T Cell Recognition and Killing of Vascular Smooth Muscle Cells in Acute Coronary Syndrome

Sergey Pryshchep, Kayoko Sato, Jörg J. Goronzy, Cornelia M. Weyand

Abstract—Loss of vascular smooth muscle cells (VSMCs) has been proposed to destabilize the atherosclerotic plaque and contribute to plaque rupture, superimposed thrombosis, and acute coronary syndromes (ACSs). We examined whether VSMCs are susceptible to T cell–induced apoptosis and found that CD4 T cells are highly effective in establishing cell–cell contact with VSMCs and triggering apoptotic death. Visualization of the T cell–VSMC contact zone on the single-cell level revealed that both patient-derived and control CD4 T cells reorganized their cell membrane to assemble an immunologic synapse with the VSMCs. Within 4 to 10 minutes, the membrane proximal signaling molecule ZAP-70 was recruited and phosphorylated. However, only patient-derived CD4 T cells sustained an intact immunologic synapse beyond 10 minutes and generated intracellular calcium signals. CD4 T cells that maintained a synaptic contact and appeared to be responsible for VSMC apoptosis accounted for approximately 20% of the circulating memory T cell population in ACS patients and were rare in the blood of age-matched controls. CD4 T cells from ACS patients were also hyperresponsive to T cell receptor–mediated stimulation when triggered by a superantigen and non-VSMC target cells. Lowered setting of the T cell activation threshold, attributable to excessive amplification of proximal CD3-mediated signals, may contribute to CD4 T cell-mediated killing of VSMCs and promote plaque instability. (Circ Res. 2006;98:1168-1176.)

Key Words: inflammation ▪ lymphocyte ▪ unstable angina ▪ vascular inflammation ▪ vascular smooth muscle cells

Atherosclerosis is initiated by deposition and oxidative modification of lipids in the subendothelial layer of blood vessels. The evolving atherosclerotic plaque can remain a stable lesion in which a cap of matrix and vascular smooth muscle cells (VSMCs) covers the lipid core. Alternatively, the plaque can develop into a chronic inflammatory lesion with accumulation of T cells and macrophages, neointegnosis, and matrix remodeling. Inflammatory destruction in the plaque, resulting in rupture of the fibrous cap, exposure of prothrombotic material, atherothrombosis, and luminal occlusion, represents an immediate threat to the host. Plaque rupture is the principal mechanism underlying acute coronary syndromes, such as unstable angina, non-ST and ST elevation myocardial infarction, and sudden cardiac death. Molecular pathways causing plaque rupture are incompletely understood, but plaque-residing macrophages produce numerous tissue-injurious mediators, all of which may increase the risk of plaque destabilization.

CD4 T cells, the dominant lymphocyte population in the inflamed plaque, are activated, suggesting stimulatory signals in the atheroma microenvironment. CD4 T cells from coronary lesions display a unique phenotype and functional profile. Specifically, they lack the costimulatory molecule CD28 and have entered the program of cellular senescence. Senescent CD4 T cells not only lose CD28 but also gain expression of novel immunoreceptors that fundamentally change their responsiveness to environmental cues. Newly acquired immunoreceptors on senescent CD4 T cells include the killer immunoglobulin–like receptor KIR2DS2, NKG2D, the fractalkine receptor CX3CR1, and others. In acute coronary syndrome (ACS) patients, CD4“CD28” KIR2DS2 T cells have cytolitic function and lyse endothelial cells, without a need for antigen recognition.

VSMCs make critical contributions to keeping the atheroma stable and protected. They produce matrix proteins and form a fibrous cap covering the lipid core. VSMC apoptosis has been implicated in destruction of the plaque surface. Whereas endothelial cells are known to serve as antigen-presenting cells (APCs) regulating the functional activity of T cells, much less is known regarding interaction between VSMCs and plaque-infiltrating T cells. In abdominal aortic aneurysms, effector T cells accumulate in the wall lesion, mediating VSMC apoptosis. However, in vitro studies of VSMCs interacting with CD4 T cells have emphasized that VSMCs and endothelial cells are fundamentally different as

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T-cell partners. VSMCs insufficiently supported costimulation of T cells and inhibited T-cell proliferation.20

The current study explored on a single-cell level how CD4 T cells communicate with VSMCs and whether they can induce VSMC death. CD4 T cells isolated from inflamed carotid plaque and from the peripheral blood of ACS patients rapidly triggered VSMC apoptosis, whereas CD4 T cells from healthy controls left VSMCs unharmed. Early molecular events, including the clustering of T-cell receptors (TCRs) and phosphorylation of membrane-proximal signaling molecules at the T-cell–VSMC contact zone, were indistinguishable in patient-derived and control T cells. However, only patient-derived T cells formed a sustained immunological synapse that persisted for more than 45 minutes and mediated VSMC death.

