Insufficient Deactivation of the Protein Tyrosine Kinase Lck Amplifies T-Cell Responsiveness in Acute Coronary Syndrome

Sergey Pryshchep, Jörg J. Goronzy, Susmita Parashar, Cornelia M. Weyand

Rationale: In the vulnerable atherosclerotic plaque, T cells may destabilize the tissue structure through direct cell-injurious effector functions. T cells transmit environmental signals, such as recognition of antigen, into cellular responses through regulated phosphorylation of cytoplasmic proteins, with the Src family kinase Lck (lymphocyte-specific protein tyrosine kinase) in critical membrane-proximal position of the T-cell receptor (TCR) signaling cascade. The balance between protein phosphorylation and dephosphorylation defines the signal transduction threshold and determines appropriate T-cell responses.

Objective: We have examined whether abnormal calibration of intracellular signaling pathways renders acute coronary syndrome (ACS) patients susceptible to disproportionate T-cell responses.

Methods and Results: Intracellular signaling cascades were quantified in CD4 T cells from ACS patients and control individuals after stimulation with major histocompatibility complex class II–superantigen complexes. ACS T cells mobilized more intracellular calcium and accumulated higher levels of phosphotyrosine than control T cells. Proximal steps in TCR signaling, such as recruitment of ZAP-70 and clustering of TCR complexes in the immune synapse, were abnormally enhanced in ACS T cells. Acceleration of the signaling cascade derived from a proximal defect in ACS T cells, which failed to phosphorylate Lck at Tyr505, extending activation of the Src kinase. Abnormalities in TCR signaling did not correlate with systemic inflammation as measured by C-reactive protein.

Conclusions: An intrinsic abnormality in the signaling machinery of ACS T cells resulting in the accumulation of active Lck lowers the TCR threshold and renders lymphocytes hyperreactive and capable of unwanted immune responses. (Circ Res. 2010;106:769-778.)

Key Words: calcium signaling • protein tyrosine kinase • signaling pathways • T cell

Atherosclerosis is now viewed as an immunoinflammatory syndrome, and chronic autoimmune disorders such as systemic lupus erythematosus and rheumatoid arthritis are recognized as risk factors for accelerated atherosclerosis.1–4 Vulnerable atherosclerotic plaque is occupied by tissue-injurious macrophages and CD4 T cells that destabilize tissue integrity through multiple damage pathways.5 CD4 T cells from acute coronary syndrome (ACS) patients produce proinflammatory cytokines and are cytotoxic toward vascular smooth muscle cells and endothelial cells, directly implicating them in vascular injury and plaque destabilization.6–8 Generally, CD4+ T cells are activated when the T-cell receptor (TCR) recognizes a complex of antigen and major histocompatibility complex class II molecules.9 Thus, encounter of disease-relevant antigen may drive excessive T-cell activity in atherosclerotic lesions. Alternatively, threshold setting of the TCR is altered, and T cells in ACS patients respond to suboptimal stimuli, including minimally altered tissue antigens.

TCR engagement by antigen–human leukocyte antigen complexes presented by antigen-presenting cells (APCs) initiates a cascade of biochemical events culminating in T-cell activation.10 Upstream steps involve phosphorylation of the Src family protein tyrosine kinase Lck (lymphocyte-specific protein tyrosine kinase),11 which phosphorylates immunoreceptor tyrosine-based activation motifs in the TCR CD3 zeta chains, enabling the docking of Syk family kinases, such as ZAP-70.12,13 ZAP-70, in turn, activates a variety of downstream signals resulting in formation of an immunologic synapse12 and translocation of the microtubule-organizing center toward the activation clusters.14 Formation of the immunologic synapse involves reorganization of the membrane and the submembrane cytoplasm with accumulation of tyrosine-phosphorylated Lck (Tyr394), ZAP-70, and other membrane adaptors, such as LAT, and excludes the large transmembrane molecules CD45 and CD43.14,15 Activation signals are subsequently transmitted by coordinated phosphorylation of mitogen-activated protein kinases. Another core aspect relates to the regulated metabolism of inositol phospholipids and the production of 2 key lipid second messengers (polyunsaturated diacylglycerols and phosphatidyl-
Functional T-Cell Assays

Target cell apoptosis was identified as previously described. To quantify T-cell proliferation, CD4 T cells labeled with 1 μmol/L carboxyfluorescein diacetate succinimidyl ester (CFSE) were stimulated with SEB-loaded (0.5 to 10 ng/mL) APCs at a 1:1 ratio. Seventy-two hours later, frequencies of proliferating cells were assessed by CFSE dilution using the LSRII flow cytometer (BD Biosciences, San Diego, Calif).

