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Myocardial Ischemia and Reperfusion Leads to Transient CD8 Immune Deficiency and Accelerated Immunosenescence in CMV-Seropositive Patients

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Rationale: There is mounting evidence of a higher incidence of coronary heart disease in cytomegalovirus-seropositive individuals.

Objective: The aim of this study was to investigate whether acute myocardial infarction triggers an inflammatory T-cell response that might lead to accelerated immunosenescence in cytomegalovirus-seropositive patients.

Methods and Results: Thirty-four patients with acute myocardial infarction undergoing primary percutaneous coronary intervention were longitudinally studied within 3 months after reperfusion (Cohort A). In addition, 54 patients with acute myocardial infarction and chronic myocardial infarction were analyzed in a cross-sectional study (Cohort B). Cytomegalovirus-seropositive patients demonstrated a greater fall in the concentration of terminally differentiated CD8 effector memory T cells (T_{EMRA}) in peripheral blood during the first 30 minutes of reperfusion compared with cytomegalovirus-seronegative patients (−192 versus −63 cells/μL; P=0.008), correlating with the expression of programmed cell death-1 before primary percutaneous coronary intervention (r=0.8; P=0.0002). A significant proportion of T_{EMRA} cells remained depleted for ≥3 months in cytomegalovirus-seropositive patients. Using high-throughput 13-parameter flow cytometry and human leukocyte antigen class I cytomegalovirus-specific dextramers, we confirmed an acute and persistent depletion of terminally differentiated T_{EMRA} and cytomegalovirus-specific $CD8^+$ cells in cytomegalovirus-seropositive patients. Long-term reconstitution of the T_{EMRA} pool in chronic cytomegalovirus-seropositive postmyocardial infarction patients was associated with signs of terminal differentiation including an increase in killer cell lectin-like receptor subfamily G member 1 and shorter telomere length in $CD8^+$ T cells (2225 versus 3397 bp; P<0.001).

Conclusions: Myocardial ischemia and reperfusion in cytomegalovirus-seropositive patients undergoing primary percutaneous coronary intervention leads to acute loss of antigen-specific, terminally differentiated CD8 T cells, possibly through programmed cell death-1–dependent programmed cell death. Our results suggest that acute myocardial infarction and reperfusion accelerate immunosenescence in cytomegalovirus-seropositive patients. (*Circ Res*. 2015;116:87-98. DOI: 10.1161/CIRCRESAHA.116.304393.)

Key Words: aging ■ cytotoxic T-lymphocytes ■ human cytomegalovirus ■ myocardial infarction ■ programmed cell death 1 ■ reperfusion ■ telomere

Human cytomegalovirus is a ubiquitous herpes virus, which is not cleared after initial infection.[1] Cytomegalovirus infection predominantly affects the $CD8^+$ T-cell compartment by memory inflation, characterized by an excessive increase in cytomegalovirus-specific effector memory T cells (T_{EMRA}) and a decrease in naive cells. Cytomegalovirus infection has been linked with a higher incidence of coronary heart disease (CHD) and may, in part, contribute to the higher

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Nonstandard Abbreviations and Acronyms	
CHD	coronary heart disease
HCA	hierarchical cluster analysis
KLRG1	killer cell lectin-like receptor subfamily G member 1
MFI	mean fluorescence intensity
PBMCs	peripheral blood mononuclear cells
PD-1	programmed cell death-1
PPCI	primary percutaneous coronary intervention
TL	telomere length
T_{EM}	effector memory T cells
T_{EMRA}	CD45RA+ effector memory T cells

burden of cardiovascular disease in areas of socioeconomic deprivation.[2]

Previously, only reactivation of cytomegalovirus in immunocompromised hosts was thought to be clinically relevant. However, a growing body of evidence now suggests an important role for cytomegalovirus during aging.[3] Cytomegalovirus seropositivity has been estimated to shorten median life expectancy by 4 years in patients aged >65 because of a higher risk of cardiovascular death.[3] Patients with CHD are known to have shorter telomere length (TL) in their peripheral blood leukocytes compared with age-matched healthy adults.[4,5] We have previously shown in patients with chronic myocardial infarction (MI) that the TL of $CD8^+$ T cells is shorter than that of all other myeloid and lymphoid leukocyte populations, and in comparison with age-matched seropositive healthy individuals without previous MI.[5] $CD8^+$ T-cell responses in elderly cytomegalovirus-seropositive patients are further characterized by an accumulation of replicatively senescent dysfunctional T cells, also with short TL.[6] The inverse correlation of telomere shortening in $CD8^+$ T cells with left ventricular function additionally suggests a link between a history of MI and immunosenescence.[5] The aim of this study was to investigate the effect of acute myocardial infarction (AMI) on immunosenescence and cytomegalovirus-specific immunity.

Methods

Please see the Online Data Supplement for further details.

Study Population

Cohort A

A time-course study was performed in 34 patients with ST-segment–elevation myocardial infarction (STEMI; mean age, 59.9±11.1 years) who were treated by primary percutaneous coronary intervention (PPCI; Table 1). Blood samples were taken before reperfusion and at 15, 30, and 90 minutes and 24 hours after reperfusion. Additional dextramer substudies were undertaken at 3 months post-MI in 8 cytomegalovirus-seropositive patients.

Cohort B

Fifty-four male patients with angiographically confirmed CHD were included in the main study population. Of these, 28 patients had acute STEMI (AMI; mean age, 56±5.8 years) and 26 were stable patients with previously treated STEMI (chronic MI; ≥3 months post-MI; mean age, 61±6 years) and were analyzed 24 hours after

Table 1. Baseline Characteristics of Cytomegalovirus Subgroups (Cohort A: Time-Course Reperfusion Substudy)

	AMI (n=34)		
	Cytomegalovirus Negative Median (IQR)	Cytomegalovirus Positive Median (IQR)	*P* Value
N	15	19	
Age, median (range)	55 (48; 68)	66.0 (58; 72)	0.06
Sex, male/female	13/2	10/9	0.06
Previous MI, %	0	0	1.0
Ejection fraction, %	53 (48; 66)	54 (43; 61)	0.54
No. of vessel-disease, 1/2/3	15/0/0	17/2/0	0.49
Serum creatinine, μmol/L	81 (69; 95)	75 (63; 88)	0.31
HDL cholesterol, mmol/L	1.2 (1.1; 1.4)	1.2 (1.0; 1.4)	0.94
LDL cholesterol, mmol/L	4.1 (3.1; 5.0)	3.6 (3.2; 4.7)	0.57
Triglycerides, mmol/L	1.9 (0.9; 2.7)	1.3 (0.8; 2.2)	0.39
BMI	26 (23; 29)	25 (23; 28)	0.99
Diabetes mellitus, %	0	5.3	1.0
Hypertension, %	26.7	36.8	0.72
Active smokers, %	53.3	52.6	1.0
Statin treatment, %	20	21.1	1.0
Initial troponin T, ng/L	40 (17; 132)	61 (33; 141)	0.35
Peak troponin T, ng/L	4371 (2102; 6200)	4395 (1933; 8408)	0.9
Onset-to-balloon time, min	135 (112; 219)	164 (114; 211)	0.63
Door-to-balloon time, min	24 (17; 28)	22 (18; 34)	0.82

All indicated *P* values (control vs CHD and cytomegalovirus positive vs cytomegalovirus negative) were calculated by nonparametric *t* test or χ^2 and Fisher exact test. AMI indicates acute myocardial infarction; BMI, body mass index; CHD, coronary heart disease; HDL, high-density lipoprotein; IQR, interquartile range; LDL, low-density lipoprotein; and MI, myocardial infarction.