Materials and Methods

Materials
Monoclonal antibodies (mAbs) specific for human CD3, CD4, CD11a (LFA-1), CD45RO, CD45RA, and phospho–ZAP-70 were purchased from BD Biosciences (San Diego, Calif). Zenon Antibody Labeling Kits with AlexaFluor 488 and 555, Annexin V (Alexa Fluor 488), and fura-2 acetoxymethyl ester (fura-2/AM) were purchased from Molecular Probes (Eugene, Ore). 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI), purified CaCl2 solution, EGTA, dimethyl sulfoxide (DMSO), and propidium iodide (PI) were purchased from Sigma-Aldrich (St Louis, Mo).

Patients and Cells
Demographics of ACS and stable angina (SA) patients are given in the online data supplement available at http://circres.ahajournals.org (Table I). Healthy individuals (mean age 68.2 years, 61% male) did not have any cardiovascular risk factors. CD4+ T cells were isolated from freshly drawn blood using the RosetteSep CD4 T Cell Enrichment Kit (StemCell Technologies, Vancouver, Canada). Purity of cell populations assessed by flow cytometry was higher than 95% (supplemental Figure I).

Healthy controls left VSMCs unharmed. Early molecular events, including the clustering of T-cell receptors (TCRs) and phosphorylation of membrane-proximal signaling molecules at the T-cell–VSMC contact zone, were indistinguishable in patient-derived and control T cells. However, only patient-derived T cells formed a sustained immunological synapse that persisted for more than 45 minutes and mediated VSMC death.

Materials and Methods

Measurement of [Ca2+]i Concentration
CD4+ T cells were loaded with fura-2/AM (4 μmol/L in DMSO), incubated for 30 minutes at 37°C, washed twice with PBS buffer (pH 7.4), and cocultured with VSMCs as described for the apoptosis assays. Intracellular free Ca2+ concentrations were measured using the AttoFluor Digital Fluorescence System (AttoFluor Ratiovision, Carl Zeiss) at an emission wavelength of 520 nm and alternating excitatory wavelengths of 343 and 380 nm. The AttoFluor system was calibrated as described by the manufacturer of the Calcium Calibration Buffer Kits (Molecular Probes). Video images were recorded using an intensified charge-coupled device Zeiss camera system.

Confocal Microscopy of the Immunological Synapse
Anti-CD3 mAbs were labeled with AlexaFluor 488, and anti-CD11a (LFA-1) mAbs were labeled with AlexaFluor 555 (Molecular Probes). CD4+ T cells (0.04×10^6/dish in experiments with VSMCs and 0.4×10^6/dish in experiments with THP-1 cells) were incubated with the labeled antibodies for 30 minutes and added to VSMCs (0.02×10^6/dish) or staphylococcal enterotoxin B–coated (10 ng/mL) THP-1 (0.2×10^6/dish; American Type Culture Collection, Manassas, Va) monolayers. The temperature of the sample was maintained at 37°C. Samples were illuminated with either the 488 nm or 543 nm laser lines from the Argon/Helium Neon laser. Live images were collected as vertical Z-stacks and projected in 3 dimensions or subsampled for the dish plane. All images were collected using a ×100/1.4 Plan-Apochromat oil immersion objective. Image capture was done at the indicated time points with a Zeiss confocal laser-scanning LSM 510 META inverted Axiovert-200 microscope.

In other experiments, T-cell–VSMC conjugates were fixed with 2% paraformaldehyde, permeabilized with 0.2% Triton X-100, and stained with PE-labeled anti–phospho-ZAP-70–conjugated antibodies and anti-CD11a AlexaFluor 488-conjugated antibodies for 1 hour. Cells were washed 3 times before analysis. Image analysis and reconstruction of Z-stacks were accomplished using the public domain software ImageJ (http://rsb.info.nih.gov/ij) and LSM-510 Expert Mode software (Carl Zeiss). (See supplemental Figure II for details.)

Statistical Analysis
Experimental groups were compared by analysis of variance and, when appropriate, by Student t test. Data are expressed as the mean±SD. A level of P<0.05 was considered statistically significant.

