosine receptor inhibitors on proinflammatory activation of myeloid cells, investigated the effect of CD73 on production of VEGF from immune cells, and measured levels of circulating VEGF after CA.

Methods

The data that support the findings of this study are available from the corresponding author on reasonable request.

Enrollment

Research was performed in accordance with study protocols approved by the Maine Medical Center Institutional Review Board, which is accredited by the Association for the Accreditation of Human Research Protection Programs. The study was performed between February 2016 and January 2018. Post-CA subjects, aged ≥18 years, admitted to the intensive care unit after a cardiopulmonary arrest and treated with targeted temperature management were enrolled after informed consent of the legally authorized representative. The current study was designed on the basis of the suggestion that inflammatory response after CA would follow general principles of the acute systemic inflammatory response observed after ischemic injury, which is characterized by an early (within several hours after injury) increase in the number of peripheral white blood cells (WBCs), followed by a "slow" decrease during the next 7 days. Because the amplitude and direction of changes in the number of WBCs are important factors indicating the degree of inflammation, the patients not anticipated to survive ≥48 hours were excluded from study. In addition, patients were excluded if informed consent could not be obtained within 12 hours of ROSC. Whenever possible, subjects underwent phlebotomy at 6, 12, 24, 48, 72, and 168 hours after ROSC.

To obtain the closest possible match on immune system activity before CA, we have used blood samples obtained from patients with coronary artery disease anticipating cardiac surgery, who share many comorbid conditions and risk factors with those who experience CA. Blood draws from subjects who underwent coronary artery bypass grafting (CABG) surgery, obtained before surgery, were used as control samples. Inclusion criteria for CABG surgery included patients aged ≥18 years and scheduled for open heart surgery supported by cardiopulmonary bypass at Maine Medical Center. Exclusion criteria for controls included known active myocarditis, hypertrophic cardiomyopathy, constrictive pericarditis, significant pericardial disease, severe pulmonary hypertension, severe ventricular arrhythmias, significant hypotension (systolic blood pressure <90 mm Hg), pregnancy, known malignancy other than nonmelanoma skin cancers, and expected survival <1 year. Pertinent clinical data were collected from the electronic medical record for all study participants.

Reagents

Lipopolysaccharide (from *Escherichia coli*, serotype 055:B5), AMP, adenosine 5′-(α, β-methylene) diphosphate (APCP), erythro-9-(2-hydroxy-3-nonyl)−adenine hydrochloride, and dimethyl sulfoxide were purchased from Sigma (St. Louis, MO). ZM 241385 was obtained from Tocris Bioscience/Bio-Techne. When used as a solvent, final dimethyl sulfoxide concentrations in all assays did not exceed 0.1%; and the same dimethyl sulfoxide concentrations were used in vehicle controls.

Blood Sample Collection

Venous blood (10 mL) was collected from CA and control CABG subjects using BD Vacutainer ACD tubes. The total number of WBCs was determined after erythrocyte lysis with ammonium chloride lysing solution (150 mmol/L NH_4Cl, 10 mmol/L $NaHCO_3$, and 1 mmol/L EDTA, pH 7.4). Blood plasma was prepared at room temperature using 2-step centrifugation, each at 2000*g* for 20 minutes. After preparation, plasma was stored at −80°C until further analysis.

Flow Cytometric Analysis

After red blood cell lysis, WBCs (10^6/mL) were treated with Human TruStain FcX (BioLegend, San Diego, CA) to prevent nonspecific binding, followed by incubation with relevant antibodies for 25 minutes at 4°C. Subpopulations of WBCs were analyzed using the following antibodies: fluorescein isothiocyanate–conjugated CD3 (UCHT1), PE (Phycoerythrin)-conjugated CD73 (AD2), CD19-PE/Cy7 (HIB19), CD39-APC (Allophycocyanin) (A1), and CD45-APC/Cy7 (HI30) (all from BioLegend).

For intracellular staining, cells were fixed and permeabilized using Cytofix/Cytoperm kit (BD Biosciences, San Jose, CA). PE-conjugated anti-human TNF-α (MAb11) and IgG1-PE (MOPC-21) isotype-matched control antibodies (BioLegend) were used to determine intracellular level of TNF-α protein. Data acquisition was performed on a MacsQuant Analyzer 10 (Miltenyi Biotec, Inc), and the data were analyzed using WinList 5.0 software. Viable and nonviable cells were distinguished using 4',6-diamidino-2-phenylindole or LIVE/DEAD Fixable Blue Stain kit (Life Technologies, Carlsbad, CA).

ROS Production

The oxidation-sensitive dye 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (Invitrogen-Molecular Probes, Eugene, OR) was used for the measurement of ROS production. Cells (10^6/mL) were incubated in serum-free RPMI 1640 medium containing 2 μmol/L 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate in the absence or presence of 10 ng/mL lipopolysaccharide at 37°C for 30 minutes, washed with PBS, and analyzed using flow cytometry. To determine effect of CD73 on ROS, 100 μmol/L

AMP and 100 μmol/L APCP were added 30 minutes before lipopolysaccharide.

Production of TNF-α and VEGF by WBCs

WBCs were resuspended in serum-free RPMI 1640 medium, containing 10 μmol/L erythro-9-(2-hydroxy-3-nonyl)−adenine hydrochloride, at a concentration of 10^7 cells/mL and stimulated with 10 ng/mL lipopolysaccharide alone or in combination with 100 μmol/L AMP, 100 μmol/L APCP, and 300 nmol/L ZM 241385. Secretion of TNF-α and VEGF in culture medium was measured using ELISA kits (Bio-Techne/R&D Systems).

For flow cytometric analysis, WBCs were resuspended in serum-free RPMI 1640 medium, containing 0.5% BSA, 3 μg/mL brefeldin A, and 10 μmol/L erythro-9-(2-hydroxy-3-nonyl)−adenine hydrochloride. Cells were incubated in the absence or presence of lipopolysaccharide alone, or in combination with AMP, APCP, and ZM 241385, at the concentrations indicated above, for 5 hours and analyzed for intracellular TNF-α.

Analysis of Circulating TNF-α and VEGF

Plasma levels of TNF-α and VEGF were measured using ELISA kits (Bio-Techne/R&D Systems).

Statistical Analysis

In the current article, we describe findings from an exploratory study, designed to analyze the relationship between immune/inflammatory response and outcome after CA. Initially, to determine sample size, we used measurements of neuregulin-1 protein level (biomarkers of cardiac and neurological injury) and the number of neutrophils (a marker of systemic inflammation) in patients who underwent CABG surgery. We determined that a sample size of 40 subjects would provide us with 80% power to detect a correlation coefficient of 0.23 with a 0.05 2-sided Fisher's z test (Pearson correlation). The sample size was adjusted to 50 subjects to account for potential sample loss (inability to obtain informed consent before early time points and patient death within 48 hours). Normally distributed variables are expressed as mean±SEM. Data are expressed as median values when distributions are skewed. Comparisons between 2 groups were performed using 2-tailed unpaired *t* tests (normal distribution) or Mann-Whitney test for variables with skewed distributions. Comparisons between ≥3 groups were performed using ordinary 1-way ANOVA with Tukey's multiple-comparisons posttest for normally distributed data or Kruskal-Wallis test with Dunn's multiple-comparisons posttest for skewed distribution. For continuous variables, correlation analysis was performed using a Pearson (normal distribution) or Spearman (skewed distribution) correlation. $P<0.05$ was considered significant.