Table 2. Baseline Characteristics of Cytomegalovirus Subgroups (Cohort B)

	AMI (n=28)			Chronic MI (n=26)		
	Cytomegalovirus Negative Median (IQR)	Cytomegalovirus Positive Median (IQR)	*P* Value	Cytomegalovirus Negative Median (IQR)	Cytomegalovirus Positive Median (IQR)	*P* Value
N	14	14		11	15	
Age, median (range)	57.2 (52; 61)	57.9 (50; 60)	0.87	59.9 (50; 64)	63.0 (61; 64)	0.15
Sex, male/female	14/0	14/0	1.0	11/0	15/0	1.0
Previous MI, %	0	7	0.33	100	100	1.0
Ejection fraction, %	42 (32; 47)	52 (43; 59)	0.01	27 (17; 47)	47 (20; 60)	0.38
No. of vessel-disease, 1/2/3	11/2/1	7/5/2	0.29	2/2/7	5/2/8	0.69
Serum creatinine, μmol/L	94 (88; 100)	92 (88; 103)	0.96	95 (78; 110)	108 (97; 113)	0.18
HDL cholesterol, mmol/L	1.1 (0.9; 1.2)	1.1 (1.0; 1.3)	0.44	1.1 (0.9; 1.3)	1.1 (1.0; 1.4)	0.41
LDL cholesterol, mmol/L	4.1 (3.5; 4.8)	3.9 (3.5; 4.3)	0.52	2.4 (1.9; 3.6)	3.1 (2.4; 3.7)	0.27
Triglycerides, mmol/L	1.3 (0.9; 2.0)	1.4 (0.9; 2.4)	0.98	1.9 (1.3; 2.8)	2.3 (1.4; 2.5)	1.0
BMI	28 (26; 31)	27 (25; 34)	0.92	28 (21; 30)	29 (27; 36)	0.1
Diabetes mellitus, %	0	21	0.07	36	40	0.85
Hypertension, %	21	21	1.0	55	67	0.23
Active smokers, %	50	50	1.0	18	7	0.36
Statin treatment, %	7	29	0.16	100	7	<0.0001
Initial troponin I, ng/L	0.5 (0.2; 11.8)	1.0 (0.1; 1.8)	0.66	…	…	NA
Peak troponin I, ng/L	50 (28; 50)	40 (19; 50)	0.18	…	…	NA
Onset-to-balloon time, min	153 (96; 240)	113 (102; 272)	0.68	…	…	NA
Door-to-balloon time, min	25 (17; 35)	19 (16; 29)	0.42	…	…	NA

All indicated *P* values (control vs CHD and cytomegalovirus positive vs cytomegalovirus negative) were calculated by nonparametric *t* test or χ^2 and Fisher exact test. AMI indicates acute myocardial infarction; BMI, body mass index; CHD, coronary heart disease; HDL, high-density lipoprotein; IQR, interquartile range; LDL, low-density lipoprotein; MI, myocardial infarction; and NA, not applicable.

PPCI or routine control coronary angiography, respectively (Table 2). Eighteen healthy male volunteers were enrolled as controls (mean age, 53.5±5.8 years).

For both the study populations, none of the patients were knowingly affected by neoplastic, autoimmune, or chronic infectious disease. All subjects with recent infections were also excluded. The study protocol was approved by the institutional ethical committee of Newcastle University (REC 12/NE/0322, cohort A, and REC 09/H0905/50, cohort B). Written informed consent was obtained from all patients and healthy volunteers.

Blood Collection

Up to 80 mL of peripheral blood was obtained by venopuncture. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation, cryopreserved, and stored at −80°C as previously described.[7]

Enumeration of Peripheral Blood Leukocyte Subsets

Absolute counts of peripheral blood leukocyte subpopulations were determined using 5-color BD TruCount-based flow cytometry assay as described previously.[7]

Determination of Donor Cytomegalovirus Serostatus

Cytomegalovirus status was determined by enzyme-linked immunosorbent assay (CMV IgG Enzyme Immunoassay Kit; GenWay Biotech, San Diego, CA). Cytomegalovirus IgG concentration was measured in stored (−80°C) serum. Cytomegalovirus seropositivity was defined as an IgG Index of >1, as per manufacturer's instructions.

Eleven-Color Flow Cytometry

For Cohort B, high-throughput 11-color flow cytometry assays were performed as previously described.[7] $CD8^+$ T-cell subset counts were calculated as total cells multiplied by percent cells within the subset gate. Gating strategy for 11-color flow cytometry is shown in Online Figure I.

Hierarchical Cluster Analysis of Flow Cytometry Data

After data acquisition, files were exported from FACSDiva software and saved as fcs version 3.0 files. The analyzed parameters were 11-color channels (killer cell lectin-like receptor subfamily G member 1 [KLRG1], CD3, CD28, CCR7, CD45RA, CD57, CD27, CD4, CD8, programmed cell death-1 [PD-1], and Aqua Dye) and 3 to 4 parameters based on forward and side scatters (FS-A, SS-A, SS-H, and [SS-W]). The raw or viable $CD3^+CD8^+$ T-cell pregated data were extracted from.fcs files and imported into R environment, where all subsequent analysis was performed. Compensation matrix (as present in.fcs files) was applied to the data followed by biexponential-like transformation and normalization (*z* score). Hierarchical cluster analysis (HCA) was then performed using our own previously described algorithm.[8] Representative HCA graphical output of the gated viable $CD8^+$ T cells and the heatmaps/dendrograms from all analyzed patients and healthy controls are shown in Online Figures II and III, respectively.

Dextramer Staining

Cytomegalovirus-specific T cells were detected in PBMC samples using a Dextramer CMV Kit (Immudex, Denmark). Patient human leukocyte antigen (HLA) typing was performed by National Health Service Blood and Transplant, Newcastle. Each allele matching the HLA-type of the patient was analyzed separately. Cells were assessed by multiparametric flow cytometry (BD FACS Canto II).

Seven-Color Flow Sorting of $CD8^+$ T Cells

Cell sorting was performed on a BD FACS Aria-II cell sorter. Viable $CD8^+$ T-cell subsets were directly sorted and aliquots spun down and dry stored at −80ºC until DNA isolation.

DNA Isolation and TL Real-Time Polymerase Chain Reaction Assay

DNA was extracted from sorted $CD8^+$ T cells using a QIAamp DNA Mini Kit (Qiagen Ltd, Crawley, United Kingdom). TL was measured by quantitative real-time polymerase chain reaction with modifications as described previously.[9]

Enzyme-Linked–Immunospot Analysis of CD8-Cytomegalovirus–Specific Antigens

PBMCs were isolated and cryopreserved as for dextramer staining. Enzyme-linked–immunospot analysis was carried out as previously described.[10]

IL-7, IL-15, and Interferon-γ ELISA

Serum IL-7 and IL-15 concentration was determined using MSD 96 Multiarray human IL-7, IL-15, and interferon-γ assays on an SECTOR Imager instrument (Meso Scale Discovery) according to manufacturers' protocol.

Th1, Th2, and Th17 Response

Th1, Th2, and Th17 T-cell responses were assessed by measuring the frequencies of interferon-γ–, IL-5–, and IL-17–secreting cells, respectively, using enzyme-linked–immunospot assays.

Proliferation of $CD8^+$ T cells (Ki-67)

Intracellular Ki-67 T-cell staining was performed on whole-blood samples before (0 minutes), at 90 minutes, and 24 hours after reperfusion. Samples were analyzed by flow cytometry (BD FACS Canto II).