Results

CD4 T Cells Induce VSMC Apoptosis
To examine whether plaque-infiltrating CD4 T cells kill VSMCs, carotid endarterectomy tissues from 10 patients were dissected, and CD4 T-cell lines and VSMC lines were established. Incubation of CD4 T cells on autologous VSMC monolayers resulted in prompt VSMC apoptosis (Figure 1A). Within 60 minutes of adding CD4 T cells, the nuclei of VSMCs changed shape, and condensed chromatin became arranged around the nuclear membrane, as visualized by DAPI staining. Eventually, the cell body contracted and separated from the anchoring surface. Apoptotic rates of DAPI-stained VSMCs correlated highly with the rates obtained by AlexaFluor 488-labeled Annexin V/PI staining (Figure 1A). Background VSMC apoptosis was distinctly low (data not shown). Plaque-derived CD4 T cells induced apoptosis in 35% to 45% of autologous VSMC (P=0.003) (Figure 1B). CD4 T cells also induced death of HLA-mismatched VSMC lines (data not shown).
To address the question of whether VSMC-killing CD4 T cells were restricted to inflamed atheroma or also present in other compartments, peripheral blood CD4 T cells from 11 ACS patients, 6 patients with SA (matched for traditional risk factors of coronary artery disease), and 12 healthy age-matched controls were examined using the same VSMC line (Figure 1C). In the presence of CD4 T cells from healthy controls or SA patients, a low percentage of VSMCs developed typical apoptotic nuclei within 60 minutes. CD4 T cells from ACS patients more effectively induced apoptosis, with an average frequency of 26% of the VSMCs undergoing cell death ($P = 0.03$, ACS versus SA; $P = 0.04$, ACS versus controls). Thus, ACS patients, but not those with SA, have increased frequencies of CD4 T cells capable of killing VSMCs. Such apoptosis-inducing CD4 T cells reside in the unstable atheroma but also circulate in the blood.

**Kinetics of T Cell–VSMC Interaction**

To decipher the mechanisms involved in cytotoxicity and the signals required for T-cell triggering, video images of T-cell–VSMC cocultures were recorded over a period of 60 minutes (supplemental Figure III). Videos were analyzed for duration of T-cell–VSMC attachment and occurrence of apoptosis. Immunophenotyping of CD4 T cells demonstrated that stable attachment and apoptosis induction were features of memory, but not naïve, CD4 T cells. CD4 T cells forming a contact zone with VSMC membranes for more than 40 minutes were enriched in patients and accounted for 35% to 40% of contacting memory CD4 T cells (supplemental Figure III; $P < 0.05$). Attachment of a CD4 T cell to the VSMC membrane for 40 to 50 minutes was an absolute requirement for apoptosis induction. Termination of T-cell–VSMC interactions after 10 and 30 minutes by physically removing T cells prevented VSMC death (supplemental Figure III).

**Molecular Components and Organization of the T-Cell–VSMC Contact Region**

To elucidate the differences between CD4 T cells from healthy controls and ACS patients, we analyzed the molecular components of the T-cell–VSMC contact site by confocal microscopy. TCR/CD3 complexes were stained, and their distribution within the T-cell membrane was examined. On contact with the VSMC surface, CD3 rapidly localized to the contact zone and within 2 minutes was clustered within the T-cell–VSMC interface (Figure 2A), with no noticeable difference between control and patient-derived CD4 T cells.

To determine whether the clustering of LFA-1 and TCR/CD3 complexes at the T-cell–VSMC interface was functional in transmembrane signaling, we stained for phosphorylated ZAP-70 (pZAP70) as 1 of the key proximal transmitters of TCR signaling. In control and patient-derived T cells, pZAP-70 was detected in the CD3 cluster (Figure 2B), indicating TCR triggering and signal transmission. The dynamics of ZAP-70 phosphorylation were distinct in patients and controls. In control T cells, pZAP-70 was found in the TCR cluster at 2 and 10 minutes and was no longer detectable at 15 minutes. In patient-derived T cells, the lag period between initial contact and the emergence of pZAP-70 in the TCR cluster took approximately 4 minutes; however, pZAP70 persisted for at least 20 minutes.