Results

Study Subjects

A total of 204 patients were admitted with encephalopathy after resuscitation from CA during the study period. Eighty-nine patients could not be enrolled because of unavailability of study personnel, 37 had no legally authorized representative available for informed consent, 18 were ineligible because of impending death (10), admission >12 hours after resuscitation (5), non-English speaking (2), or enrolled in a competing trial (1), and 7 legally authorized representatives refused informed consent, leaving 53 patients with informed consent and 48 subjects with adequate samples to warrant inclusion (at least 3 temporally distinct values of all measured parameters available). Among 48 patients included in the analysis, 35 had a pure cardiac cause of their arrest (myocardial infarction or primary arrhythmia), 9 had primary respiratory arrest followed by CA, 2 had primary circulatory collapse, 1 had pulmonary embolism, and 1 cause was unknown. Blood samples with at least 3 values of all measured parameters were available from 48 patients (100%). Because of delayed consent or clinical circumstances, blood samples were missing in 17% of subjects with CA at 6 hours, 4% at 12 hours, 6% at 48 hours, 17% at 72 hours, and 48% at 168 hours after ROSC. Subjects with CA were cooled to 32°C to 34°C for 24 hours beginning minutes to hours after ROSC, then gradually rewarmed over 12 to 18 hours, and maintained normothermic until 72 hours post-ROSC; our standardized postresuscitation care protocol has been described elsewhere.[50]

A total of 138 patients underwent CABG surgery during study period. Of these patients, 30 were enrolled in this study. Reasons for failure to enroll included the following: known active myocarditis, hypertrophic cardiomyopathy, constrictive pericarditis, significant valvular and/or pericardial disease, severe pulmonary hypertension, significant hepatic disease or renal impairment (creatinine >2.5 mg/dL), severe ventricular arrhythmias, malignancy other than nonmelanoma skin cancers, expected survival <1 year, and emergency surgery. Blood samples from noncooled subjects who underwent CABG were obtained immediately before surgery.

There were no differences in age or sex between CA and control groups. A comparison of the demographics and clinical characteristics of CA and control subjects is provided in Table.

Table. Characteristics of Control Subjects and Subjects With CA

Characteristic	Control Subjects (n=30)*	Subjects With CA (n=48)
Age, median (IQR), y	64 (57–72.5)	62 (49–68)
Male, n (%)	16 (54)	36 (75)
Witnessed, n (%)	…	29 (60)
Bystander CPR, n (%)	…	44 (92)
Time to ROSC, median (IQR), min	…	15.5 (8.3–23)
Out-of-hospital CA, n (%)	…	38 (80)
Shockable rhythm, n (%)	…	30 (62)

CA indicates cardiac arrest; CPR, cardiopulmonary resuscitation; IQR, interquartile range; ROSC, return of spontaneous circulation.
*Plasma samples from control (patients undergoing coronary artery bypass grafting) were taken immediately before surgery (before heparin administration).

CA Induces Changes in the Numbers of Circulating Neutrophils and Lymphocytes But Not Monocytes for 3 Days After ROSC

We used flow cytometric analysis to determine number of CD45-positive ($CD45^{pos}$) immune cells after CA, as shown in Figure 1A. To characterize time-dependent changes in major subpopulations of peripheral blood cells, we initially analyzed the numbers of cells in neutrophil, monocyte, and lymphocyte gates at different time points after ROSC in comparison to blood samples obtained from subjects undergoing CABG. As shown in Figure 1B, total number of WBCs was significantly increased during 48 hours after ROSC compared with control subjects. Although not statistically significant, subjects with CA also demonstrated a trend toward higher number of WBCs at 72 and 168 hours compared with control subjects. Further analysis revealed that neutrophils are the major subpopulation of immune cells contributing to the increased number of WBCs (Figure 1C). Circulating monocytes were increased only briefly, at 6 hours after ROSC, with no statistically significant differences for the rest of examined time compared with control subjects (Figure 1D). In contrast to myeloid cells, the number of cells in the lymphocyte gate was decreased starting at 6 hours and persisting through 72 hours after ROSC (Figure 1E).

To determine if changes in neutrophils or lymphocytes were associated with survival after CA, we compared number of cells in groups of survivors and nonsurvivors after intensive care unit discharge. As shown in Figure 1F and 1G, no statistically significant differences were found in number of neutrophils or lymphocytes between 2 groups of subjects with CA. Peripheral blood lymphocytes include subpopulations of T cells, B cells, and natural killer cells. To determine number of subpopulation lymphocytes and their association with survival, we then performed flow cytometric characterization of CD3 T and CD19 B lymphocytes.

Numbers of CD3 T Cells and CD19 B Cells Are Decreased After CA

The major subpopulation of circulating cells within $CD45^{high}$/side scatter (SSC)-low lymphocyte gate is represented by CD3 T cells in control subjects (Figure 2A). Both the percentage and number of CD3 T cells were decreased early after CA and remained low during next 72 hours after ROSC compared with controls (Figure 2B and 2C). In addition, the number of CD19 B cells was decreased at 12, 48, and 72 hours after ROSC when compared with control subjects (Figure 2D). The decline in B-cell numbers after CA was less dramatic compared with the decrease in CD3 T cells. In contrast to T and B cells, the number of CD3-negative/CD19-negative cells (mostly natural killer cells) did not change after CA and was similar to the number of double-negative cells in control subjects (Figure 2E).

Comparative analysis revealed that the number of T cells differed between survivors and nonsurvivors after CA. CD3 T cells were higher in survivors at 12, 24, and 48 hours after ROSC compared with nonsurvivors (Figure 2F). In contrast to CD3 T cells, no differences were found in CD19 B cells between survivors and nonsurvivors (Figure 2G).

Higher Number of CD73 Lymphocytes Is Associated With Survival After CA

Lymphocytes play a dual role in the regulation of inflammation, contributing to both amplification of inflammation,[51] and protection of tissue from excessive inflammatory response.[52] One mechanism contributing to lymphocyte-dependent down-regulation of inflammation is related to expression of CD39 and CD73 and production of the anti-inflammatory purine nucleoside, adenosine.[40] Because our data demonstrated an association between the number of lymphocytes and survival, we analyzed time-dependent changes in CD39- and CD73-expressing cells. We found that CD3 T and CD19 B lymphocytes represented only a minor subpopulation of cells expressing CD39 in both control subjects and subjects with CA (Figure 3A). Most CD39-expressing cells were neutrophils and monocytes (data not shown). The number of CD39-expressing cells was increased at 6 and 12 hours after ROSC (Figure 3B). No differences were noted in numbers of CD39-expressing cells between survivors and nonsurvivors after CA (Figure 3C).