T-Cell Apoptosis Studies

For spontaneous apoptosis experiments, PBMCs obtained from STEMI patients before PPCI were incubated in 96-well plates (2×10^5 cells per well) for 16 hours at 37°C. Cells were washed and stained with anti-CD8 and anti-PD-1 monoclonal antibodies, followed by staining with Annexin V and 7-AAD. For PD-1 blocking experiments, PBMCs were cultured in 24-well culture plates (10^6 cells per well). Cells were stimulated with anti-CD3 monoclonal antibody (Mabtech)

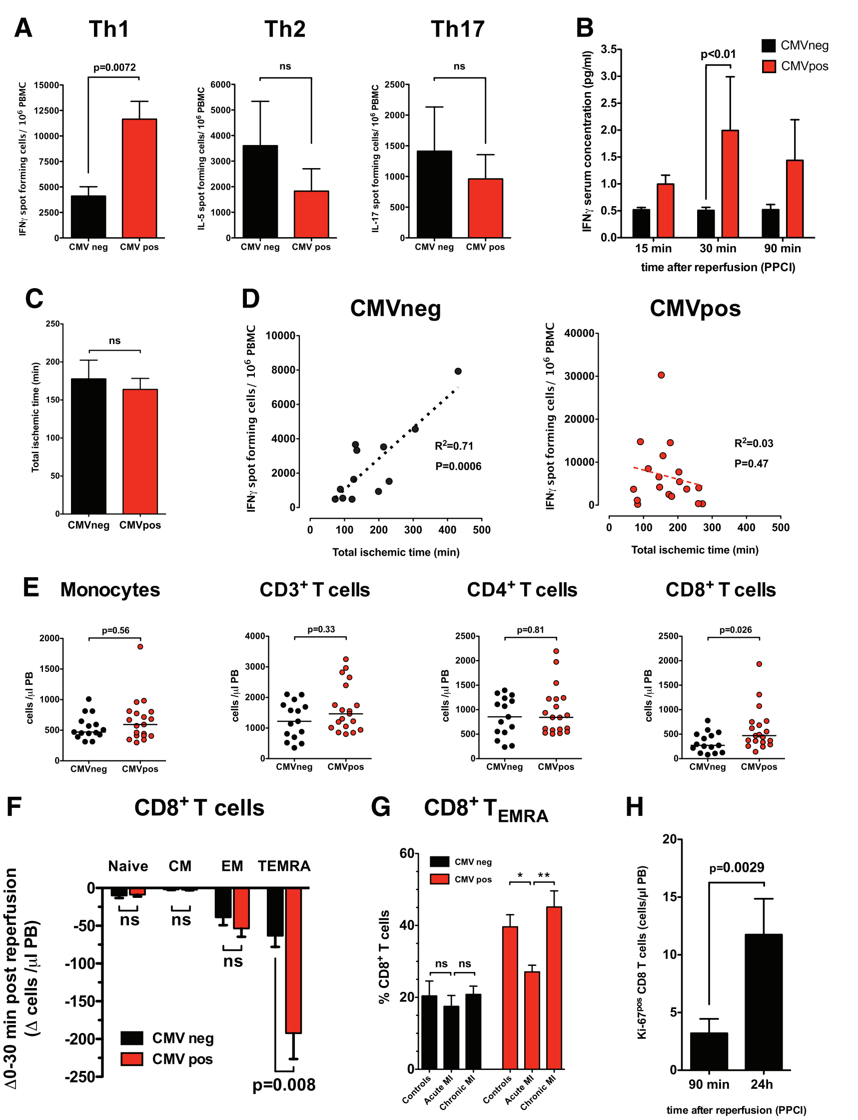

Figure 1. Myocardial ischemia/reperfusion triggers acute depletion of circulating $CD8^+$ effector memory cells in cytomegalovirus (CMV)-seropositive patients. A, Peripheral blood mononuclear cells (PBMCs) from CMV-seropositive patients with acute myocardial infarction (MI) displayed significantly higher T-helper type-1 (Th1) response (interferon [IFN]-γ enzyme-linked–immunospot assay after PHA stimulation) than PMBCs from CMV-seronegative MI patients already before primary percutaneous coronary intervention (PPCI; Th2 response [IL-5], Th17 response [IL-17]). **B**, CMV-seropositive MI patients showed increased serum levels of IFN-γ after reperfusion (intraindividual time course from n=15 patients). **C** and **D**, Magnitude of Th1 response depends on total ischemic time (onset-to-balloon) in CMV-seronegative MI patients. **E**, Absolute numbers of leukocyte populations before reperfusion. **F**, Absolute changes in circulating $CD8^+$ T-cell subpopulations during the first 30 minutes of reperfusion. **G**, Loss of CD8 T_{EMRA} cells 24 hours postmyocardial infarction (acute myocardial infarction), compared with healthy controls and patients with chronic MI (Cohort B). **H**, Increase in proliferating $CD8^+$ T cells 24 hours postreperfusion, as quantified by intracellular staining for Ki-67 and flow cytometry. All bars are mean±SEM. *$P<0.05$; **$P<0.01$. ns indicates not significant.

Table 3. Leukocyte Characteristics of Cohort B

	Healthy Controls	AMI (24 hours)	Chronic MI	*P* Value 1-Way ANOVA
N	18	28	26	
Total leukocyte count, cells/μL	6645±462	12533±757	8312±401	<0.0001
Total granulocyte count, cells/μL	3732±301	9088±659	5692±280	<0.0001
Total monocyte count, cells/μL	474±45	874±62	610±48	<0.0001
Total lymphocyte count, cells/μL	2302±219	2298±167	1885±124	0.13
Total $CD4^+$ T cells, cells/μL	970±78	1077±92	890±64	0.24
Total $CD8^+$ T cells, cells/μL	609±95	458±45	507±60	0.27
CD4/CD8 ratio	1.89±0.15	3.06±0.48	2.38±0.30	0.11
$CD8^+$ NAIVE count, cells/μL	127±21	82±11	108±14	0.12
$CD8^+$ CM count, cells/μL	24±6	75±14	28±4	0.0007
$CD8^+$ EM count, cells/μL	191±33	172±18	139±18	0.27
$CD8^+$ T_{EMRA} count, cells/μL	243±59	108±15	215±41	0.03

AMI indicates acute myocardial infarction; and MI, myocardial infarction.

at 5 μg/mL alone or in the presence of 10 μg/mL of blocking anti-PD-1 monoclonal antibody (eBioscience), for 1 or 4 days. Cells were washed and stained with annexin-V, anti-CD3-FITC, and propidium iodide. PBMCs were analyzed using a BD FACSCanto II cytometer.

Statistical Analysis

Data are reported as mean±SE. Comparison of 2 groups was performed using either the Mann–Whitney *U* test or an unpaired *t* test, if normal probability–probability plots demonstrated approximate normality. Comparison of 3 means was performed by ANOVA, followed by Tukeys' post hoc test. Wilcoxon and Friedman tests were used to compare the means of 2 or 3 matched groups, respectively. Correlation analyses were performed with the use of linear regression and Spearman rank coefficient. $P<0.05$ was considered statistically significant. All statistical tests were performed using GraphPad Prism version 6 for Macintosh (www.graphpad.com).

Results

Myocardial Ischemia/Reperfusion Causes Acute Depletion of CD8 Effector Memory Cells

Seropositive patients displayed a significantly stronger T-helper type-1 (Th1) response ($P=0.0072$ versus cytomegalovirus-seronegative patients; Figure 1A) immediately before reperfusion. Subsequently, we detected a significant rise in systemic levels of interferon-γ, the signature Th1 cytokine, in cytomegalovirus-seropositive patients after reperfusion ($P<0.01$ versus seronegative patients at 30 minutes post-PPCI; Figure 1B). The magnitude of Th1 response correlated with total ischemic time in cytomegalovirus-seronegative patients ($r^2=0.71$; $P=0.0006$; Figure 1C–D). In contrast, Th1 response in cytomegalovirus-seropositive patients was higher and did not correlate with ischemic time ($r^2=0.03$; $P=0.47$; Figure 1C–1D).

Quantification of blood leukocyte subsets before reperfusion revealed significantly increased concentrations only of cytotoxic $CD8^+$ T cells in cytomegalovirus-seropositive patients (median, 470 versus 272/μL; $P=0.026$; Figure 1E). Therefore, we focused on the $CD8^+$ T-cell compartment for more detailed study.