To examine whether the differences in TCR signaling events were related to distinct molecular organization of the T-cell–VSMC contact zone, LFA-1 and CD3 molecule distributions were monitored at 5-minute intervals by confocal laser-scanning microscopy. At baseline, both molecules were...
Figure 2. Recruitment of TCR/CD3 complexes to the T-cell–VSMC contact site and clustering of membrane-proximal signaling molecules. TCR/CD3 complexes were labeled with AlexaFluor 488–conjugated anti-CD3 antibodies (green), and their relocation was assessed by confocal microscopy. A, Typical digital interference contrast (DIC) images of T-cell–VSMC membrane contact (left). After 2 minutes, TCR/CD3 molecules (green) are relocated to the contact site. Accumulation of TCR/CD3 (see supplemental Figure II) is quantified by plotting the intensity for AlexaFluor 488 across the arrow as line-intensity histograms (right). B, Phosphorylation of the signaling protein ZAP-70 was visualized in control (top 2 rows) and patient-derived (bottom 2 rows) CD4 T cells. CD4 T cells were added to VSMC monolayers, centrifuged at 37 degrees for 1 minute, and fixed at the indicated time points. Cultures were stained with PE-conjugated anti-pZAP-70 (red) and AlexaFluor 488 labeled anti–LFA-1 (green). Yellow lines indicate the T-cell–VSMC interface. Results are representative of 3 independent experiments. Scale bar=5 μm.
diffusely distributed in the cell membrane. By 5 minutes, LFA-1 and CD3 molecules had clustered in the contact zone and were arranged in a structured pattern (Figure 3). LFA-1 molecules arrayed in a ring surrounding a center composed of CD3/TCR molecules. This organization of a central supramolecular activation complex (c-SMAC) and a peripheral p-SMAC is typical for an immunologic synapse.24 Whereas at 10 minutes, the c-SMAC/p-SMAC compartmentalization was maintained, T cells from controls displayed a different structure from those of ACS patients by 15 minutes (Figure 3A and 3B). In control T cells, the immunologic synapse did not mature but, rather, dissolved (Figure 3C). In patient-derived T cells, the formation of the immunologic synapse progressed and by 60 minutes of T-cell–VSMC interaction, CD3/TCR complexes were clustered in the central region in more than 25% of the conjugating T cells (Figure 3D). During the later phase of synapse formation, an area not occupied by either LFA or CD3 became visible, reminiscent of a secretory synapse in which a membrane channel is built between 2 cell partners, allowing for the expulsion of granules into the target cell.25 Indeed, pretreatment of CD4 T cells with strontium abrogated the cytotoxic function, supporting the notion that release of cytotoxic granules is the underlying mechanism of apoptosis (supplemental Figure IV).

**Kinetics of T-Cell Activation Signals**

To identify differences in transmembrane signaling, CD4 T cells from controls and patients were monitored longitudinally for intracellular calcium levels (Figure 4). All CD4 T cells making contact with a VSMC membrane (short-term, intermediate, and long-term attaching T cells) responded with an initial rise of intracellular free calcium. This response never exceeded 200 nmol/L and was followed by a return to baseline by 8 minutes. In control T cells, calcium levels subsequently oscillated around average concentrations of less than 200 nmol/L. In contrast, conjugate formation of patient-derived T cells with VSMCs led to rising intracellular [Ca^{2+}] signals. In T cells maintaining contact sites for 20 to 30 minutes, Ca^{2+} signals followed a rhythmic wave-like pattern with peak responses reaching 250 nmol/L (Figure 4B). A clear distinction emerged for long-attaching apoptosis-inducing T cells. Global Ca^{2+} signals with sudden peaks reaching 400 nmol/L were first recorded after 6 minutes. Such Ca^{2+} peaks recurred over the next 20 minutes. In an amazingly synchronized response pattern, intracellular Ca^{2+} concentration rose beyond 400 nmol/L at 28 to 30 minutes, giving rise to a sustained increase in Ca^{2+} concentrations. Parallel tracking of fura-loaded VSMCs showed an abrupt, dramatic increase in calcium concentrations (Figure 4C) up to 1500 nmol/L at 40 minutes when Ca^{2+} in the adjacent T cells was at its peak. Such extensive calcium fluxes are characteristic for apoptotic cells.26 In contrast, cytoplasmic Ca^{2+} in VSMCs cocultured with normal T cells remained unchanged.