As seen in Figure 3D, only CD3 T and CD19 B lymphocytes expressed CD73 in both controls and subjects with CA. The CD3 T lymphocytes thus represented a major subpopulation of circulating CD73-expresing immune cells. The percentage of CD73/CD3 T lymphocytes and total number of

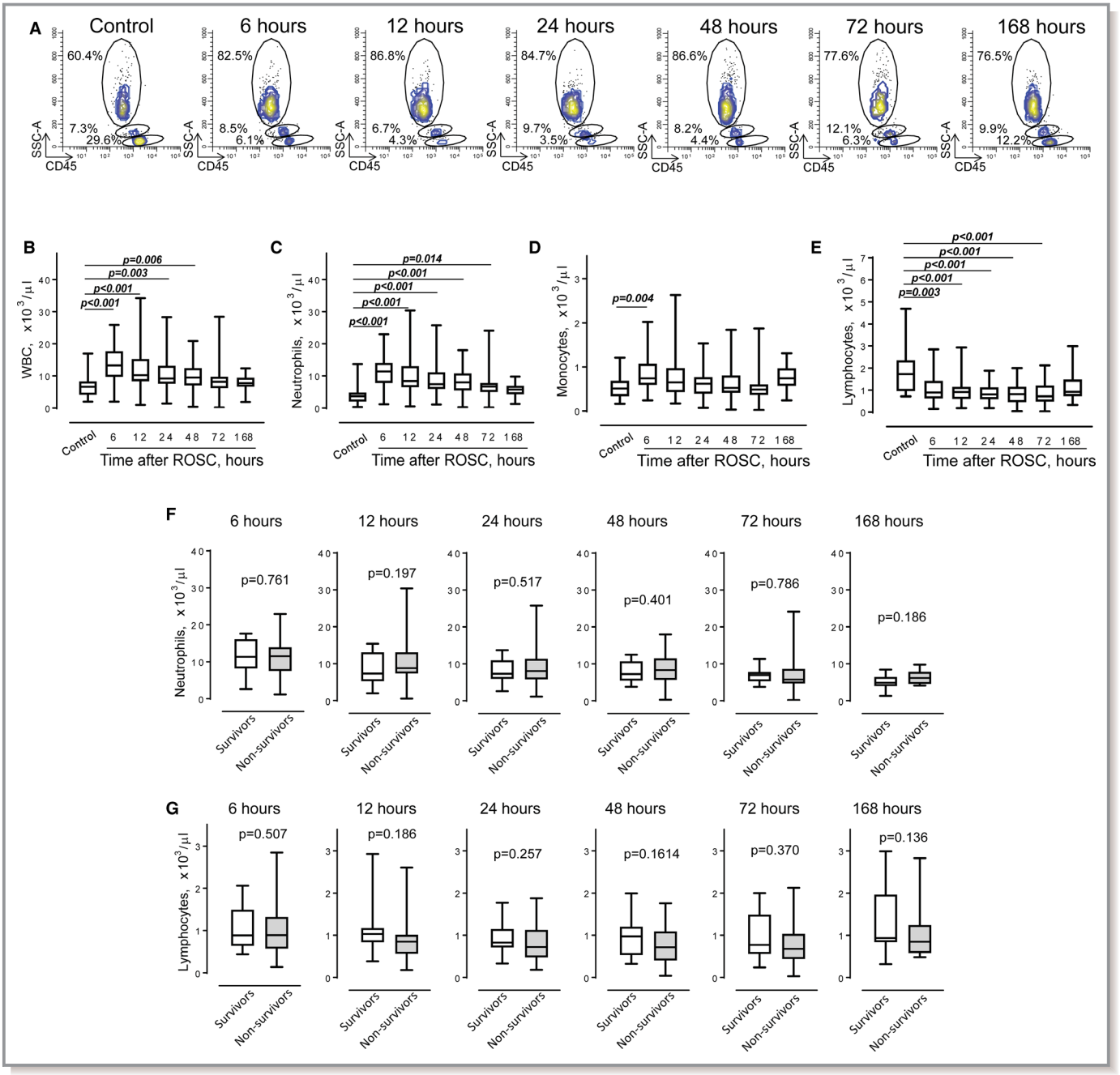

Figure 1. Time-dependent changes in subpopulations of white blood cells (WBCs) after cardiac arrest (CA). **A**, Representative flow cytometric contour plots demonstrating percentages of neutrophils (upper gate), monocytes (middle gate), and lymphocytes (lower gate) in peripheral blood of subjects who underwent preoperative coronary artery bypass grafting (control) and subjects with CA on different time points after return of spontaneous circulation (ROSC). **B** through **E**, Graphical representation of flow cytometry data showing total number of WBCs (**B**) and cells in neutrophil (**C**), monocyte (**D**), and lymphocyte (**E**) gates in groups of control subjects (n=30) and subjects with CA (n=48). Data are presented in standard percentile format (minimum value, 25th percentile; median, 75th percentile; and maximum value). Statistical significance was calculated using Kruskal-Wallis test with Dunn's multiple-comparisons posttest, and *P* values are indicated. **F** and **G**, The number of cells in neutrophil (**F**) and lymphocyte (**G**) gates in survivors (n=19) and nonsurvivors (n=29) after CA. Mann-Whitney test was used, and *P* values are indicated. SSC indicates side scatter.

CD73-expressing cells were significantly decreased after CA (Figure 3E). Thus, our data show that CA is associated with reduced number of CD73-expressing cells, which mediate hydrolysis of AMP. Comparative analysis showed that survivors have higher number of CD73-expressing cells compared with nonsurvivors after CA (Figure 3F), suggesting a potential role for CD73 in the pathophysiological features of post-CA syndrome.