Flow Cytometry

CD4 T cells labeled with anti-CD3 (APCs) were stimulated with SEB-loaded (10 ng/mL) APCs at a 1:1 ratio. For intracellular staining, cells were fixed (2% paraformaldehyde) at different time points (0, 0.5, 2, and 10 minutes) after T cell–APC coculture, permeabilized (0.01% Triton X-100), blocked with 1% BSA for 30 minutes, and stained with anti-pLck (Tyr505).

Enzyme-Linked Immunosorbent Assay

To determine ZAP-70 phosphorylation kinetics, purified CD4 T cells were cocultured with SEB-loaded APCs (10 ng/mL) for 1 to 10 minutes at 37°C and lysed, and phospho-ZAP-70 (Tyr319) was measured by ELISA (Cell Signaling Technology).

Confocal Microscopy

Freshly isolated CD4 T cells were stained and incubated with SEB-loaded APCs. For intracellular protein staining, CD4 T-cell/APC conjugates were fixed at different time points after contact initiation, permeabilized (0.01% Triton X-100), blocked with 1% BSA for 30 minutes, and stained. All antibodies were labeled with AlexaFluor (488 or 546 nm) by Zenon technology kits (Invitrogen). All images were captured by a Zeiss confocal LSM 510 META (Carl Zeiss, Thornwood, NY) using a Plan-Apochromat ×63/1.4 oil differential interference contrast objective. Intracellular calcium concentrations were measured with 2 Ca²⁺ indicator dyes, Fluor-4/AM and Fura-red/AM (Molecular Probes). T cells were stimulated with SEB-loaded APCs (10 ng/mL), soluble anti-CD3 (3 μg/mL), phorbol 12-myristate 13-acetate (PMA) (25 ng/mL), ionomycin (50 ng/mL), or anti-CD3/CD28 beads. Fluorescence images were collected with a confocal microscope using 488 nm excitation light and emission at 520 to 550 nm and at 580 to 600 nm, for Fluor-4 and Fura-red, respectively.

Quantitative Image Analysis

Image analysis was accomplished using the LSM software (Carl Zeiss), Imaris (Bitplane, St Paul, Minn) and ImageJ software (NIH, http://rsb.info.nih.gov/ij). To estimate protein accumulation in the synapse, mean fluorescence intensities (MFIs) in the quarter of the T cell directed toward the APCs were measured in 12 focal z-series. Fold enrichment of molecules at the synapse was calculated as the ratio between the MFI of the contact region and the region outside the synapse after background subtractions.

The CD3 cluster at the center of the contact zone with MFIs more than 2-fold above background was defined as the central supramolecular activation complex (c-SMAC); the peripheral SMAC (p-SMAC) was localized through the leukocyte function-associated antigen (LFA)-1–expressing ring-shaped structure surrounding the CD3 cluster.

Statistical Analysis

Experimental groups were compared by analysis of variance and, when appropriate, by Student t test or ANOVA. All data are expressed as means±SD. A level of P<0.05 was considered statistically significant.