Experiments in Ca^{2+}-depleted medium confirmed that the Ca^{2+} in CD4 T cells derived from extracellular sources

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**Figure 3.** Formation of immunologic synapses between CD4 T cells and VSMCs. CD4 T cells were isolated from 8 healthy controls and 8 age-matched ACS patients, stained with anti-CD3 antibodies (green) and anti-LFA-1 (red), and added to VSMC monolayers. The distributions of CD3 and LFA-1 molecules on the T-cell surface were captured every 5 minutes by confocal microscopy. A minimum of 30 T-cell–VSMC conjugates were analyzed at each time point. The top rows show representative DIC images of T-cell–VSMC contact sites of a control (A) and a patient-derived (B) T cells. Scale bars=10 μm. In the middle rows, overlay images demonstrate the redistribution of TCR/CD3 complexes and LFA-1. Scale bars=5 μm. The bottom rows show Z-axis reconstruction images demonstrating formation of immunologic synapses. Conjugates of CD4 T cells from 8 controls (C) and 8 ACS patients (D) with VSMCs were examined for the presence of multifocal or central accumulation of CD3/TCR complexes (see supplemental Figure II for definitions) or the absence of any clusters; results are given as percentages of conjugates. For each time point, 70 to 100 individual T cell/VSMC conjugates were analyzed.
Figure 4. Intracellular \([\text{Ca}^{2+}]\) signals in CD4 T cells interacting with VSMCs. CD4 T cells from 5 ACS patients and 5 age-matched controls were loaded with fura-2/AM and added to VSMC monolayers, and \([\text{Ca}^{2+}]\) in individual cells was recorded by fluorescent microscopy. A, Typical ratiometric images of intracellular \([\text{Ca}^{2+}]\) recorded at excitation 343/380 nm from healthy donors (top row) and patients (bottom row) are shown. B, \([\text{Ca}^{2+}]\) traces from CD4 T cells derived from control donors (left column) and patients (right column) are shown as mean (black lines)±SD from a minimum of 20 individual T cells recorded in 5 independent experiments. Results are shown for T cells forming short-term conjugates (top), intermediate-term conjugates (middle), and long-term conjugates (bottom). C, Monitoring of intracellular \([\text{Ca}^{2+}]\) levels in fura-2–loaded VSMCs demonstrated collapse of \([\text{Ca}^{2+}]\) homeostasis at 40 to 50 minutes after initiation of contact with patient-derived T cells (right), but not control T cells (left). \([\text{Ca}^{2+}]\) levels, measured in 20 individual VSMCs from 2 independent experiments, are shown as mean (dark line)±SD. D, Intracellular \([\text{Ca}^{2+}]\) traces derived from healthy (left) and patient (right) long-term conjugating CD4 T cells in the absence of extracellular \([\text{Ca}^{2+}]\) are shown.
accompanied by marked reduction in VSMC apoptosis when TCR signaling with anti-HLA class II antibodies was interrupted by adding antibodies blocking HLA-DR molecules to the T-cell–VSMC cocultures (supplemental Figure V). Interrupting TCR signaling with anti-HLA class II antibodies was accompanied by marked reduction in VSMC apoptosis (P = 0.004).

Reduced Activation Threshold in T Cells From ACS Patients

One possible explanation for the observed differences in T-cell–VSMC interaction is that ACS patients have higher frequencies of T cells that recognize antigens on VSMCs with high affinity. Alternatively, patients may have a T-cell population that is hyperresponsive to low-affinity signals. To address this question, we compared T-cell responsiveness in an experimental system that is independent of an endogenous antigen. THP-1 tumor cells were loaded with the superantigen staphylococcal enterotoxin B and incubated with freshly isolated CD4 T cells at a ratio of 1:2 to trigger the TCR. Formation of an organized immunologic synapse was monitored after 10 and 30 minutes (Figure 5A). During the initial phase of T-cell–APC interaction, control and patient-derived CD4 T cells were indistinguishable. After 10 minutes, approximately 1/3 of T cells from both sources formed conjugates, and CD3 and LFA-1 molecules polarized toward the contact zone, with CD3/TCR complexes clustered in the c-SMAC and LFA-1 in the pSMAC. However, control and patient-derived T cells were clearly different after 30 minutes. Organized synapses were found in 45% of control cells and 63% of patient-derived T cells (P = 0.03). To quantify the biological consequences of sustained synapse formation, induction of THP-1 apoptosis was measured (Figure 5B). Healthy CD4 T cells infrequently induced THP-1 apoptosis; approximately 5% of the target cells underwent programmed cell death. CD4 T cells from ACS patients consistently induced THP-1 cell death with a median frequency of 17% of all target cells (P < 0.05).

Discussion

VSMCs secure the stability of the atherosclerotic plaque by covering the lipid core and shielding procoagulant mediators and surfaces. Here we show that such VSMCs are highly susceptible to CD4 T cell–mediated apoptosis. CD4 T cells with VSMC-killing ability reside in the inflamed atheroma and, more importantly, are highly frequent among circulating CD4 T cells in patients experiencing plaque instability. Imaging of the T-cell–VSMC contact zone on the single-cell level demonstrated that apoptosis induction involved signaling of TCR-CD3 complexes and recognition of HLA class II molecules. Comparison of patients and healthy controls indicated a lowered intrinsic T-cell activation threshold in patients which translated into prolonged synapse stability and eventual induction of target cell apoptosis.