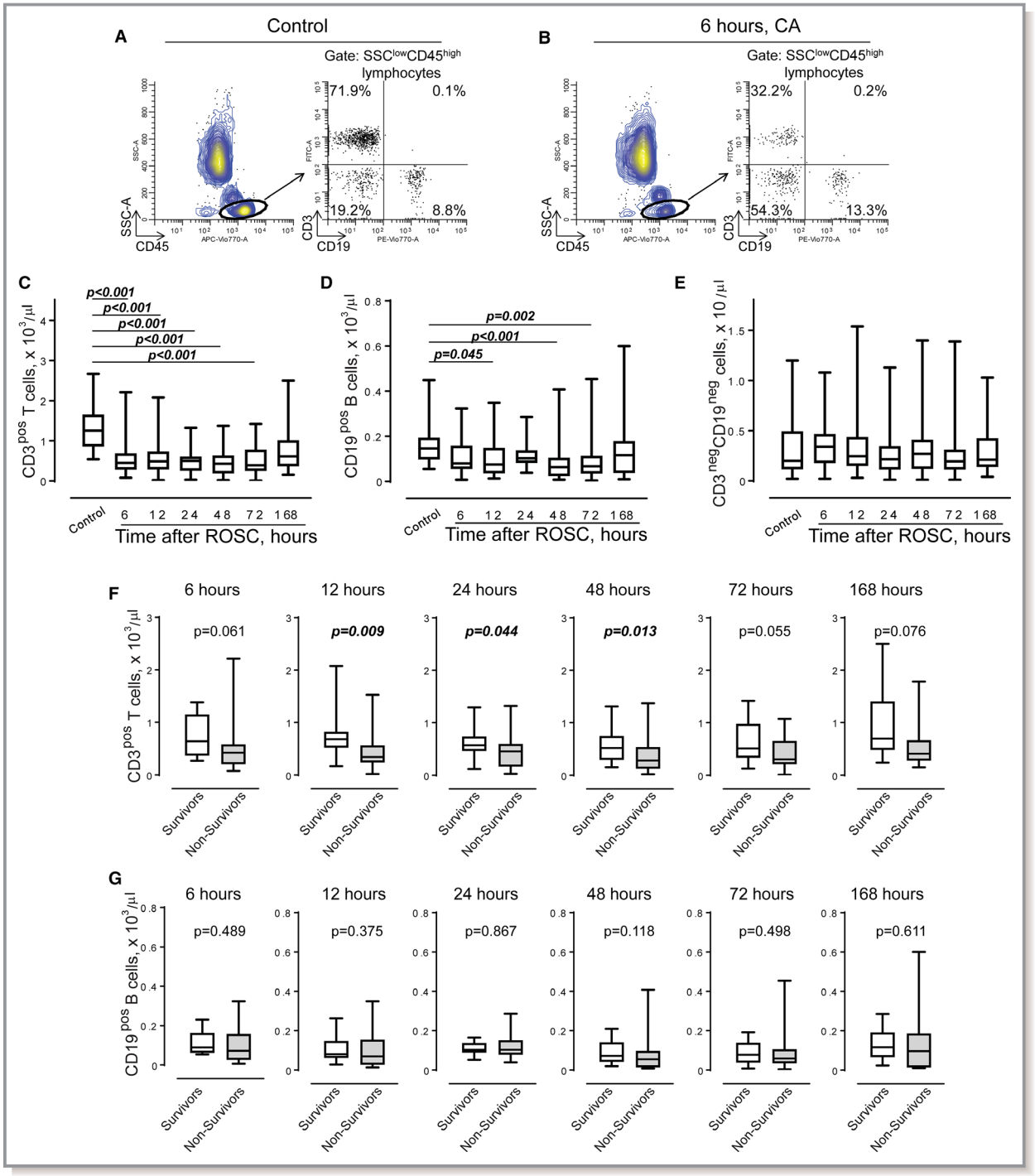

Figure 2. Number of CD3 T cells is significantly decreased after cardiac arrest (CA) and associated with survival. **A** and **B**, Representative flow cytometric plots demonstrating percentage of CD3-positive ($CD3^{pos}$; upper left quadrant), CD19-positive ($CD19^{pos}$; lower right quadrant), and CD3-negative ($CD3^{neg}$)/CD19-negative ($CD19^{neg}$; where negative indicates no expression; lower left quadrant) cells within the side scatter (SSC)$^{low}CD45^{high}$ lymphocyte gate (contour plots) in control subjects (**A**) and subjects with CA (**B**). **C** through **E**, Graphical representation of flow cytometry data showing total number of $CD3^{pos}$ T cells (**C**), $CD19^{pos}$ B cells (**D**), and $CD3^{neg}/CD19^{neg}$ cells (**E**) in groups of control subjects (n=30) and subjects with CA (n=48). The number of cells was calculated using total number of white blood cells, percentage of cells in the lymphocyte gate, and percentage of cells corresponding to specific cell subpopulation. Statistical significance was calculated using Kruskal-Wallis test with Dunn's multiple-comparisons posttest, and *P* values are indicated. **F** and **G**, The number of $CD3^{pos}$ T cells (**F**) and $CD19^{pos}$ B cells (**G**) in survivors (n=19) and nonsurvivors (n=29) after CA. Mann-Whitney test was used. ROSC indicates return of spontaneous circulation.

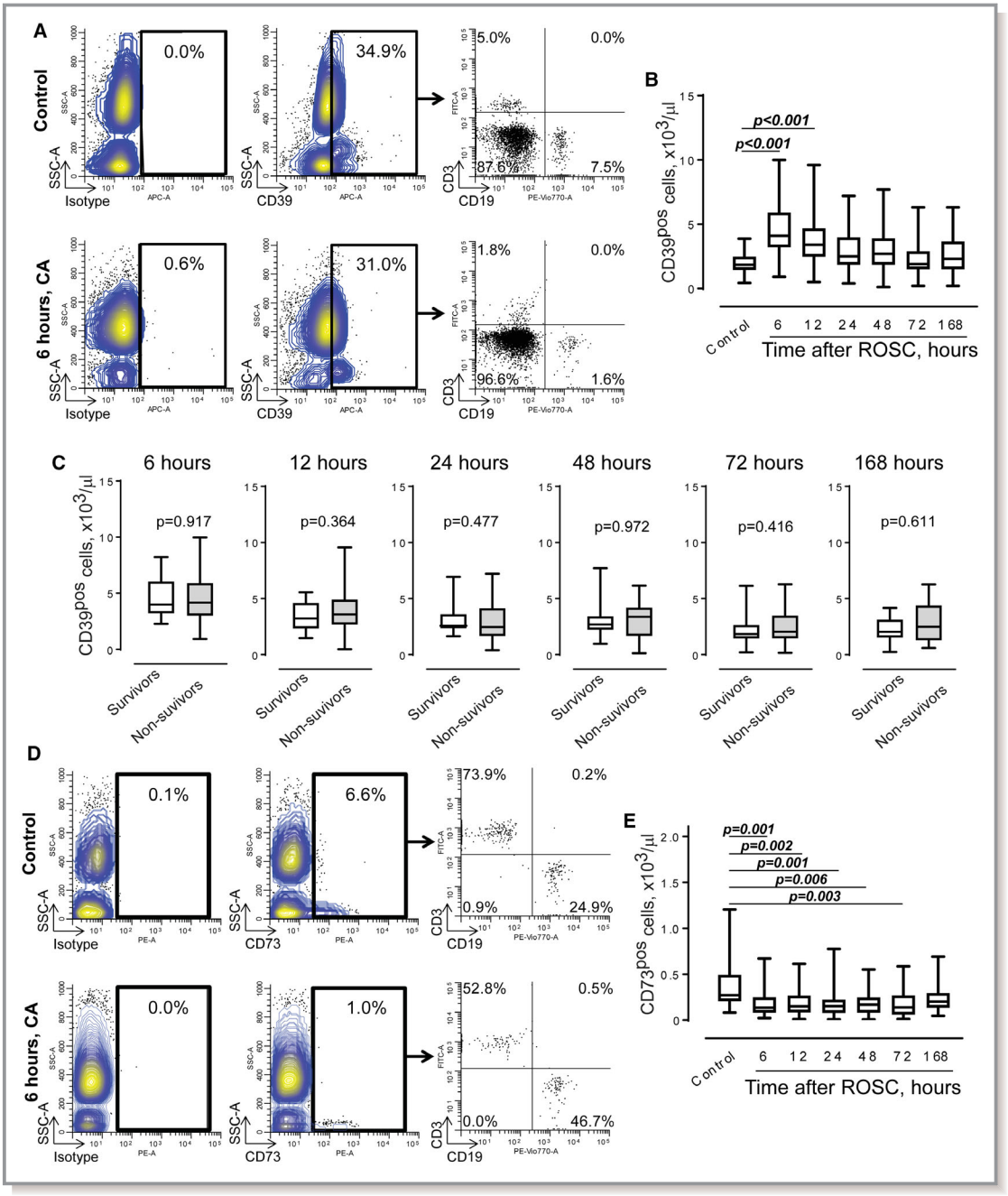

Figure 3. Higher number of CD73-positive ($CD73^{pos}$) lymphocytes but not CD39-positive ($CD39^{pos}$) cells is associated with survival after cardiac arrest (CA). **A**, Representative flow cytometric plots showing total percentage of $CD39^{pos}$ cells (left and middle contour plots) and subsets of CD39-expressing CD3-positive ($CD3^{pos}$), CD19-positive ($CD19^{pos}$), and CD3-negative ($CD3^{neg}$)/CD19-negative ($CD19^{neg}$) cells (where negative indicates no expression; right dot plots) in control subjects (**top**) and subjects with CA (**bottom**; 6 hours after return of spontaneous circulation [ROSC]). **B**, Graphical representation of flow cytometry data showing total number of CD39-expressing cells in control subjects (n=30) and subjects with CA (n=48) at different times after ROSC. Kruskal-Wallis test and Dunn's multiple-comparisons posttest were used. **C**, The number of $CD39^{pos}$ cells in survivors (n=19) and nonsurvivors (n=29) after CA. Mann-Whitney test was used. **D**, Flow cytometric plots showing total percentage of $CD73^{pos}$ cells in peripheral blood and subsets of CD73-expressing $CD3^{pos}$ and $CD19^{pos}$ cells. **E**, Graphical representation of flow cytometry data showing total number of CD73-expressing cells in control subjects (n=30) and subjects with CA (n=48) at different times after ROSC. Kruskal-Wallis test and Dunn's multiple-comparisons posttest were used. **F**, The number of $CD73^{pos}$ cells in survivors (n=19) and nonsurvivors (n=29) after CA. Mann-Whitney test was used. FITC indicates fluorescein isothiocyanate; SSC, side scatter.