Results

Increased Mobilization of Intracellular Calcium Levels in ACS T Cells After TCR Ligation

Fluctuations of intracellular calcium ions is an immediate consequence of TCR engagement. To quantify TCR signal strength intensity, we monitored intracellular...
calcium levels in ACS T cells triggered with several modes of T-cell activation: superantigen-loaded APCs, cross-linking antibodies against CD3, and pharmacological stimulation with PMA/ionomycin (Figure 1). Engagement of CD4 T cells by SEB-loaded APCs promptly increased intracellular free calcium (Figure 1A and 1B). TCR ligation produced a sustained elevation of \( \text{Ca}^{2+} \) concentrations that were higher in ACS T cells than controls for at least 10 minutes (Figure 1B). The enhanced signal intensity in ACS T cells was independent from CD28-mediated costimulation (Figure 1C and 1D) because response patterns of increased \( \text{Ca}^{2+} \) concentrations were similar following stimulation with soluble anti-CD3 antibodies. In contrast, circumventing TCR signaling by activating T cells through PMA/ionomycin yielded similar kinetics of intracellular calcium levels in control and ACS T cells (Figure 1E) localizing the defect in patient-derived T cells to membrane-proximal phosphorylation, upstream of calcium mobilization.

Enhancement of Early Tyrosine Phosphorylation Events in ACS T Cells

To assess the most proximal stages of TCR signaling, we quantified phosphorylation of tyrosine residues in adaptor molecules transmitting TCR-initiated signals (Figure 2A). In ACS patients, elevated total pTyr concentrations (Figure 2B) at the TCR cluster site indicated more efficient transmission of TCR signals and suggested acceleration of membrane-proximal steps as the underlying mechanism. The rapid phosphorylation of Tyr319 in ZAP-70 is required for phospholipase C-γ1 activation and calcium mobilization. Monitoring of phosphorylated (p)-ZAP70 over the first 10 minutes after TCR stimulation demonstrated a very early rise in the ACS T cells with a biphasic curve whereas control T cells responded monophonically (Figure 2C).

Enhanced Accumulation of TCR/CD3 Complexes in the Immunologic Synapse of ACS T Cells

Acceleration of TCR-induced tyrosine phosphorylation reflects the strength of the initial TCR-mediated signal or results from amplification loops augmenting the T-cell activation cascade. We tested whether enhanced signaling waves in ACS T cells were associated with altered clustering of TCR complexes in the immunologic synapse. T-cell/APC conjugate formation was equally effective in control and ACS T cells (Figure 3A); however, the molecular composition and compartmentalization of the immunologic synapse were distinct. CD3 accumulation in the synapse was accelerated in ACS T cells at a SEB concentration of 10 ng/mL (Figure 3B). Three-dimensional interface reconstructions demonstrated typically arranged synapses, with CD3 clustering in the central SMAC and LFA molecules positioned in the peripheral SMAC (Figure 3C). Time kinetics of CD3 recruitment appeared similar in control and ACS T cells, but densities of CD3 complexes accumulating in the c-SMAC were significantly higher in the patients (Figure 3D; \( P = 0.001 \)) compared to both healthy control T cells and T cells from patients with stable coronary artery disease. Abnormal accumulation of CD3 complexes in the synapse occurred in naïve, as well as memory T cells, excluding prior antigenic experience as the underlying mechanism (Figure 4A and 4B).
Defective Regulation of Lck Activity in the Immunologic Synapse of ACS T Cells

At the top of the T-cell activation cascade, the Src family protein kinase Lck phosphorylates the TCR/CD3 chain, allowing for the recruitment/activation of ZAP-70. For Lck to adopt an “open” and enzymatically active configuration, Tyr505 needs to be dephosphorylated, and the activation loop containing the activating Tyr394 needs to be exposed. Transphosphorylation of Tyr394 residue promotes kinase activity. Rephasphorylation of Tyr505 functions as a negative-feedback loop slowing down signal transmission. As shown by flow cytometric analysis of intracellular p-Lck (Tyr505), CD4 T cells rapidly lost phosphorylation of Tyr505 after activation to then reaccumulate the “restrained” configuration of Lck (Figure 5A and 5B). In control T cells, levels of p-Lck (Tyr505) were higher at 2 and 10 minutes after TCR engagement (Figure 5B). In ACS patients, rephosphorylation of the negative regulatory Tyr 505 site of Lck was delayed and blunted (Figure 5B).