Single-cell analysis of VSMCs revealed that target cell apoptosis occurred subsequent to LFA-1 and TCR/CD3 complex reorganization on the T-cell surface. These experiments established that VSMC killing was a receptor-mediated event, not attributable to nonspecific cytoxicity. Apoptosis occurred in only those cells with a stable attachment to T lymphocytes for 40 to 50 minutes. Short-term interactions between CD4 T cells and VSMCs did not cause VSMC death, even when a transient immunological synapse was formed (supplemental Figure III). The critical roles of TCR triggering and sustained signaling were directly demonstrated by monitoring intracellular concentrations of free calcium in individual T cells and linking sustained calcium fluxes with subsequent target cell death (Figure 4).

Initial data raised the questions of whether the CD4 T cells of patients were primed against VSMCs and whether increased efficiency of VSMC killing reflected clonal expansion of autoreactive CD4 T cells. Several lines of evidence refuted this interpretation. Patient-derived CD4 T cells were superior to control T cells in VSMC apoptosis induction when tested on allogeneic VSMCs disparate for MHC class II polymorphisms, suggesting an intrinsic T-cell abnormality independent of a specific antigen presented on VSMCs. Freshly isolated CD4 T cells from all patients and controls were tested on the same VSMC cell line, making it highly unlikely that specific alloantigens were recognized. Also, patients with acute myocardial infarction do not show enrichment for any particular HLA-DR haplotype (data not shown).
Conclusive evidence was obtained in experiments that used a myelomonocytic cell line as target cell. By introducing a superantigen in these cocultures, an identical antigen-independent trigger was provided to the patient-derived and control CD4 T cells; yet the T cells of patients promoted target cell apoptosis significantly more efficiently.

Direct visualization confirmed that the T-cell–VSMC contact zone resembled an immunologic synapse. Membrane LFA-1 redistribution started within 1 minute of contact. By 5 minutes, TCR/CD3 complexes had moved into the cSMAC. Surprisingly, early synapse formation was indistinguishable between patients and controls. Membrane proximal signaling events including the recruitment of ZAP-70, if anything, occurred earlier in controls than patients. However, by 10 minutes after contact, the CD4 T cells of patients displayed different behavior. Whereas control CD4 T cells aborted synapse formation and ZAP-70 phosphorylation, these events continued in patients. Two mechanisms could account for this difference. Either negative signals actively disrupting TCR signaling in control CD4 T cells counteract T-cell activation or amplification loops boost the proximal steps in CD3-mediated signal transduction more efficiently in the CD4 T cells of patients. Inhibitory receptors play a critical role in fine-tuning T-cell and NK cell activation thresholds, and patients may fail to recruit inhibitory receptors that prevent productive T-cell activation. Killer immunoglobulin–like receptors can inhibit NK cell stimulation and are also expressed on T-cell subsets. However, inhibitory killer immunoglobulin–like receptors are infrequently expressed in memory T cells from healthy individuals; in fact, the expression appears to be increased in ACS patients (data not shown). Several other inhibitory receptors have been described, and it is possible that 1 of them is defective in ACS patients.

Alternatively, the T-cell activation threshold for positive signals may be lowered in ACS patients. Mechanisms regulating the setting of the T-cell activation threshold are not entirely understood. T-cell hypersensitivity has been induced by knocking out DRAK2, a member of the death-associated kinase family. T cells from DRAK2−/− mice exhibited enhanced sensitivity to TCR-mediated stimulation, with reduced requirement for costimulation providing evidence that DRAK2 raises the T-cell activation threshold through negative regulation of TCR signaling. Additional mechanisms of tuning T-cell activation thresholds have been attributed to the ubiquitin ligase cbl-b. CD28 and CTLA-4 were found to have opposing effects on cbl-b protein levels, indirectly regulating TCR molecule degradation and their density on the cell surface. Proximal defects in CD3-mediated signal transduction have been described in MRL mice prone to develop systemic lupus erythematosus. Naive CD4 T cells from such mice respond to lower doses of anti-CD3 or antigenic peptides with enhanced and prolonged rise in intracellular calcium, similar to results seen in the present study. Obviously, T-cell hypersensitivity could easily override tolerance mechanisms and result in autoimmunity.