The 4 main subsets of circulating human $CD8^+$ T cells (naive $CD45RA^+CCR7^+$, central memory (T_{CM}) $CD45RA^+CCR7^-$, effector memory (T_{EM}) $CD45RA^-CCR7^-$, and $CD45RA^+$ effector memory (T_{EMRA}) $CD45RA^+CCR7^-$) were sequentially analyzed in STEMI patients after PPCI. Only $CD8^+$ effector memory (T_{EM} and T_{EMRA}) cell concentration fell (−47 and −135 cells/μL, respectively) during the initial 30 minutes after reperfusion. When taking cytomegalovirus status and absolute cell concentrations into account, cytomegalovirus-seropositive patients had a 3-fold greater drop in T_{EMRA} cell concentration than cytomegalovirus-seronegative patients (−192 versus −63 cells/μL; $P=0.008$; Figure 1F). Other T-cell subsets did not differ. At 24 hours postinfarction, absolute cell numbers of $CD8^+$ T_{EM}, but not T_{EMRA}, had fully recovered (Tables 2 and 3). The percentage of $CD8^+$ T_{EMRA} cells 24 hours postinfarction was significantly lower in acute STEMI patients compared with healthy controls or patients with chronic MI. However, T_{EMRA} cells were reduced in cytomegalovirus-seropositive AMI patients only and not in cytomegalovirus-seronegative patients (27% versus 40%; $P<0.05$; Figure 1G). To investigate whether homeostatic proliferation might contribute to the recovery in T_{EM} cells, we quantified IL-7 and IL-15 after reperfusion. Although IL-7 serum levels did not change significantly over time, IL-15 increased from 0.9 to 1.7 pg/mL after 24 hours ($P<0.001$; data not shown). As expected, proliferating $Ki67^+$ CD8 T cells increased from 3 to 12 cells/μL PB between 90 minutes and 24 hours ($P=0.0029$; Figure 1H).

It has been shown previously that primary CMV infection (as measured by seroconversion) may occur lifelong and the rate of seroconversion increases with age, with a peak at the age of 30 to 35 years.[11] The time point of primary infection determines the duration of virus persistence and exposition to viral antigens, which may largely influence the currently measured immune parameters. To address this question, we performed additional analyses of the CD8 T-cell subset changes after reperfusion studying a larger group of cytomegalovirus-seropositive patients with acute STEMI undergoing PPCI. Patients were divided into 2 groups, such as ≥55 years (young, n=12) and >55 years (old, n=25). Interestingly, although there was no difference in infarct size between groups (reflected by peak troponin T serum levels after infarction), younger patients (≤55 years of age) demonstrated a more pronounced loss of senescent CD8$^+$ T$_{EMRA}$ cells (delta %CD8$^+$CD27$^-$ T$_{EMRA}$ 0–24 hours: −35% versus −0.5%; *P*=0.04; Online Figure IV). In fact, CD8$^+$CD27$^-$ T$_{EMRA}$ cells seemed reconstituted in older patients after 24 hours. There was no significant effect of diabetes mellitus on acute changes in the T-cell compartment, as shown in Online Figures V and VI.

Cytomegalovirus-Specific CD8 T$_{EMRA}$ Cells are Persistently Lost After Myocardial Ischemia/Reperfusion

To phenotype the CD8$^+$ T-cell memory subset that was depleted 24 hours after reperfusion, we used a computerized algorithm for 13-parameter polychromatic analysis, followed by HCA in 10 cytomegalovirus-seropositive patients and 10 cytomegalovirus-seropositive controls (Figure 2A and 2B). The algorithm identified 3 CD8$^+$ clusters (naive, T$_{EM}$, and senescent T$_{EMRA}$ cells) that were present in all 20 samples (Figure 2C). Cluster 9, which contained terminally differentiated T$_{EMRA}$ cells was contracted in patients 24 hours post-MI compared with controls (median, 8% versus 23%; *P*=0.0021; Figure 2D). We then compared the frequencies of cells resembling the phenotype of cluster 9 (CD3$^+$CD4$^-$CD8$^+$CD27$^-$CD28$^-$CD57$^+$KLRG1$^+$) in cytomegalovirus-seropositive and cytomegalovirus-seronegative patients, to determine whether this cluster harbored mainly cytomegalovirus-specific cells. We found that CD8$^+$ T$_{EMRA}$ cells of this phenotype were increased ≤90 cells/µL in cytomegalovirus-positive CHD patients after MI, compared with 6 cells/µL in cytomegalovirus-negative patients (*P*<0.0001; Figure 2E). Using conventional gating strategies, we then investigated whether it was only specific subsets of CD8$^+$ T$_{EMRA}$ cells that were lost after MI. Surprisingly, CD27$^+$CD28$^+$ T$_{EMRA}$ cells were unaffected, while CD27$^-$CD28$^-$ T$_{EMRA}$ cells showed the largest decrease compared with controls (11.6% versus 21.9%; *P*=0.009; Figure 3A). To then prove the loss of cytomegalovirus virus–specific memory T cells, we used HLA class I cytomegalovirus–specific dextramers (Figure 3B). The percentage of cytomegalovirus-specific CD8$^+$ T cells that could be detected with individual HLA-specific dextramers ranged from 0.2% to 18% between patients and HLA-specific epitopes (Figure 3C). Furthermore, compared with their relative proportion before reperfusion, we saw a significant decline in cytomegalovirus-specific CD8 T cells to 24% at 90 minutes, 53% at 24 hours, and 54% at 3 months (Figure 3B and 3D). In addition, we found that the number of cytomegalovirus-specific CD8$^+$ T cells did not correlate with the magnitude of memory cells reacting to cytomegalovirus antigen in the enzyme-linked–immunospot assay. This was illustrated by an inverse relationship between dextramer-positive cells and the proportion of functional cells among them (*r*=−0.79; *P*=0.006; Figure 3E).

PD-1$^+$ T$_{EMRA}$ Cells Become Depleted During AMI

Attrition of virus-specific CD8$^+$ T cells has been linked to PD-1 (programmed death-1), which can be upregulated on the surface of memory T cells on activation. We, therefore, determined if PD-1 was responsible for the selective loss of CD8$^+$ T$_{EMRA}$ cells in STEMI patients undergoing PPCI. Costaining of CD8 T-cell populations with CD69 showed that