Figure 2. Altered dynamics of tyrosine phosphorylation in ACS CD4 T cells. A, CD4 T cells were isolated from ACS patients and controls (n=5 each) and added to SEB-loaded APCs (10 ng/mL). At the given time points, cells were fixed and stained with anti-pTyr. Top images represent the density profiles of p-pTyr fluorescence. B, Data are presented as means of MFI±SD from a minimum of 20 cell conjugates analyzed for each time point in 5 independent experiments. C, Phospho–ZAP-70 (Tyr319) protein was quantified in cell lysates by ELISA. Phosphorylation kinetics of ZAP-70 are shown as fold increases relative to levels in resting T cells. Data from 5 independent experiments are presented as means±SD.

Figure 3. Enhanced recruitment and retention of TCR/CD3 complexes in the immunologic synapse in ACS. CD4 T cells from patients with ACS, stable coronary artery disease (CAD), and age-matched controls (n=10 each) were stained with anti-CD3 (AlexaFluor 488 [green]) and anti-CD11a (LFA-1, AlexaFluor 555 [red]) and stimulated with SEB-loaded APCs. Percentages of T-cell/APC conjugates were measured after 30 minutes. A, Data from 6 independent experiments are shown as means±SD. B, Recruitment of TCR/CD3 molecules to the immunologic synapse after 6 minutes of T cell–APC interaction. MFI±SD for anti-CD3 (AlexaFluor 488) are shown. C, Representative images of CD3 (green) and LFA-1 (red) molecular arrangements in the immunologic synapse. Left, color-merged projection image of the z-stacks with maximal intensity CD3 in green and LFA-1 in red; right, reconstructed interface view. D, Real-time recruitment of CD3 molecules to the T cell–APC interface over time. MFI recordings from 5 independent experiments are given as means±SD. *P<0.001.
The enrichment of inactivated Lck p-Tyr505 within the immunologic synapse was monitored by single-cell microscopy. In control T cells, phosphorylation of Lck at Tyr505 started at 1 minute, with a steady increase over the next 5 minutes (Figure 5C and 5D). ACS T cells failed to accomplish Lck deactivation at a similar rate and quantity. Starting at 2 minutes, p-Tyr505 levels were significantly lower in the synapse of ACS T cells and did not exceed baseline levels at any time point of the observation period, suggesting a failure of the Lck inactivation machinery (Figure 5D). Rearrangement of total Lck molecules in the immunologic synapse was examined by confocal microscopy, comparing patient-derived and control CD4 T cells undergoing stimulation. Within 0.5 to 1 minute after TCR engagement, Lck molecules clustered in the subsynaptic region (Figure 5E and 5F). Lck enrichment followed a similar course in patient and control T cells for the first 2 minutes. In the controls, Lck recruitment subsequently reverted and by 6 minutes most Lck molecules had left the subsynaptic compartment. In ACS, Lck accumulation peaked at 4 minutes, remaining significantly higher at 6 minutes. These data suggested that insufficient inhibition of Lck at Tyr505 enhanced recruitment of active Lck in ACS T cells.

CD4 T Cells From ACS Patients Hyperreact to TCR-Mediated Signals

To test whether defective downregulation of Lck and enhanced TCR-mediated signaling leads to more vigorous T-cell responses, we assessed 2 major functional outcomes of TCR engagement: T-cell clonal expansion and T cell-mediated toxicity. Patient-derived CD4 T cells more effectively killed SEB-loaded APCs, particularly at superantigen concentrations of 10 ng/mL and lysed twice as many targets as control T cells ($P \leq 0.002$) (Figure 6A). In the absence of T cells apoptosis of SEB-coated target cells was explicitly low (Online Figure I). Besides cytotoxic capability, proliferative expansion of the CD4 T cells of patients 72 hours after stimulation was significantly higher than in controls (Figure 6B and 6C). At all superantigen concentrations tested (0.5 to 10 ng/mL), the CD4 T cells of patients proliferated to a higher population size ($P < 0.001$). In essence, ACS CD4 T cells outperformed control T cells in both cytotoxicity and proliferative expansion.

The distribution of naïve and memory CD4 T cells was examined by flow cytometry analysis for CD45RO (Online Figure II). Also, surface CD3 expression on CD4 T cells (Online Figure III) and the repertoire of TCR Vβ elements relevant for SEB binding were indistinguishable in patients and controls (Online Figure IV).