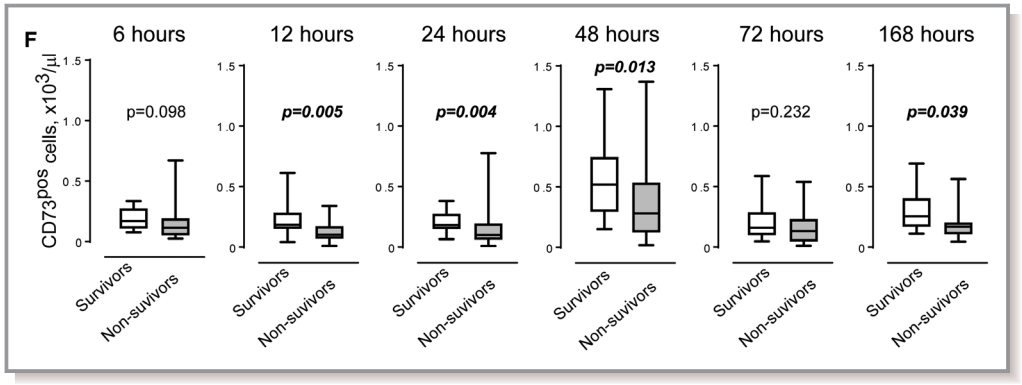

Figure 3. Continued.

Different causes of CA may affect clinical outcome. To determine the relationship between causes of CA and CD73-expressing lymphocytes, we analyzed the cause of CA in study subjects. Among 48 patients included in the analysis, 35 (73%) had a pure cardiac cause of their arrest (myocardial infarction or primary arrhythmia), with 49% mortality in this group. As shown in Figure S1, the number of CD73-expressing lymphocytes in patients with pure cardiac cause was significantly higher in group of survivors compared with nonsurvivors at 12, 24, 72, and 168 hours after ROSC, indicating that CD73-expressing cells may play an important role in survival after CA induced by myocardial infarction or arrhythmia. Further investigation in larger cohort of patients is required to make reliable conclusions about clinical significance of CD73-expressing lymphocytes in patients with primary respiratory arrest (n=9, 100% mortality), circulatory collapse (n=2), pulmonary embolism (n=1), and unexplained CA (n=1).

CD73-Expressing Lymphocytes Mediate Inhibition of TNF-α Production and ROS Generation by Myeloid Cells

To determine whether CD73, expressed on lymphocytes, plays a role in controlling overactivation of myeloid cells, we investigated the effect of AMP on inhibition of lipopolysaccharide-induced TNF-α production by WBCs obtained from subjects with CA. Myeloid cells are a well-known source of TNF-α,[53] and adenosine is a potent endogenous inhibitor of TNF-α production.[36] The effect of adenosine on TNF-α is mediated through activation of A_{2A} adenosine receptors.[36,54]

The involvement of CD73/adenosinergic signaling was analyzed in WBC isolates from 5 subjects with CA (at 24 hours after ROSC) with numbers of CD73-expressing lymphocytes whose values are between 162 and 300 cells/µL blood (median value, 177 cells/µL blood; IQR, 162–300 cells/µL blood), which closely resembles the median value and interquartile range found in a group of survivors (median value, 182 cells/µL blood; IQR 150–275 cells/µL blood) at 24 hours after ROSC. As shown in Figure 4A and 4B, lipopolysaccharide induced significant accumulation of $CD45^{pos}$/SSC-intermediate monocytes expressing high level of TNF-α protein. No $CD45^{pos}$/SSC-high neutrophils or $CD45^{pos}$/SSC-low lymphocytes with high expression of TNF-α protein after stimulation with lipopolysaccharide were found in peripheral blood obtained from subjects with CA.

Incubation of cells with CD73 substrate, AMP, abolished the lipopolysaccharide-induced TNF-α expression in $CD45^{pos}$/SSC-intermediate cells (Figure 4B and Figure S2). Then, both APCP, an inhibitor of CD73, and ZM 241385, a potent adenosine receptor antagonist, ablated the inhibitory effects of AMP on lipopolysaccharide-induced TNF-α production, indicating that CD73, expressed on lymphocytes, is functionally active and may limit proinflammatory activation of $CD45^{pos}$/SSC-intermediate monocytes in response to AMP. To validate flow cytometric data, we performed analysis of TNF-α secretion and demonstrated effects of AMP, APCP, and ZM 241385 on lipopolysaccharide-induced TNF-α protein level in supernatant from WBCs using ELISA (Figure 4C). We also measured level of TNF-α in plasma. However, no difference in levels of circulating TNF-α protein was found between subjects with CA and control subjects (Figure 4D).

To further characterize the effect of CD73 in immune cells from subjects with CA, we evaluated the effect of AMP on lipopolysaccharide-induced generation of ROS. Short-time incubation of immune cells with lipopolysaccharide increased generation of ROS, resulting in a right shift in 2′,7′-dichlorodihydrofluorescein diacetate fluorescence, in neutrophils and monocytes but not in lymphocytes (Figure 4E). AMP inhibited the effect of lipopolysaccharide in myeloid cells. The inhibitory effect of AMP was blocked with APCP in both neutrophils and monocytes, demonstrating a role of CD73 in inhibition of lipopolysaccharide-induced ROS generation (Figure 4F).