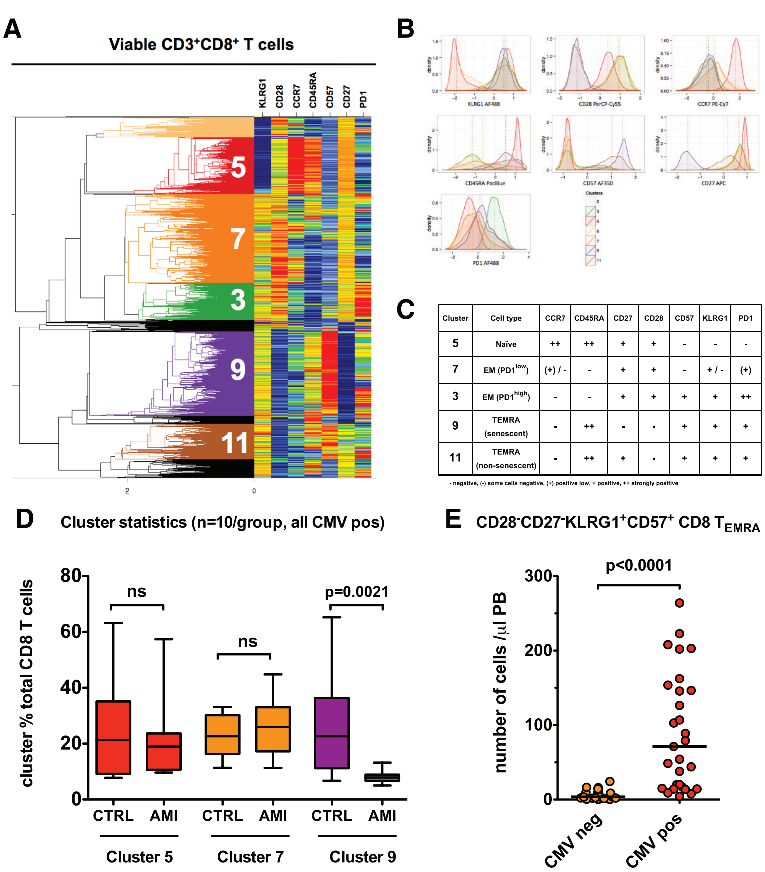

Figure 2. Hierarchical cluster analysis (HCA) of CD8$^+$ T cells in cytomegalovirus (CMV)-seropositive acute myocardial infarction (MI) patients and controls (Cohort B). A, Dendrogram with heatmap-HC of the gated viable CD3$^+$CD8$^+$ T cells acquired from peripheral blood mononuclear cells of healthy control and ST-segment–elevation myocardial infarction patients (24 hours after primary percutaneous coronary intervention). Heatmap shows relative levels of selected parameters (columns) in all gated events (rows) in color coding (blue, low expression and red, high expression). Dendrogram shows the hierarchy of CD8$^+$ T cells based on their similarity in all parameters measured. Colored branches of the dendrogram are selected clusters. **B**, Histogram cluster representation. **C**, Immunophenotypic summary of the selected clusters. **D**, HCA clusters 5, 7, and 9 in CMV-seropositive patients (n=10) and healthy controls (n=10). **E**, CD28$^-$CD27$^-$KLRG1$^+$ CD57$^+$ senescent T$_{EMRA}$ cells are the predominant CD8 effector cell subpopulation in CMV-positive coronary heart disease patients with chronic MI. AMI indicates acute myocardial infarction; CTRL, control; KLRG1, killer cell lectin-like receptor subfamily G member 1; and PB, peripheral blood.

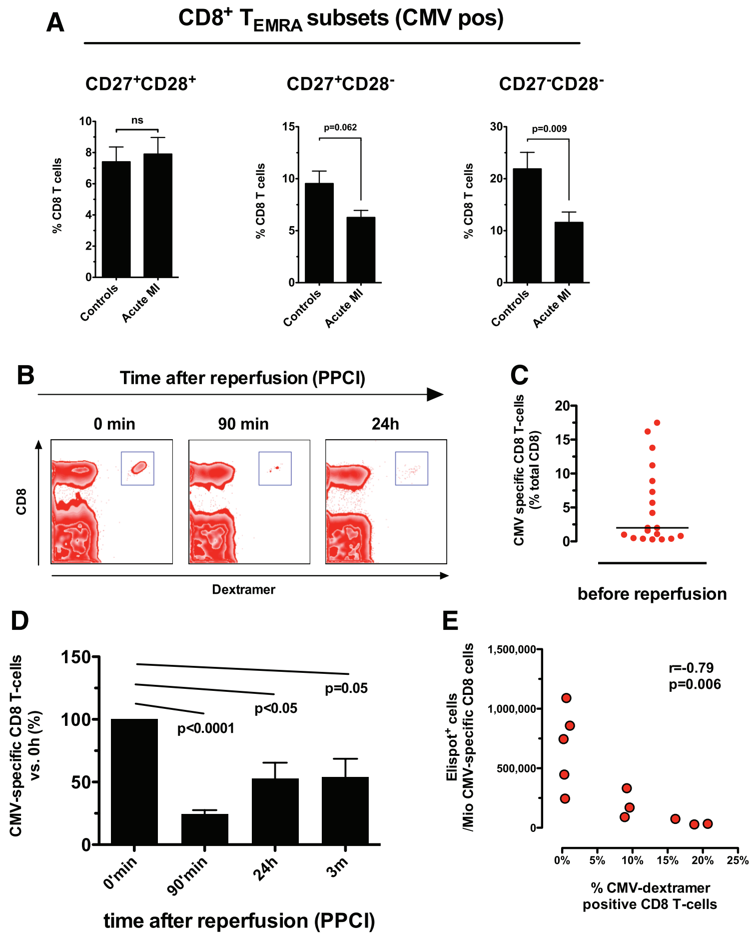

Figure 3. Loss of cytomegalovirus (CMV)-specific cells during reperfusion. A, Conventional gating analysis confirmed specific depletion of the CD28⁻CD27⁻ subset among CD8⁺ T_{EMRA} cells in CMV-seropositive patients. **B**, CMV-specific CD8⁺ T cells are depleted during acute myocardial infarction (MI; human leukocyte antigen class I CMV-specific dextramers). Representative flow cytometry scatter plots show gating of dextramere-positive CD8⁺ T cells in the peripheral blood after reperfusion. **C**, Variability of CMV-specific CD8 T cells in patients undergoing primary percutaneous coronary intervention (PPCI). **D**, Relative changes in the circulating CMV-specific cell counts. Dextramer-positive cells were rapidly depleted during the first 90 minutes and remained significantly decreased after 24 hours and 3 months after PPCI. **E**, Inverse correlation between functionality of CMV-specific cells and number of CMV-specific CD8⁺ T cells in CMV-seropositive patients with acute myocardial infarction (ELISPOT⁺=interferon-γ release after ex vivo stimulation with CMV peptides). ELISPOT indicates enzyme-linked immunospot.

10% of T_{EMRA} cells were activated before reperfusion, as opposed to only 2% of naive cells (*P*=0.016; Figure 4A). PD-1⁺ T_{EMRA} cells were specifically depleted during the first 90 minutes of reperfusion (*P*<0.01; Figure 4B). Twenty-four hours after reperfusion, cytomegalovirus-seropositive patients had a smaller proportion of PD-1⁺ cells among their CD8⁺ T_{EMRA} cells than cytomegalovirus-seronegative patients (23% versus 34%; *P*=0.04; Figure 4C). Accordingly, the percentage of PD-1⁺ cells before PPCI strongly correlated with the relative loss of circulating CD8⁺ T cells within 24 hours after reperfusion (*r*=−0.8; *P*=0.0002; Figure 4D).

PD-1 expression has been associated with a higher susceptibility of CD8 T cells to apoptosis ex vivo.[12] We, therefore, sought to investigate the role of PD-1 in spontaneous and induced apoptosis of T cells from STEMI patients. Our results show that apoptosis sensitivity depends on PD-1–expression level on CD8⁺ T cells (Figure 4E). Accordingly, PD-1^{dim} and PD-1^{high} cells displayed significantly higher susceptibility to spontaneous apoptosis ex vivo, when compared with PD-1^{neg} cells (*P*<0.05 for % early apoptosis PD-1^{dim} versus PD-1^{neg}, and *P*<0.001 for % late apoptosis PD-1^{high} versus PD-1^{neg} CD8⁺ T cells, accordingly; Figure 4F–4H). Moreover, ex vivo PD-1 blockage significantly reduced the rate of CD3-induced apoptosis in T cells from cytomegalovirus-positive patients (32% versus 16%; *P*=0.03; Figure 4I). We further tested PD-1 expression levels on cytomegalovirus-specific CD8⁺ T cells (Figure 4J). Here, cytomegalovirus dextramer–positive cells showed a higher mean percentage of PD-$1^{med/high}$ subsets than dextramer-negative CD8⁺ T cells (*P*=0.04; Figure 4K). STEMI patients with a higher percentage of PD-1⁺ cytomegalovirus-specific cells before reperfusion displayed a significantly higher loss of CD8⁺ T_{EMRA} cells within 24 hours post-PPCI (*P*=0.02; Figure 4L).

Although selective loss of PD-1⁺ cells during reperfusion (via apoptosis) might in part explain PD-1 deficiency in CD8 memory compartments, it might also indicate a limited ability of cytomegalovirus-seropositive patients to suppress T-cell responses in a PD-1–dependent manner. We, therefore, determined the immunophenotypic characteristics of PD-1^{pos} and PD-1^{neg} CD8⁺ effector memory cell subsets (Figure 5A). Here, we observed that PD-1^{neg} T_{EMRA} cells from cytomegalovirus-seropositive patients showed no significant difference in the expression of differentiation and senescence markers CD27, CD28, CD57, and KLRG1 when compared with cytomegalovirus-seronegative patients (Figure 5B).

