Clinical Investigation and Reports

Heat-Shock Protein 60–Reactive CD4⁺CD28null T Cells in Patients With Acute Coronary Syndromes

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Background—CD4⁺CD28null T cells are present in increased numbers in the peripheral blood of patients with acute coronary syndrome (ACS) compared with patients with chronic stable angina (CSA). The triggers of activation and expansion of these cells to date remain unclear.

Methods and Results—Twenty-one patients with ACS and 12 CSA patients with angiographically confirmed coronary artery disease and 9 healthy volunteers were investigated. Peripheral blood leukocytes were stimulated with human cytomegalovirus (HCMV), Chlamydia pneumoniae, human heat-shock protein 60 (hHSP60), or oxidized LDL (ox-LDL). CD4⁺CD28null cells were separated by flow cytometry and assessed for antigen recognition using upregulation of interferon-γ and perforin mRNA transcription as criteria for activation. CD4⁺CD28null cells from 12 of 21 patients with ACS reacted with hHSP60. No response was detected to HCMV, C pneumoniae, or ox-LDL. Incubation of the cells with anti–MHC class II and anti-CD4 antibodies but not anti–class I antibodies blocked antigen presentation, confirming recognition of the hHSP60 to be via the MHC class II pathway. Patients with CSA had low numbers of CD4⁺CD28null cells. These cells were nonreactive to any of the antigens used. Circulating CD4⁺CD28null cells were present in 5 of the 9 healthy controls. None reacted with hHSP60.

Conclusions—We have shown that hHSP60 is an antigen recognized by CD4⁺CD28null T cells of ACS patients. Endothelial cells express hHSP60 either constitutively or under stress conditions. Circulating hHSP60-specific CD4⁺CD28null cells may, along other inflammatory mechanisms, contribute to vascular damage in these patients. (Circulation. 2004;109: 1230-1235.)

Key Words: angina ■ lymphocytes ■ antigens

Patients with unstable angina (UA) but not chronic stable angina (CSA) experience expansion of a subset of CD4⁺ T cells that lack the CD28 marker.1 These CD4⁺CD28null cells, which in UA may constitute up to 50% of the total CD4⁺ compartment, express killer immunoglobulin-like receptors, a characteristic of natural killer cells, and have cytolytic function releasing perforin on activation.2 Their ability to produce high levels of interferon (IFN)-γ and perforin mRNA transcription as criteria for activation.2 Their ability to produce high levels of interferon (IFN)-γ together with the finding that CD4⁺CD28null cells can be isolated from ruptured unstable atherosclerotic plaques, supports the notion that alongside other proinflammatory mechanisms, they may have a role in the events leading to plaque destabilization and acute coronary syndromes (ACS).2 The triggers of activation and expansion of CD4⁺CD28null cells to date remain unclear, although restricted T-cell receptor-β usage points to stimulation by a specific antigen.3

Several antigens, in particular Chlamydia pneumoniae, human cytomegalovirus (HCMV), oxidized LDL (ox-LDL), and human heat-shock protein-60 (hHSP60), have been implicated in the pathogenesis of coronary artery disease.4–8 Persistence of these antigens is considered instrumental in the initiation of inflammatory responses within the coronary arteries via activation of either macrophages or humoral and T cell–mediated response to stimulation. Previous studies have shown that T lymphocytes reactive to C pneumoniae as well as chlamydial and hHSP60 and ox-LDL are present within atherosclerotic tissue.6–9 The objective of the present study was to investigate whether any of these antigens act as targets for the CD4⁺CD28null cells. An understanding of antigen-mediated emergence and expansion of these cells may provide an invaluable insight into a possible contributory pathway of plaque destabilization.