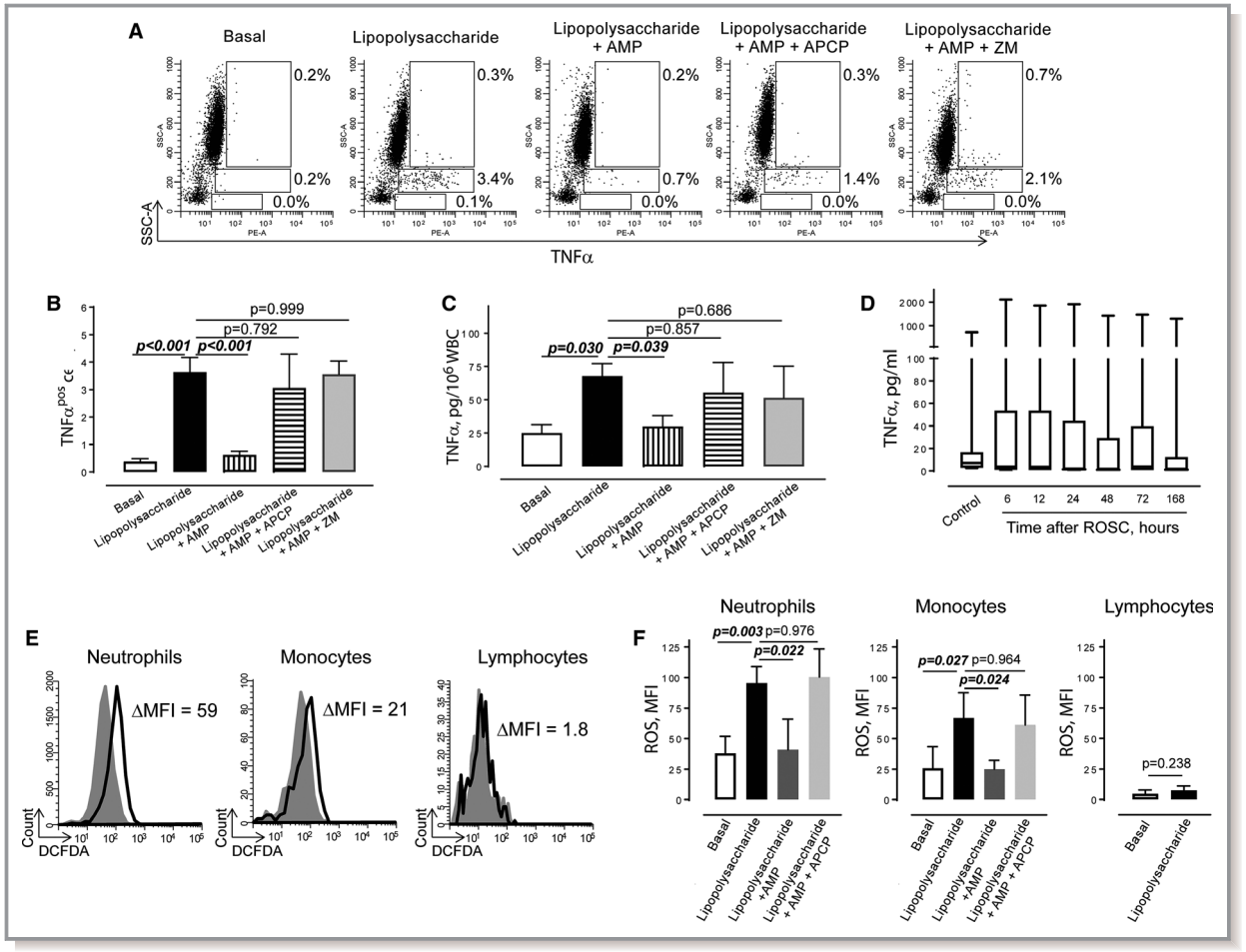

Figure 4. CD73 mediates inhibition of lipopolysaccharide-induced production of tumor necrosis factor-α (TNF-α) and generation of reactive oxygen species (ROS) by myeloid cells. **A** through **C**, **E**, and **F**, White blood cells (WBCs) were isolated 24 hours after return of spontaneous circulation (ROSC) from 5 subjects with cardiac arrest (CA) with a median value of 177 (interquartile range [IQR], 162–300) CD73-expressing lymphocytes/μL of blood, which closely resembles the median value and IQR found in a group of survivors (median, 182; IQR, 150–275 CD73-expressing lymphocytes/μL of blood) at 24 hours after ROSC. **A**, Representative flow cytometric plots showing cells with high levels of TNF-α protein production in subpopulations of neutrophils (upper gate), monocytes (intermediate gate), and lymphocytes (lower gate) in the absence (basal) or presence of 10 ng/mL lipopolysaccharide alone or in combinations with 100 μmol/L AMP (lipopolysaccharide+AMP), 100 μmol/L of CD73 inhibitor, adenosine 5′-(α, β-methylene) diphosphate (APCP; lipopolysaccharide+AMP+APCP), and adenosine receptor inhibitor, 300 nmol/L ZM 241385 (lipopolysaccharide+AMP+ZM). Cells were incubated for 6 hours in the presence of brefeldin A to prevent secretion of TNF-α from cells and 10 μmol/L erythro-9-(2-hydroxy-3-nonyl)−adenine hydrochloride to prevent degradation of adenosine in cell culture. **B**, Graphical representation of flow cytometric data demonstrating percentage of cells with high expression of TNF-α (n=5); 1-way ANOVA was used, and *P* values from Tukey's multiple-comparisons test are shown. **C**, Levels of TNF-α protein in supernatant of WBCs measured by ELISA (n=5); 1-way ANOVA was used with Tukey's multiple-comparisons test. **D**, Levels of TNF-α protein in the peripheral circulation of control subjects (n=30) and subjects with CA (n=48). Kruskal-Wallis test and Dunn's multiple-comparisons posttest were used. **E**, Representative flow cytometric histograms demonstrating basal (gray-shaded) and 10 ng/mL lipopolysaccharide-induced (open histogram) levels of ROS generation in major subpopulations of WBCs obtained from subjects with CA. **F**, Graphical representation of ROS generation in the absence (basal) or presence of lipopolysaccharide alone or in combinations with 100 μmol/L AMP (lipopolysaccharide+AMP) and 100 μmol/L of CD73 inhibitor, APCP (lipopolysaccharide+AMP+APCP) (n=5); 1-way ANOVA with Tukey's multiple-comparisons test was used. MFI indicates mean fluorescence intensity.

CD73 Activation Promotes VEGF Secretion From Immune Cells Obtained From Subjects With CA

To determine if CD73 expressed on lymphocytes could directly contribute to secretion of prosurvival factors, we incubated WBCs obtained from subjects with CA in the absence or presence of AMP and then measured secretion of VEGF. As shown in Figure 5A, AMP induced an increase in the production of VEGF. This effect was abolished by APCP, a CD73 inhibitor. Because our data demonstrated that levels of VEGF protein are significantly increased in the plasma of subjects with CA, at 6 and 12 hours after ROSC, compared with controls (Figure 5B),

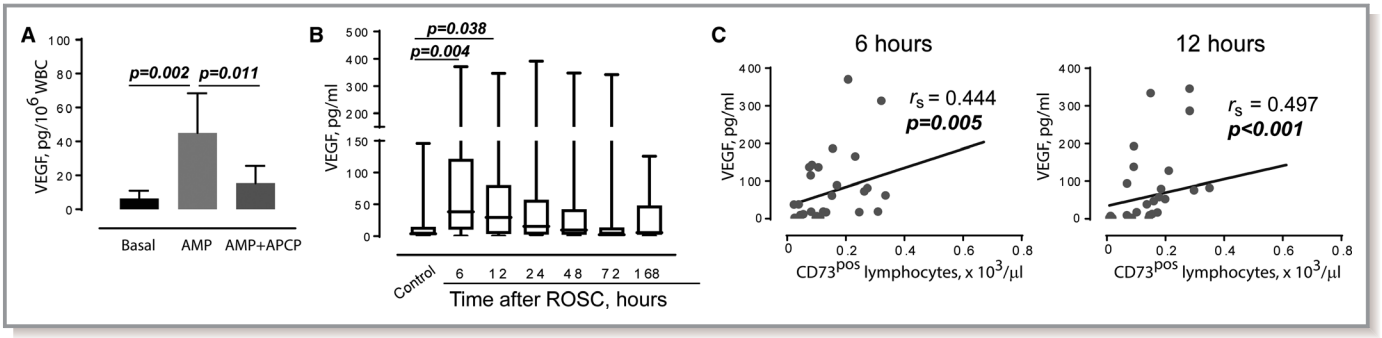

Figure 5. CD73 mediates upregulation of vascular endothelial growth factor (VEGF) protein secretion from white blood cells (WBCs) of subjects with cardiac arrest (CA). **A**, Level of VEGF protein in supernatant of WBCs obtained from subjects with CA and incubated for 6 hours in the absence (basal) or presence of 100 μmol/L AMP alone or in combination with 100 μmol/L adenosine 5′-(α, β-methylene) diphosphate (APCP; AMP+APCP). WBCs were isolated 24 hours after return of spontaneous circulation (ROSC) from 5 subjects with CA (n=5), with a median value of 177 (interquartile range [IQR], 162–300) CD73-expressing lymphocytes/μL of blood, which closely resembles the median value and IQR found in a group of survivors (median, 182; IQR, 150–275 CD73-expressing lymphocytes/μL of blood) at 24 hours after ROSC. Incubation medium contained 10 μmol/L erythro-9-(2-hydroxy-3-nonyl)−adenine hydrochloride, an adenosine deaminase inhibitor, to decelerate adenosine catabolism in cell culture. Data presented as mean±SEM (n=5); 1-way ANOVA with Tukey's multiple-comparisons test was used. **B**, Levels of VEGF protein in peripheral circulation of control subjects (n=30) and subjects with CA (n=48); Kruskal-Wallis test was used, and *P* values are indicated (Dunn's multiple-comparisons test). **C**, The correlation between levels of VEGF protein and number of CD73-positive ($CD73^{pos}$) lymphocytes in blood of subjects with CA at 6 and 12 hours after ROSC (n=48). Spearman's correlation coefficient and *P* values are as indicated.