Whatever mechanism is responsible for reducing the activation threshold of the T cells of patients to TCR ligation, the defect was not universal to all T cells but limited to a subset of memory T cells, with almost 10% of circulating CD4 T cells functioning as effective VSMC killers. The large size of the responding CD4 T-cell population excludes antigen specificity as the sole mechanism driving this activity. Rather, many different TCR specificities, all defective in fine-tuning the threshold for TCR signaling, must be involved. T-cell samples were harvested when patients were admitted with the first episode of plaque instability, within hours of the cardiac ischemia. It is unlikely that ischemia affected T-cell threshold control. Instead, abnormalities in T-cell function must have developed over a longer time period. This interpretation is consistent with studies identifying several aspects of T-cell function as abnormal in ACS patients. Most remarkably, CD4 T cells from such patients have been described to lyse endothelial target cells, lack CD28, display autoreactivity against HSP, and use KIR and DAP12 to kill target cells in antigen-independent pathways.

This study has immediate implications for our understanding of immune-recognition events in the atherosclerotic plaque and their clinical consequences. Under physiologic conditions, T cells do not enter the vessel wall and have no access to VSMCs. In contrast, neovascularization in the atheroma provides access for lymphocytes directly to the niche occupied by VSMCs. T-cell hypersensitivity combined with effective cytolytic machinery could have obvious deleterious effects as CD4 T cells invading into the atheroma would no longer obey typical tolerance mechanisms. Protecting the patient from nondiscriminatory T-cell effector functions could possibly be achieved by correcting the molecular settings of the T-cell activation threshold.

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Supplemental Figure 1. Quality control of CD4^+ T-cell purification. CD4^+ T cells were isolated from the freshly drawn blood of healthy donors and ACS patients using the RosetteSep™ CD4^+ T Cell Enrichment Kit (StemCell Technologies, Vancouver, Canada). Purity of isolated cells was monitored by flow cytometry of isolated cells following staining with anti-CD3-FITC, anti-CD8 PE, and anti-CD4 APC monoclonal antibodies. Representative density plots from two different donors are shown. Contaminating CD8 T cells were distinctly infrequent.
Supplemental Figure 2

A

B

C
Supplemental Figure 2. Assessment of the molecular components and organization of the synapses formed between CD4 T cells and VSMC membranes.

CD3/TCR complexes were labeled with AlexaFluor 488-conjugated anti-CD3 antibodies (green); LFA-1 was identified by AlexaFluor 555-labeled antibodies (red). Relocation of these molecules on the surface of CD4 T cells and clustering in the immunologic synapse were assessed by a Zeiss confocal laser-scanning LSM 510 META inverted microscope (Carl Zeiss, Thornwood, NY.).

(A) Confocal images representative of different arrangements of CD3/TCR complexes at the interface between T cells and VSMCs are shown (upper panels). White lines indicate the membrane contact zone and plane of interface z-axis reconstruction. Interface images (insets) depict the distribution of CD3/TCR complexes. Lower panels show the green fluorescence as a heat blot for better quantitative visualization. Left: No clustering of labeled TCR molecules. Middle: Multifocal clustering with green CD3 complexes in an extended region of the T-cell membrane. Right: Central clustering with accumulation of TCR complexes (green) in a central position (c-SMAC) surrounded by a ring of LFA-1 molecules (red) (p-SMAC).

(B) To quantify the distribution of CD3 molecules in the T-cell membrane, the cell surface was manually marked, and the fluorescence intensity of labeled molecules was recorded around the cell perimeter. The cellular ‘background’ level for CD3 was defined as the maximal intensity of AlexaFluor 488 in isolated, non-attached T cells (dashed lines). The upper panel shows a T cell which did not show any evidence of CD3 clustering. Multifocal clustering of CD3/TCR was defined as a multi-peak accumulation
of CD3 in the cell-cell contact area with fluorescence intensities less than twofold above the cellular background (middle panel). Central TCR accumulation was defined as the clustering of labeled CD3 within the central 25% of the interface area of the synapse and fluorescence intensities greater than twofold above the cellular background (lower panel). Only those contact zones that fulfilled the criteria of central clustering were considered to represent synapses. Central and, to some extent, multifocal clustering was associated with a compensatory decrease in CD3 fluorescence in depleted areas.