Methods

Patients Studied
Thirty-three patients (18 men) admitted to St George’s Hospital for assessment of angina chest pain were assessed. Of these, 12 had CSA and 21 ACS. CSA was defined as typical exertional chest pain

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Peripheral blood mononuclear cells (PBMCs) were separated by use of Lymphoprep (Nycomed Pharma AS) and resuspended in RPMI 1640 containing 10% human AB serum, 1% penicillin/streptomycin, and 1% L-glutamine. Antigen stimulation was performed with 1 mL of the cell suspension containing 4×10^6 cells per antigen. The optimum concentrations of antigens to be used were predetermined to be as follows: 10 μg/mL of purified whole HCMV antigen (Abingdon Oxon), 10 μg/mL of C pneumoniae elementary bodies (University of Washington Department of Pathobiology), 5 μg/mL of hHSP60 (Bioquote UK), and 10 μg/mL of LDL (Biodesign USA) oxidized according to Stemme et al.\(^\text{6}\) Because hHSP60 is known to be contaminated with lipopolysaccharides (LPS), Escherichia coli LPS (Sigma) at concentrations ranging from 0 to 640 pg/mL, equivalent to 4 times the level of LPS present within the hHSP60 preparation, was used as control. The cells were incubated overnight at 37°C and 5% CO\(_2\) followed by a wash in RPMI and maintenance culture in a further 24 hours. The 48-hour culture ensured that transient downregulation of the CD28 marker after antigen encounter would not interfere with the separation of the CD4^+/-CD28^- cells. Positive and negative controls included phytohemagglutinin (PHA)-stimulated PBMCs (10 μg/mL) and unstimulated cells, respectively.

The cells were subsequently washed in PBS containing 1% FCS and labeled with phycoerythrin-conjugated anti-CD4 antibodies (Becton Dickinson) and fluorescein isothiocyanate (FITC)-conjugated anti-CD28 antibodies (Becton Dickinson) for 30 minutes on ice. Isotype-matched monoclonal antibodies were included as controls in the assays. The CD4^+/-CD28^- as well as the CD4^+/-CD28^+ populations were sorted using a MoFlo High speed Cell Sorter (DakoCytometry).

**Reverse-Transcriptase Polymerase Chain Reaction**

Cellular RNA was extracted with Trizol reagent and 20 μg glycogen followed by cDNA synthesis with Superscript II reverse transcriptase and random hexamers (Life Technologies). Amplification of IFN-γ and perform mRNA was performed with 1 μg of cDNA and AmpliTag Gold polymerase with a 40-cycle amplification protocol and annealing temperatures of 55°C (Perkin Elmer). The primers used for IFN-γ were 5’-AGTTATATCTGCGTTCTTA-3’ and 5’-ACCGAATAATTACGTGC-3’,\(^\text{12}\) Perforin primers were as follows: 5’-GTCGCTCTTCGCGCATCTCC-3’ and 5’-CGGGGGAGTGGTGAACCATGGAAA-3’,\(^\text{13}\)

The CD4^+/-CD28^- phenotype was further confirmed by reverse-transcriptase polymerase chain reaction (RT-PCR) analysis of mRNA for the CD4 and CD28 markers using primers for CD4 (5’-TGGCTTGGAACCTCACACT-3’ and 5’-GACACTGGCAAG-TCTCTCTC-3’) and CD28 (5’-GTTGTAAGGCTCTGATGATT-3’ and 5’-GCGGCTACTGCTGTACAAATC-3’).\(^\text{14,15}\)

**Blocking MHC Class I and II Antigen Presentation**

MHC-restricted antigen presentation was assessed by incubating the PBMCs with blocking antibodies for 40 minutes at room temperature before antigen stimulation. The antibodies used were 10 μg/mL of anti–human MHC class I (HLA A, B, and C; Dako), anti–human CD4, anti-human MHC class II (HLA DR, DP, DQ), or a combination of both anti–CD4 and anti–human class II antibodies (Becton Dickinson). Stimulation with hHSP60 was subsequently performed as described above. Cells blocked with isotype control antibodies as well as PHA-stimulated and unstimulated cells were included as controls.