we performed correlation analysis to determine whether CD73-expressing lymphocytes contributed to plasma level of VEGF at these time points. As seen in Figure 5C, plasma levels of VEGF displayed low positive correlation with the number of $CD73^{pos}$ lymphocytes at 6 and 12 hours after ROSC, indicating that lymphocyte-dependent adenosinergic signaling may contribute to upregulation of VEGF secretion after CA.

Discussion

This study demonstrates that the peri-CA period is characterized by peripheral lymphopenia and that a reduced number of circulating CD3-positive lymphocytes is associated with worse outcome. It furthermore shows that higher levels of circulating CD73-expressing cells during the postresuscitation syndrome are associated with survival, and CD3 T lymphocytes are the major subpopulation of immune cells expressing functionally active CD73 in circulation. Lymphocytes expressing CD73 mediate a transition from proinflammatory activation of neutrophils and monocytes toward protective paracrine signaling after CA.

It is well-known that targeted temperature management can affect inflammation and survival after CA. In the current study, resuscitated patients after CA, but not subjects after CABG, were cooled to 32°C to 34°C and rewarmed, suggesting the potential effect of temperature on the level of the immune activation between 2 groups of patients. However, analysis of CD73-expressing lymphocytes in relation to survival after CA was only performed in the CA patient group, not compared against the CABG group.

Cardiopulmonary arrest treated with cardiopulmonary resuscitation induces a shift toward an increased number of neutrophils accompanied by a decreased number of lymphocytes in the peripheral circulation, suggesting predominant activation of the innate branch of the immune system.[55] Both lymphopenia and increased neutrophil/lymphocyte ratio associated with poor outcome.[24–26] In agreement with above-mentioned studies, we found that CA induced high neutrophil counts accompanied by low numbers of lymphocytes. Our data showed no definitive association between numbers of neutrophils or lymphocytes and survival after CA. We found, however, a trend toward a higher number of lymphocytes consistently present for 3 days after CA; and a significantly higher number of CD3 T cells was noted in the survivors' group. These data suggest that lymphocytes may exert protective effects after CA.

To investigate mechanisms underlying the association between CD3 T lymphocytes and survival after CA, we performed analyses of CD39 and CD73 expression. It has been shown previously that both CD39 and CD73 are expressed on T-regulatory cells, a well-characterized anti-inflammatory subset of lymphocytes, and mediate generation of an immunosuppressive purine nucleoside, adenosine.[40,41] Our data showed that CD39 is expressed on the cell surface of peripheral immune cells, including neutrophils, monocytes, and lymphocytes, and that the number of CD39-expressing cells increased after CA. CD39 mediates hydrolysis of ATP, which can induce neuronal cell death.[56] It is possible, therefore, that an increased number of CD39 cells is protective, mediating the removal of ATP released from apoptotic and dying cells.[57] In contrast to CD39,

expression of CD73 was found only on lymphocytes and not on myeloid cells.

CD73 is a key enzyme in the generation of adenosine from AMP.[57] To study the effect of CD73 on inflammation, we induced proinflammatory activation of immune cells using lipopolysaccharide, a well-characterized activator of TLRs. Our data demonstrated that activation of CD73 resulted in inhibition of lipopolysaccharide-induced TNF-α and ROS production from myeloid cells. However, no increase in the level of TNF-α was found in plasma obtained from subjects with CA. This might be explained by the fact that plasma contains large amounts of molecules that bind and neutralize endotoxin.[58] Further study is needed to determine the role of CD73-expressing lymphocytes in the regulation of local production of proinflammatory cytokines and ROS in injured tissues after CA.

The proinflammatory role of T cells after CA has been previously demonstrated in a mouse model of CA.[59] CD4 lymphocytes rapidly infiltrated into the brain after experimental CA and contributed to neuronal injury through secretion of TNF-α and interferon-γ. However, the number of anti-inflammatory lymphocytes, and specifically CD73-expressing cells, was not determined in that study. It has been recently reported that the number of anti-inflammatory T-regulatory cells is decreased in subjects with CA. However, survivors have a higher T-regulatory cell count compared with nonsurvivors.[60] Further animal studies will help to determine if CD73-expressing cells can reduce brain tissue inflammation and neuronal death after CA.

Our data demonstrate activation of CD73 induced production of VEGF from immune cells obtained from subjects with CA. We also demonstrate a correlation between the VEGF level and the number of CD73-expressing cells, indicating that adenosine generated by CD73 lymphocytes is a major contributor to the level of circulating VEGF after CA. On the basis of the previously described adenosine effects in inhibition of acute inflammation and new data obtained in this study, we propose a model of adenosinergic regulation of immune responses by CD73-expressing lymphocytes after CA (Figure 6). According to this model, generation of adenosine by CD73 expressed on peripheral blood lymphocytes has a dual effect on both inhibition of inflammation and promotion of cell survival after CA. Acting on A2 adenosine receptors, adenosine suppresses proinflammatory activation of myeloid cells and induces production of prosurvival growth factor VEGF, contributing to better outcomes after CA.

Although our current study focused primarily on CD73-dependent regulation of peripheral immune cells, the role of CD73 and adenosine in the modulation of neuronal survival has been described.[61–63]

These new data contribute to a better understanding of the complex nature of the immune response after CA and highlight the importance of further studies to investigate the role of CD73-expressing cells in the regulation of inflammation. The proposed model identifies CD73 lymphocytes as a key modulator of immune response after CA and stimulation of CD73/adenosine A_2 receptor axis as a potential therapeutic approach to improve outcomes.

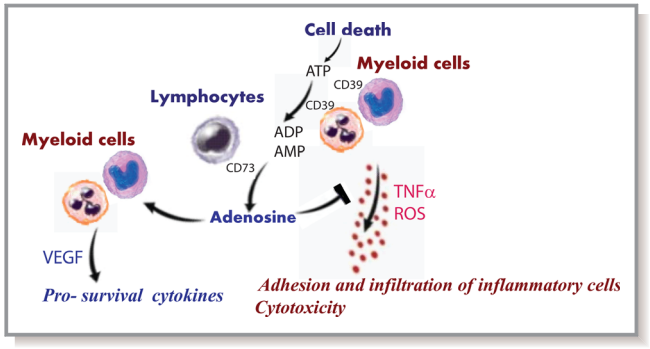

Figure 6. Proposed role of CD73/5′-nucleotidase–expressing lymphocytes in the control of immune response after cardiac arrest. Myeloid cells (neutrophils and monocytes) express CD39 ectonucleotidases that mediate hydrolysis of ATP, released from dead and apoptotic cells, to ADP and AMP. Then, AMP is hydrolyzed by CD73/5′-nucleotidase, expressed on lymphocytes, to adenosine, which suppresses proinflammatory activation of myeloid cells, secretion of tumor necrosis factor-α (TNF-α), and production of reactive oxygen species (ROS) and promotes production of prosurvival vascular endothelial growth factor (VEGF).