Accelerated Telomere Shortening and Increased KLRG1 Expression in CD8 T_{EMRA} Cells in Chronic MI Patients

Because T_{EMRA} cells seemed to have been restored in patients with chronic MI, we wanted to know if they were more senescent compared with at the time of AMI. For this, we specifically looked at the coexpression of CD57 and KLRG1 in CD8 subpopulations. Although naive CD8⁺ cells expressed both the

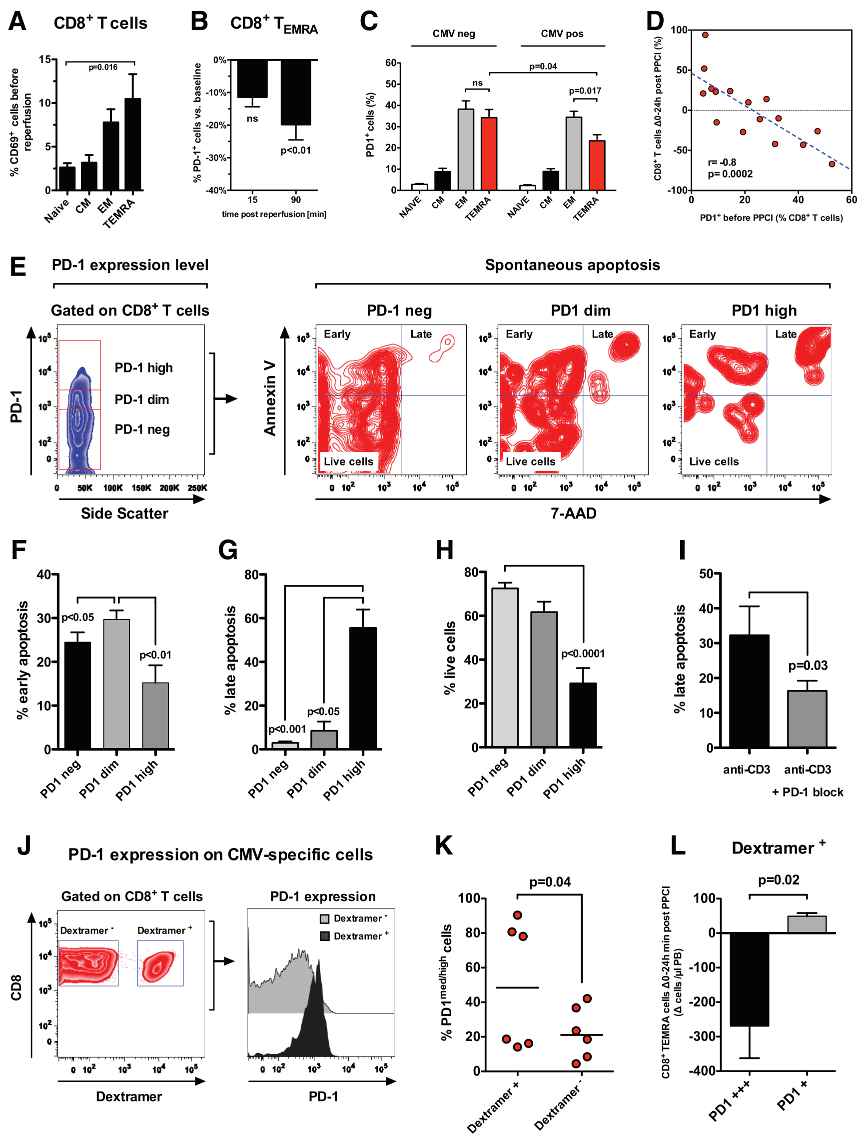

Figure 4. Loss of PD-1⁺ effector memory cells during reperfusion. A, FACS analysis of activated CD8 T-cell populations (CD69 staining) before reperfusion. **B**, Loss of PD-1⁺ CD8 T_{EMRA} cells during the first 90 minutes of reperfusion. **C**, Percentage of PD-1⁺ cells among T_{EMRA} cells is significantly lower in cytomegalovirus (CMV)-seropositive patients after 24 hours after reperfusion. **D**, Percentage of PD-1⁺ cells before primary percutaneous coronary intervention (PPCI) correlates with a relative loss of CD8⁺ T cells during 24 hours after reperfusion. **E**, Expression level of programmed cell death-1(PD-1) (left panel) determines susceptibility toward spontaneous ex vivo apoptosis of CD8⁺ from ST-segment–elevation myocardial infarction patients before reperfusion. **F–H**, Percentages of early (Annexin-V⁺/7-AAD⁻) and late apoptosis (Annexin-V⁺/7-AAD⁺), and live cells (Annexin-V⁻/7-AAD⁻) among $PD1^{neg}$, $PD1^{dim}$, and $PD1^{high}$ CD8⁺ T cells (n=10 pts.). **I**, Blockage of PD-1 inhibits CD3-induced late apoptosis (Annexin-V⁺/PI⁺) of T cells from CMV-seropositive patients (n=6, before PPCI). **J** and **K**, CMV-dextramer-positive CD8⁺ T cells display higher PD-1 expression than dextramer-negative population. **L**, Higher PD-1 expression on dextramer-positive cells determines loss of CD8 T_{EMRA} cells during the 24 hours after reperfusion (PD-1⁺⁺⁺–high percentage of PD-1–positive cells and PD-1⁺–low percentage of PD-1–positive cells). CM indicates central memory; EM, effector memory; and PI, propidium iodide.

markers at low level, all T_{EMRA} cells and the majority of T_{EM} cells expressed high levels of KLRG1 (Figure 6A). However, CD57, which also reflects the proliferative history of cells, was specifically upregulated in T_{EM} cells of patients 24 hours after myocardial infarction. In T_{EMRA} cells, KLRG1 expression was highest in patients with chronic MI (Figure 6A). To quantify mean telomere length in different CD8⁺ subsets, we used 7-color flow cytometric cell sorting, followed by TL real-time polymerase chain reaction (Figure 6B). TL of CD8⁺ T cells in cytomegalovirus-seropositive individuals declined from healthy controls to patients with AMI and chronic MI (3397 versus 2814 versus 2225 bp; $P<0.001$; Figure 6C). This was mainly attributed to chronic patients with previously large heart attacks, complicated by congestive heart failure (Figure 6C). Finally, we measured TL of effector memory populations (CD8⁺ T_{EM} and T_{EMRA} cells) in relation to the same patients' naive cell TL, the latter serving as an intraindividual baseline for the longest telomeres of any CD8 subset. TL was ≈1000 to 1400 bp shorter in T_{EM} cells between controls and chronic MI patients ($P=0.21$), but significantly shorter in T_{EMRA} cells from patients with chronic MI (2000 versus 600 bp; $P=0.0015$; Figure 6D).

Discussion

In this study, we demonstrate the depletion of circulating CD8 memory T cells after AMI and reperfusion. These quantitative changes seem to be reversible for all but terminally differentiated memory (T_{EMRA}) cells in cytomegalovirus-seropositive patients. Our results suggest that PD-1, the programmed death protein-1, might be involved in the persistent loss of T_{EMRA} cells in cytomegalovirus-seropositive patients. We have used 3 different methods to confirm the specific depletion of T_{EMRA} cells, which have previously been shown to harbor the majority of cytomegalovirus-specific cytotoxic cells.[13] Polychromatic flow cytometry identified a reduction in CD27⁻CD28⁻ double negative CD8 T_{EMRA} cells during AMI, and HCA yielded a selective depletion of CD27⁻CD28⁻CD57⁺KLRG1⁺ CD8⁺ T_{EMRA} cells. Finally, by using HLA type I-specific dextramers, we found a reduction in cytomegalovirus-specific cytotoxic T cells, which persisted between 24 hours and 3 months after MI.