**Enzyme-Linked Immunosorbent Assay**

A sandwich enzyme-linked immunosorbent assay (ELISA) was used to measure circulating levels of hHSP60 using monoclonal anti-hHSP60 antibody II-13 as a capture reagent\(^\text{16}\) and biotinylated antibody ML-30 (Autogen Bioclear-Stressgene)\(^\text{16}\) for detection of the bound hHSP60. Peroxidase-conjugated streptavidin and subsequently 2,2’-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (Sigma) were used as detection reagents. The detailed methodology has been described previously.\(^\text{16}\)
Categorical data were analyzed with a 2-tailed t test for unpaired observations. Results were analyzed and presented as mean ± SD for continuous variables and as percentages for categorical data. Continuous variables were analyzed with 2-tailed t test for unpaired observations. Categorical data were analyzed with a χ² test.

Results

Twelve of the 21 patients with ACS had CD4 CD28null cells reactive to hHSP60 and produced INF-γ and perforin mRNA in response to antigenic challenges. Representative results from a patient with ACS are shown in Figure 1, A and B. Eight of the patients with HSP-reactive cells had UA, 3 had ST-segment–elevation AMI, and 1 non–ST-segment–elevation AMI. No signals were detected in the CD4 CD28null cell fraction obtained from patients with ACS after stimulation with HCMV, C pneumoniae, or ox-LDL. To reaffirm nonreactivity of the CD28null fraction with ox-LDL, a series of dilutions of the antigen ranging from 0 to 10 µg/mL were also used (data not shown). No response to ox-LDL was observed in the CD28null population. Because patients with CSA had low numbers of CD4 CD28null cells, larger starting volumes of PBMCs were used in experiments to obtain sufficient cDNA quantities for PCR amplification. Despite this, no IFN-γ or perforin mRNA was detectable in the antigen-stimulated CD4 CD28null cells in CSA patients. Response was observed in the presence of PHA, a T-cell mitogen that was used as a positive control. Representative data are shown in Figure 1, D and E. Amplification of the CD4 but not CD28 mRNA confirmed the phenotype of these cells to be CD4 CD28null (data not shown).

To determine whether the ACS and CSA patients had circulating T helper cells of the CD4 CD28null phenotype, capable of responding to any of the antigens, the CD4 CD28null population was also evaluated. HCMV-, ox-LDL-, and hHSP60-reactive T helper cells were found in all patients tested, whereas 2 individuals did not respond to C pneumoniae and HCMV. Representative data from 2 patients, 1 with ACS and the other with CSA, are shown in Figure 1, C and F. Responsiveness of the CD4+ population confirms previous exposure of these patients to the antigens used and suggests that the sensitivities of the assays are such that reactive CD4+ cells can be identified. The selectivity of response to hHSP60 in these experiments was found to be limited to the CD4 CD28null population.

PBMCs obtained from 9 healthy controls were also exposed to hHSP60. Circulating CD4 CD28null cells were found in 6 individuals. None of these reacted with hHSP60 (χ²=5.32, significant at 2.5% level). PHA responsiveness confirmed the integrity of the cells (data not shown).

Incubation of the PBMCs taken from ACS patients with anti–MHC class II and anti-CD4 antibodies either alone or in combination blocked antigen presentation and IFN-γ mRNA transcription. Anti–MHC class II antibodies did not reduce the level of IFN-γ mRNA levels, confirming that recognition of the hHSP60 peptides occurs via the MHC class II pathway. When the PBMCs were incubated with isotype control antibodies, no blocking effect was observed. This suggested that anti–class II and anti-CD4 antibodies blocked antigen presentation specifically (Figure 1G). In addition, anti–MHC class II antibodies failed to block IFN-γ or perforin mRNA production after PHA stimulation (data not shown).