(C) For correct placement of z-stack reconstructions of the synapse area, T cell-VSMC contact regions were marked (yellow lines), confocal xy-plane images were collected in z-slices (at 1 µm intervals) and displayed as a series of images showing the distribution of labeled CD3 complexes (upper panels). Accumulation of CD3 was quantified by plotting the intensity for AlexaFluor 488 across a vertical line (red line). A histogram of the fluorescence intensities at the respective z-value is shown in the lower panel. Central CD3 accumulation was defined as the slice with maximal CD3 fluorescence intensity, and intensity levels of greater than twofold above the cellular background were required to be considered synapse formations. This point was then used to identify the plane for optimal reconstruction of the contact area.
Supplemental Figure 3: Kinetics of CD4 T cell – VSMC interaction.

CD4 T cells from ACS patients and age-matched controls were incubated on VSMC monolayers. Video images of individual VSMCs with interacting T cells were obtained every 10 seconds over
a time period of 60 minutes using an intensified charge-coupled device Zeiss camera system (Attofluor Ratiovision, Zeiss, Thornwood, NY). Immediately after being placed on the VSMC monolayer, CD4 T cells started scanning the target cell surface. A subset of T cells established firm contact to the VSMCs and stopped scanning. A minimum of 200 T cell-VSMC conjugates were monitored by DIC and fluorescence images for 60 min. Time periods over which conjugates persisted were recorded for each T cell analyzed.

Frequencies of CD4 T cells remaining arrested on the VSMC surface for the indicated contact period were compared in 16 patients and 19 controls and are given as means ± SD (A). About 50% of the CD4 T cells arrested on the VSMC and formed a conjugate. For about 25% of the T cells, the conjugate persisted for less than 10 minutes. A subpopulation of about 15% of T cells stayed in a fixed position for 20 to 30 minutes before detaching. Patients had increased frequencies of T cells that formed conjugates lasting for more than 40 minutes.

CD4 T cells from 6 patients and 6 controls were labeled with anti-CD45RA (AlexaFluor 488) or anti-CD45RO (AlexaFluor 555), added to the VSMC monolayer, and continually monitored for the time period of attachment to the VSMCs. Percentages of naïve CD4 T cells (B) and memory CD4 T cells (C) forming stable conjugates for the indicated time interval are given as means ± SD. CD4⁺CD45RA⁺ T cells, irrespective of whether they were derived from healthy controls or patients, made contact for less than 10 minutes, with a small fraction attaching for an intermediate period of 20-30 minutes. CD4⁺CD45RO⁺ T cells were prone to form more permanent contact with the VSMCs. CD4 T cells establishing long-term contact belonged to the memory T-cell subset, were rare in controls but frequent in patients. (D) To determine the contact time that is required for initiating VSMC apoptosis, T cells from ACS patients were cocultured with VSMC. After the indicated time span, conjugates were mechanically dissociated,
and T cells were removed. In all cultures, VSMC apoptosis was assessed after 60 minutes.

Apoptosis rates in VSMCs cocultured with T cells for 10 and 30 min were not different from spontaneous apoptosis rates in the absence of VSMCs. Apoptosis rates increased abruptly, when T cells were present for 60 minutes, suggesting that the subset of memory T cells establishing long-term contact is responsible for apoptosis induction.
Supplemental Figure 4. Granule-mediated apoptosis of VSMCs.

The granule exocytosis pathway of CD4 T cells was examined by depleting the granule components with strontium chloride. Freshly isolated CD4 T cells from the peripheral blood of 6 ACS patient and 6 age-matched controls were treated with 25 mM SrCl₂ (Sigma-Aldrich, St. Louis, MO) for 18 h (1, 2). Pre-treated T cells were added to VSMC monolayers, and apoptosis rates were determined by DAPI staining after 1 h of T cells/VSMC cells co-culture. CD4 T cell cytotoxicity in patients was sensitive to granule depletion.
Supplemental Figure 5. VSMC-mediated T-cell activation depends on recognition of MHC class II molecules.

CD4 T cells were loaded with fura-2/AM and co-cultured with VSMCs in the presence of control IgG or anti-HLA-DR mAb (L243, 10 µg/ml). (A) Intracellular [Ca²⁺]ᵢ signals were recorded in individual T cells forming stable conjugates for 30 minutes. Mean [Ca²⁺]ᵢ traces of 20 CD4 T cells for each line are shown. Results are representative of five patients. Isotype-matched control antibodies did not affect the dynamics of [Ca²⁺]ᵢ, with T cells receiving above-threshold signals. Addition of anti-HLA-DR antibodies completely abrogated calcium signaling. (B) Frequencies of apoptotic VSMCs pretreated with either isotype-matched control Ig or anti-HLA-DR mAb were determined one hour after adding T cells. Results are shown as mean ± SD of five ACS patients and five healthy controls.
## Supplemental Table 1

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