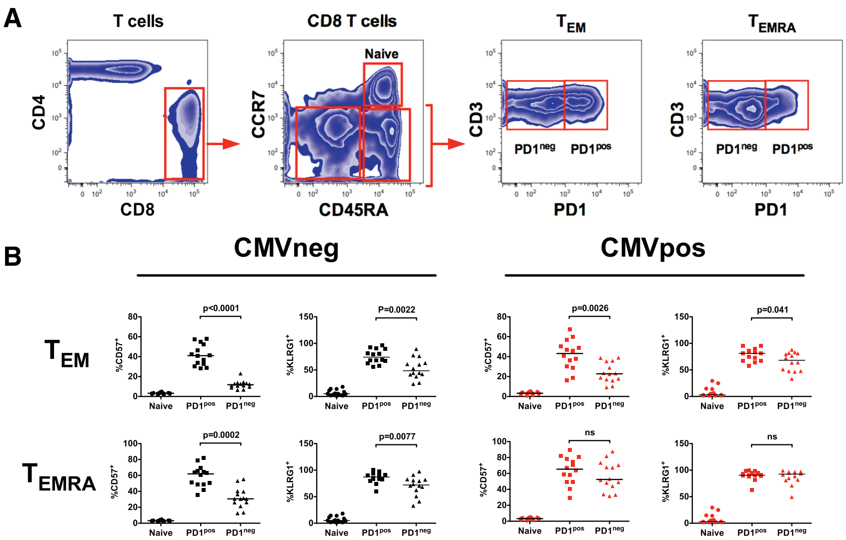

Figure 5. A, Representative gating strategy of PD-1^{pos} and PD-1^{neg} CD8 T_{EMRA} cells (CCR7^{neg}CD45RA^{pos}). Naive CD8^{+} T cells (CCR7^{pos}CD45RA^{pos}) are shown as an intraindividual reference. **B**, Phenotypic analysis of PD-1^{pos} and PD-1^{neg} CD8 T_{EMRA} subsets from cytomegalovirus (CMV)-seropositive and CMV-negative patients with acute myocardial infarction (24 hours after primary percutaneous coronary intervention; Cohort B). PD-1 indicates programmed cell death-1.

Although age seemed to have an effect on T-cell recovery by 24 hours (Online Figure IV), it is important to emphasize that the relative changes observed within 24 hours after reperfusion might indeed not reflect the cumulative effects of ischemia/reperfusion. This is most probably because lymphocyte counts at the time point of PPCI cannot be considered as a true baseline after several hours of already ongoing ischemia. Therefore, our additional cross-sectional analysis (AMI versus chronic MI) is a potentially more objective visualization of the CD8^{+} T_{EMRA} contraction and absolute T-cell losses in the course of AMI and reperfusion.

The implications of these findings are 2-fold: First, the loss of cytomegalovirus-specific memory cells may diminish the antiviral T-cell potential, enhancing latent reactivation of the virus and sustaining low-grade chronic inflammation. Second, cytotoxic T_{EMRA} cells could enhance myocardial necrosis or delay infarct healing through direct migration into peri-infarct tissue.[14]

Acute Stress–Induced Redistribution of T-Lymphocytes

A short-term decrease in circulating CD8 T-lymphocytes may reflect trafficking of cells out of the bloodstream and into different target organs, including the spleen, lung, lymph nodes, or the site of inflammation.[15] There are 2 major signaling pathways that have been shown to be involved into T-lymphocyte migration and egress. First, migration of T cells is influenced by the sphingosine-1 phosphate pathway.[16] Lymphocyte egress from lymph nodes requires the S1P1 receptor, and the transmembrane C-type lectin CD69 inhibits S1P1 chemotactic function and leads to downmodulation of S1P1.[17] Second, epinephrine has been shown to cause acute demargination of T-lymphocytes by suppressing adhesive fractalkine signaling.[18] The receptor for fractalkine, CX3CR1, is mainly expressed in cytotoxic CCR7- or CD62L-negative T-lymphocytes, coinciding with the egressing cell populations in our study.[18] In our opinion, fractalkine signaling could be a promising target to investigate the role of CD8 T cells in ischemia/reperfusion in the future.

Role of PD-1 in Persistent Loss of cytomegalovirus-Specific T Cells

PD-1 is an inhibitory surface receptor, which is upregulated on memory T cells on activation.[19] PD-1 is known to inhibit CD8^{+} T-cell effector functions by dampening T-cell responsiveness to antigenic stimulation.[20] PD-1 expression has also been linked to a proapoptotic phenotype of CD8^{+} T cells.[21] Our data show that in CD8^{+} T cells from patients with acute STEMI spontaneous apoptosis occurs mainly in cells expressing high levels of PD-1. In addition, blocking PD-1 could reduce the ratio of induced CD8^{+} T-cell apoptosis ex vivo. We, therefore, questioned whether PD-1 expression on effector memory T cells could be associated with the acute inflammatory response and CD8^{+} T-cell loss in STEMI patients undergoing PPCI. We found that 24 hours after reperfusion, cytomegalovirus-seropositive patients displayed significantly lower proportions of PD-1^{+} CD8 T_{EMRA} subsets in comparison with cytomegalovirus-seronegative patients. Furthermore, CD8 T_{EMRA} cells showed the highest amount of activation, as evidenced by CD69 staining, and the PD-1^{+} subpopulation were specifically depleted during the first 30 minutes of reperfusion. This loss was not secondary to increased senescence, as documented by similar CD27, CD28, KLRG-1, and CD57 expression in PD-1–positive versus PD-1–negative subsets.[22] Altogether, these findings are highly suggestive of a specific role of PD-1 in the permanent loss of T_{EMRA} and cytomegalovirus-specific cells during MI. Indeed, Zhang et al[23] have recently demonstrated that upregulation of PD-1 during acute viral infection is involved in the attrition of cytomegalovirus-specific cells. Accordingly, PD-1 has been shown in mouse models to limit T-cell responses in cardiomyocytes and in the arterial wall, thereby protecting from atherosclerosis and myocarditis.[24,25]

Coronary Artery Disease and Immunosenescence

There is an increasing evidence for continuous immune activation in CHD patients, characterized by accumulation of immunocompetent cells in the arterial lesion and elevated levels of inflammatory markers in the circulation.[26] A cross-sectional