Correlation of Synaptic CD3 Clustering and Serum C-Reactive Protein Levels in ACS Patients

To explore whether signaling abnormalities in T cells were associated with the systemic inflammatory syndrome of ACS, CD3 clustering in the synapse of activated T cells was correlated with serum levels of C-reactive protein CRP (Figure 7). CRP levels did not predict enhancement in membrane-proximal signaling events $r = -0.43$, $P = 0.137$.

Discussion

CD4 T cells in the atherosclerotic plaque display several tissue-damaging effector functions, including killing of both endothelial cells and vascular smooth muscle cells.7,24 How plaque-infiltrating T cells are activated to display their cytolytic capabilities remains unknown. Phosphorylation of
the cytoplasmic part of the TCR sets in place a complex cascade of signaling events involving tyrosine phosphorylation and generation of second messengers, finally culminating in the translocation and activation of the transcription factors NFAT (nuclear factor of activated T cells), activator protein 1, and nuclear factor κB.11,13 To avoid inappropriate and exacerbated T-cell activation, the TCR signaling cascade incorporates feedback loops that calibrate responsiveness and return the T cell to a resting state after antigen encounter. Here, we report that T cells from ACS patients have a defect in phosphorylating Lck at Tyr505, thus failing to deactivate this membrane-proximal Src kinase.

Impaired Lck inhibitory function affected the TCR signaling cascade at the top of the signaling hierarchy as the phosphorylation of the proximal Syk kinase ZAP-70 was already accelerated in ACS T cells. Bypassing the TCR complex with ionomycin/PMA produced identical response patterns in ACS and control T cells, localizing this defect to very early events that control signal strength intensity. Abnormalities in the signaling machinery, in particular insufficiency in this Lck-dependent negative-feedback loop, enables T cells to respond in conditions which otherwise would be ignored by the adaptive immune system.

Our studies demonstrate that Lck recruitment into the synapse was enhanced in ACS T cells (Figure 5). However, phosphorylation of inhibitory Tyr505 was hampered with continuous inclusion of the active Lck molecule into the TCR cluster site. Thus, insufficient Tyr505 phosphorylation was not attributable to a loss of recruiting Lck molecules. In normal T cells, Tyr505 is phosphorylated by Csk11,18,19 and ~50% of Tyr505 is phosphorylated in unstimulated T cells.25 T cells of both control and patients had the same Lck expression levels in the cytoplasm (data not shown). Also, T cells rapidly dephosphorylate Tyr505 after TCR triggering, demonstrating that mechanisms of Lck activation were intact in ACS T cells. Data suggest that the negative-feedback loop relying on Csk rephosphorylating Lck was defective. The most proximal stages of the TCR signaling cascade is tightly regulated through positive and negative-feedback loops (Figure 8).19 Comparing the accumulation of phosphorylated target molecules and intracellular calcium levels was sufficient to pinpoint the defect of ACS T cells to the first

Figure 5. Failure of Lck inactivation in CD4 T cells from ACS patients. CD4 T cells were isolated from ACS patients and controls (n=17 each) and added to APCs precoated with SEB (10 ng/mL). Cells were stained with anti-CD3 (APCs) and with anti-pTyr505 Lck (AlexaFluor488) after cell fixation and permeabilization. A, Representative flow cytometry histograms from control (top) and patient-derived (bottom) CD4 T cells show dephosphorylation kinetic of Tyr505. B, Fold changes of MFIs for pTyr505 after CD4 T-cell activation from 12 independent experiments are given as means±SD. C, Dynamics of phosphorylation at the negative regulatory site of Tyr-505 Lck. After T-cell stimulation by SEB-loaded APCs, cells were fixed, permeabilized, and labeled with anti-Lck (AlexaFluor 546, red) and anti–pTyr-505 Lck, (AlexaFluor 488, green). D, Phosphorylation of Tyr-505 Lck was calculated as fold changes of MFIs in the synapse divided by the average mean intensity of the remaining cells. Values were calculated on a total of 12 confocal planes. Data are presented as means±SD for each time point from 5 independent experiments. E, Cells were stained with anti-CD3 (AlexaFluor 488) and anti-Lck (AlexaFluor 546), and images were collected by confocal microscopy. The middle plane of the z axis and the maximum projection of 12 z-planes of the cell–cell conjugates are shown. The contact site is indicated as a yellow line. Scale bar=5 μm. Right images represent the density profiles of Lck fluorescence obtained at 6 minutes. F, Accumulation of Lck molecules within the contact area is measured as fold enrichment of the MFI for Lck. MFI in the synapse was divided by the average mean intensity of the remaining cells. Values were calculated on a total of 12 confocal planes. Data are presented as means±SD for each time point from 5 independent experiments.
minutes of the activation process, compatible with a failure of the negative regulatory kinase Csk to counteract the perpetual accumulation of enzymatically active Lck.