The hHSP60 used in the present study is known to be contaminated with bacterial LPS at 32 pg/µg of hHSP. To confirm the specificity of the CD4 CD28null cells, PBMCs were exposed to LPS at concentrations ranging from zero to 4 times the level present in the culture medium containing recombinant hHSP60 (0 to 640 pg/mL). No response to LPS was observed in the CD4 CD28null component of the PBMCs (Figure 2). This was a consistent and reproducible finding. As expected, the CD4 CD28+ population reacted positively to the antigen.

No correlation was observed between the presence of diabetes, hypercholesteremia, hypertension, smoking, or family history of ACS and hHSP60-reactive CD4 CD28null cells. In addition, the use of nitrates, β-blockers, calcium antagonists, aspirin, lipid-lowering drugs, ACE inhibitors, and
These proteins in atheromatous lesions, correlating with the severity of atherosclerosis, is consistent with a focal role that HSPs may play in the pathogenesis of the disease. This, combined with a growing body of evidence that suggests that risk factors commonly associated with atherosclerosis, such as infectious agents and ox-LDL, are capable of inducing HSPs, points to a link between atherogenic agents and these proteins.

The finding of CD4+CD28null cells reactive to hHSP60 during the acute phase of coronary artery disease and their absence in stable CSA and healthy individuals is of critical importance and suggests that an autoimmune T cell–mediated response may hold the balance. Although previous studies have reported the presence of HSP-reactive T cells within atheromatous lesions, this is the first investigation into antigenic specificity of the CD4+CD28null cells. The mechanism of emergence and outgrowth of these cells is unknown. Studies in rheumatoid vasculitis have shown a decline in both nuclear protein binding motifs α and β within the CD28 minimal promoter, which controls gene expression, suggesting that these cells emerge from chronic persistent in vivo hHSP60 stimulation of the CD4+CD28null cells. This pattern is different from CD28 loss by replicative senescence, in which only β-binding promoter motif is downregulated. Evidence to support the role of antigenic stimulation in exacerbated loss of the CD28 expression has also been presented in infections with the human immunodeficiency virus as well as Trypanosoma cruzi in Chagas’ disease. It is possible that in vivo hHSP60 stimulation of the CD4+CD28 null cells in patients with ACS may account for the loss of the CD28 marker and emergence of the CD4+CD28null cells. The correlation between the presence of CD4+CD28null cells and high levels of circulating hHSP60 suggests that this may be the case. How long these cells remain in circulation is unknown. Studies conducted by Liuozzo et al found that CD4+CD28null cells persisted in the peripheral blood of patients with UA for at least 3 months. Longitudinal studies are needed to evaluate the timing of appearance and decline in the numbers of this population of lymphocytes and the effect that clinical interventions may have on these parameters.

HCMV, C pneumoniae, and ox-LDL are antigens commonly thought of as culprits in atherogenesis. Reactivity to these antigens was not detectable in the CD4+CD28null cell population, even though the CD28+ T cells in the same patients showed a positive signal on challenge with these antigens, confirming previous exposure. The experimental conditions used for detection of antigen specificity were initially optimized by use of whole PBMCs, and the concentrations of antigen able to stimulate total CD4+ cell

![Figure 2](http://circ.ahajournals.org/)

**Figure 2.** CD4+CD28null circulating T lymphocytes from patients with ACS react with hHSP60 (A and B, well 6) and PHA (A and B, well 7) but show no reactivity with bacterial LPS. Concentrations used were 0, 80, 160, 320, and 640 pg/mL (wells 1 to 5, respectively). A, IFN-γ and B, perforin mRNA production in response to stimulation. CD4+CD28null cells produce IFN-γ in response to all concentrations of LPS used (C, wells 1 to 5).