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Disclosures

None.

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SUPPLEMENTAL MATERIAL

Figure S1. The number of CD73^{pos} cells in survivors (*n=18*) and non-survivors (*n=17*) after CA with pure cardiac etiology. Mann-Whitney test.

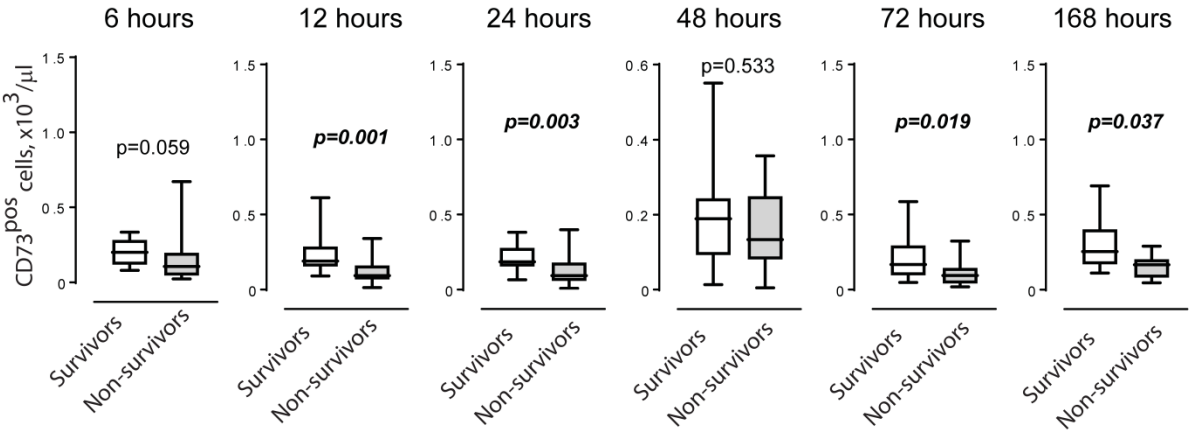
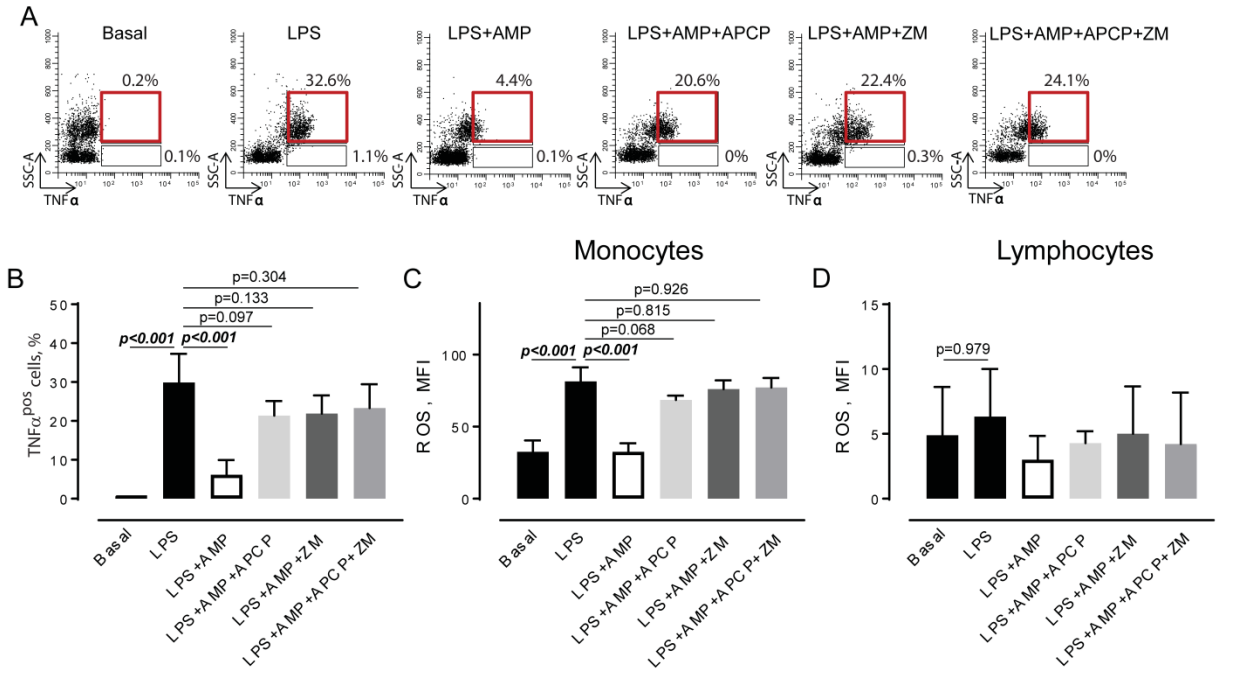


Figure S2. CD73 mediates inhibition of LPS-induced production of TNFα and generation of ROS by peripheral blood mononuclear cells (PB MNC).



Peripheral blood mononuclear cells were isolated using Ficoll-Paque™ gradient as previously described[1]. PB MNC from five CA subjects (*n=5*) with median value of 177 (*IQR, 162-300*) CD73-expressing lymphocytes per µl of blood, which closely resembles the median value and interquartile range found in a group of survivors (Me, 182; IQR 150-275) at 24 hours after ROSC, were used. **A**. Representative flow cytometric plots showing cells with high levels of TNFα protein production in subpopulations monocytes (*red gate*) and lymphocytes (*black gate*) in the absence (*basal*) or presence of 10 ng/ml LPS alone, or in combinations with 100 µM AMP (*LPS+AMP*), 100 µM of CD73 inhibitor, APCP (*LPS+AMP+APCP*) and adenosine receptor inhibitor, 300 nM ZM241385 (*LPS+AMP+ZM*), or combination of 100 µM AMP, 100 µM APCP and 300 nM ZM241385 (LPS+AMP+APCP+ZM). Cells were incubated for 6 hours in the presence of Brefeldin A to prevent secretion of TNFα from cells and 10 µM EHNA to prevent degradation of adenosine in cell culture. **B**. Graphical representation of flow cytometric data demonstrating percentage of cells with high expression of TNFα, n=5; One-way ANOVA, *p* values from Tukey's multiple comparisons test are shown. **C-D**. Graphical representation of flow cytometric data showing ROS generation by monocytes (**C**) and lymphocytes (**D**) in the absence (*Bas*) or presence of LPS alone, or in combinations with 100 µM AMP (*LPS+AMP*) and 100 µM of CD73 inhibitor, APCP (*LPS+AMP+AP*), and 100 µM AMP, 100 µM APCP and 300 nM ZM241385 (LPS+AMP+APCP+ZM), n=5; One-way ANOVA with Tukey's multiple comparisons test; *MFI* – mean fluorescence intensity.

Supplemental Reference:

1. Ryzhov S, Matafonov A, Galindo CL, Zhang Q, Tran TL, Lenihan DJ, Lenneman CG, Feoktistov I, Sawyer DB. Erbb signaling attenuates proinflammatory activation of nonclassical monocytes. *American journal of physiology. Heart and circulatory physiology*. 2017;312:H907-H918.