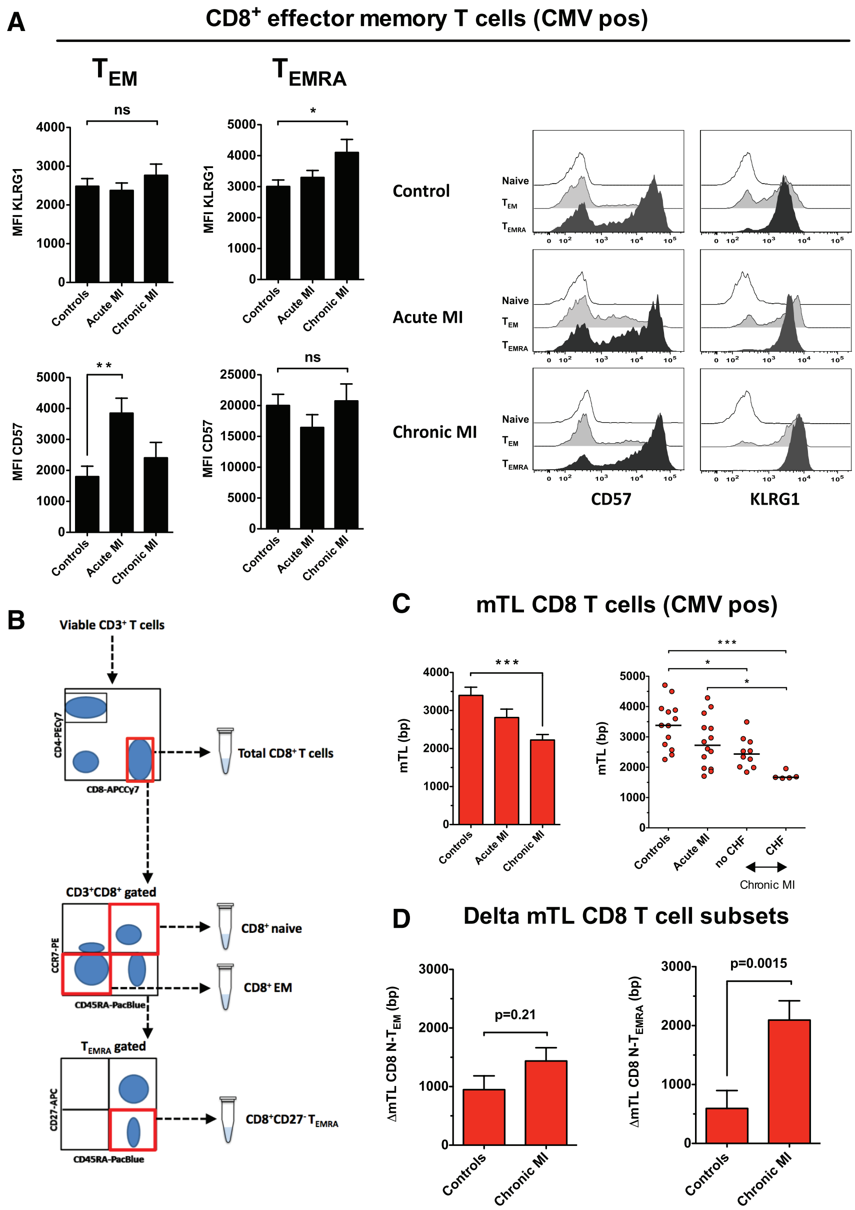

Figure 6. Accelerated immunosenescence in cytomegalovirus (CMV)-seropositive patients with chronic myocardial infarction (MI; Cohort B). A, Expression analysis of KLRG1 and CD57 in T_{EM} and T_{EMRA} cells (MFI, mean fluorescence intensity). Representative histograms show the expression of KLRG1 and CD57 on T_{NAIVE}, T_{EM}, and T_{EMRA} CD8+ cells from healthy donors, acute myocardial infarction, and chronic MI patients. **B–D**, Telomere length (TL) analysis in sorted CD8+ T-cell subpopulations: (**B**), Schematic representation of the flow sorting strategy for CD8+ T-cell subsets; (**C**) Telomere shortening in chronic MI is present in CD8+ T cells, especially in patients with larger infarcts and severely reduced left ventricular function (congestive heart failure). Mean telomere length (mTL) in total CD8+ T cells is shown for each cohort; (**D**) Accelerated telomere shortening in CD8+ T_{EMRA} cells occurs in chronic MI. TL of naive CD8+ T cells was chosen as an internal standard and intraindividually compared with mTL of T_{EM} and T_{EMRA} cell populations, rendering $\Delta TL = TL_X - TL_{CD8naive}$ (telomeric gap). **P*<0.05; ***P*<0.01; ****P*<0.001. KLRG1 indicates killer cell lectin-like receptor subfamily G member 1; and ns, not significant.

study in >7700 participants found that cytomegalovirus seropositivity was significantly associated with a history of cardiovascular disease.[2] A further longitudinal study has shown a significant increase in CHD-related mortality in cytomegalovirus positive, but otherwise healthy individuals over the course of 17 years.[3] This translated into shortening of lifespan by ≈3.7 years. We have also found a significant increase in CHD among cytomegalovirus-seropositive participants in the Newcastle 85+ study (Spyridopoulos et al, manuscript in preparation), in which we investigated 751 participants of the same age group (85 years). Interestingly, the presence of senescent CD4 and CD8 memory T cells was an independent predictor of mortality even in this age group. We have previously demonstrated that cytomegalovirus-seropositive patients with chronic myocardial infarction have increased telomere shortening in their CD8 T-cell compartment when compared with cytomegalovirus-seropositive healthy controls, indicating differences in the pathobiology of cytomegalovirus-driven immunosenescence in patients with CHD.[5,27] This effect was more pronounced in patients with more extensive infarction and more severely impaired left ventricular function. This could be explained by ≥3 reasons: (1) genetic differences, leading to better control of the virus in patients without CHD, such as is seen in offspring from longevity families, (2) underlying inflammation as present in atherosclerosis, driving latent cytomegalovirus infection, and (3) changes in the CD8 compartment that are related to AMI, particularly in younger cytomegalovirus-seropositve MI patients, as seen in our present study. Another interesting aspect in our analysis was that the proportion of functional cytomegalovirus-specific cells, as indicated by spot forming cells in response to cytomegalovirus antigen, declined sharply with the total number of dextramer-positive cytomegalovirus-specific cells. This suggests that patients with a large cytomegalovirus-specific T-cell repertoire actually still contain relatively fewer cells that can control the latent infection. It is, therefore, conceivable that the lack of effective virus control in cytomegalovirus-seropositive patients instigates further low-grade inflammation in the arterial wall, accelerating atherosclerosis.[28]

Conclusions

Our results suggest that the temporary loss of cytomegalovirus-specific cells in patients after MI diminishes the potential for the host to contain latent cytomegalovirus-infection, leading to homeostatic proliferation of T cells to replenish effector memory subsets.[29] This proposed mechanism could explain accelerated CD8 T-cell immunosenescence in cytomegalovirus-seropositive patients with MI, as well as progression of atherosclerosis. In light of an aging population and cytomegalovirus prevalence of up to 90% in the elderly, future strategies to delay or reverse T-cell immunosensecence, such as early vaccination against cytomegalovirus or pharmacological interventions aimed at direct deanergising memory T cells[30–32] but also adjuvant, anti-inflammatory, age-decelerating treatment options, including statins might prove beneficial for patient outcome in older patients.[33,34]

Limitations

The main limitation of our study is the lack of longitudinal data (beyond 4 months) in the same patient with MI. Furthermore, because of relative low number of patients within the particular study groups, only limited subanalyses of the effect of diabetes mellitus and age on the observed acute and chronic changes within the T-cell compartment after reperfusion could be performed. Finally, the value of our observations with respect to potential therapeutic interventions in cytomegalovirus-seropositive CHD patients remains uncertain. Future prospective studies are needed to prove the value of T-cell senescence as a predictor of adverse outcome in these patients and identify potential cellular targets for immunomodulatory interventions.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- Infection with cytomegalovirus is never cleared from the human body and leads over time to an aged immune system (immunosenescence), which eventually contributes to chronic low-grade inflammation.
- Chronic infection with cytomegalovirus leads to shorter life expectancy, mainly because of an increase in acute myocardial infarction.
- In cytomegalovirus -seropositive patients with previous myocardial infarction lymphocytes age faster than in those without coronary heart disease.

What New Information Does This Article Contribute?

- CD8 lymphocytes temporarily decrease by >40% in the peripheral blood after reopening of the blocked coronary artery in patients with acute myocardial infarction.
- CD8 memory lymphocytes that are directed against cytomegalovirus are selectively depleted from the blood for >24 hours, most probably because of programmed cell death (apoptosis) via programmed cell death-1 signaling.
- This triggers reconstitution of cytomegalovirus-specific cells and accelerates immunosenescence.

Chronic infection with cytomegalovirus affects the majority of the population in Western countries and is thought to instigate chronic low-level inflammation. Cytomegalovirus infection is also associated with an increase in the incidence of coronary heart disease. We have found that acute myocardial infarction with subsequent reperfusion of the infarcted tissue by stent insertion triggers a temporary decrease of all CD8 T-lymphocytes as well as a more persistent loss of cytomegalovirus-specific memory lymphocytes, most probably through apoptotic cell death via the programmed cell death-1 receptor. This leads to reactivation of the adaptive immune system with accelerated proliferation and aging of memory lymphocytes, promoting a vicious circle where aged immune cells can lead to even further inflammation and, therefore, faster progression of underlying atherosclerosis. These findings highlight the need for immune-specific interventions in cytomegalovirus-seropositive patients with acute myocardial infarction, such as anti-inflammatory drugs that can reverse aging of memory lymphocytes.