**Figure 3.** Levels of circulating hHSP60 measured by ELISA and represented as mean absorbance values with standard errors shown on each column. ACS patients with hHSP60-reactive CD4+CD28null cells are shown as group A, ACS patients without reactive cells as B, and patients with CSA as C.
population were evaluated and used throughout. It is possible that the CD28null population has different requirements and that the level or period of antigenic stimulation may need to be altered for optimum stimulation. The nonresponsiveness of the CD28null population to HCMV, C pneumoniae, and ox-LDL therefore does not detract from evidence presented by others of the involvement of these agents in coronary artery disease.\textsuperscript{22,24,25,31} The antigen concentration used in stimulation experiments is of particular significance with ox-LDL. Data published by Frostegard et al\textsuperscript{25} suggest that high concentrations of ox-LDL may be inhibitory to lymphocyte activation. For this reason, the CD4\textsuperscript{+} CD28null cells were rechallenged with serial dilutions of ox-LDL. No response was observed even at lower concentrations of this antigen (data not shown). Nine patients with ACS did not respond to hHSP60 or to any of the other 3 antigens used in these assays. Patients with nonresponsive cells had low levels of circulating hHSP60. Whether these cells expanded in number as a result of stimulation with another antigen is not known.

When CD4\textsuperscript{+} CD28null cells from patients with CSA were analyzed, no response was observed to hHSP60 or any of the other antigens used in stimulation experiments. The low numbers of cells present in these patients cannot account for this finding, because a higher total number of PBMCs was used in these experiments to allow separation of a large enough population of CD4\textsuperscript{+} CD28null cells from CSA patients and sufficient amounts of cDNA for PCR assays. Because circulating hHSP60 levels in these patients were low, it is possible that antigen-specific modulation of the CD4\textsuperscript{+} CD28null population had not occurred.

Our data did not show activation of CD4\textsuperscript{+} CD28null cells with whole undisrupted C pneumoniae, even though HSP60 is a constituent of the bacterium. This may reflect either suboptimal levels of antigen presented to T cells after in vitro antigenic processing by antigen-presenting cells or reactivity of the CD4\textsuperscript{+} CD28null cells to nonconserved epitopes of HSP60. Although HSPs are highly conserved molecules, there are regions of nonhomology within these proteins, and epitope specificity of the CD4\textsuperscript{+} CD28null cells is as yet unknown.

Recombinant hHSP60 is reported to be contaminated with bacterial LPS. This affects experiments in which macrophage stimulation is evaluated. Gao and Tsan\textsuperscript{37} have shown that tumor necrosis factor factor α (TNF-α) release by macrophages stimulated by hHSP60 is a direct result of LPS contamination of the commercially available hHSP60. The quantity of endotoxin present within 2 preparations of recombinant E coli hHSP60 was assessed by Gao and Tsan.\textsuperscript{37} Using concentrations of LPS up to 4 times higher than those present in the hHSP60, we assessed the effect of this endotoxin on T-cell specificity assays. It is our observation that CD4\textsuperscript{+} CD28null cells were not stimulated by LPS. Reactivity of the cells to hHSP60 is therefore not influenced by the contaminant present in the product.

Atherosclerosis as a chronic inflammatory disorder has been investigated extensively, and the significance of C-reactive protein, TNF-α, and interleukins, along with other markers of inflammation, has been assessed.\textsuperscript{34–36} The role of T cells in the events leading to ACS, however, is unclear. In rheumatoid arthritis, the production of proinflammatory cytokines and tissue-degrading enzymes such as metalloproteinases is T\textsubscript{helper} cell–dependent.\textsuperscript{37} Therefore, the presence of circulating autoreactive CD4\textsuperscript{+} CD28null cells in rheumatoid arthritis may have a contributory role in disease pathogenesis not only by causing perforin-mediated tissue damage but also via inflammatory mechanisms. Metalloproteinases are also involved in the degradation of the fibrous cap of atherosclerotic plaques.\textsuperscript{39} Whether the presence of these T\textsubscript{helper} lymphocytes within the circulation may encourage the production of metalloproteinases and precipitate plaque rupture is uncertain. CD4\textsuperscript{+} CD28null cells are cytotoxic lymphocytes able to kill via killer cell immunoglobulin receptors recognizing polymorphic regions on MHC class I molecules.\textsuperscript{3} The contribution of this effector function to vascular damage remains to be investigated.

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