Abnormalities in proximal TCR signaling have been implicated in pathological conditions, including autoimmunity, malignancy, and immunodeficiency.16,26 T-cell responsiveness in type 1 diabetes, systemic lupus erythematosus, and rheumatoid arthritis have been found to be abnormal, with a multitude of mechanisms involved, ranging from alterations in TCR/CD3 complex composition to excessive expression of costimulatory receptors.27,28 Defective inhibition of TCR activation leads to breakdown of self-tolerance and autoimmune disease in animal models as well. Mice deficient in the ubiquitin ligase Itch present with spontaneous autoimmunity, manifesting with diffuse tissue infiltrates and autoantibody production.29 Similarly, Cbl-deficient mice are much more sensitive to TCR stimulation.30 Mice carrying naturally arising mutations in the SHP-1 gene (moth-eaten mice), a well-established negative regulator of proximal activation-promoting signaling, have spontaneous autoimmunity.31 In Mgsat5-deficient mice, enhanced TCR clustering was associated with lowered T-cell activation thresholds rendering animals susceptible to autoimmunity.32

Inflammatory pathways contribute to atherosclerosis at all stages of the disease but are particularly important in rendering the plaque vulnerable and rupture prone.4,33,34 Macrophage-mediated effector functions are critically important,34 but regulatory, as well as tissue-damaging functions of plaque-residing T cells, are now emerging as pathogenic elements in plaque instability.35 Myeloid and plasmacytoid dendritic cells instruct intraplaque T cells, monitor the tissue microenvironment, and respond promptly to danger signals. Thus, plaque immune responses are not solely determined by antigen but reflect a multitude of factors encountered by infiltrating immune cells.

Testing antigen-driven immune response in humans is a particularly technical challenge as the frequency of antigen-specific T cells is usually lower than 1 in 10,000. For the present study, a novel test system was developed using artificial APCs and superantigen. To ensure we could compare different patients and focus on T-cell abnormalities instead of APC abnormalities, we chose a stable, nondisease APC, THP-1 cells, to present superantigen. Superantigens bind to selected TCR Vβ elements, and flow cytometry analysis with Vβ-reactive antibodies was used to ensure similar frequencies of relevant T-cell subsets in patients and controls (Online Figure IV). We also ruled out that an inherent difference in the TCR surface expression in ACS and control T cells influenced signal strength.
Abnormalities in T-cell biology in ACS patients so far have been limited to the memory T-cell compartment, suggesting that prior antigenic experiences remodel the T-cell repertoire and enrich potentially dangerous T cells.36,37 T cells accumulated in the human plaque are indeed enriched for end-differentiated CD4 memory cells,38 including CD4\(^+\)CD28\(^-\) T cells. In the present study, T-cell hyperresponsiveness was a feature of memory and naïve T cells, making it unlikely that altered TCR calibration is downstream of immunologic priming. To the contrary, the broad distribution of the defective signaling regulation within the entire T-cell compartment suggests metabolic abnormalities, such as altered membrane dynamics. Patients and controls were carefully matched for the diagnosis of hyperlipidemia and lipid-lowering therapies to exclude distorted lipid metabolism as an underlying mechanism. Most ACS patients enrolled into this study were not taking statins, which may interfere with T-cell function.39

A shift in the TCR threshold setting does not eliminate antigen as a critical checkpoint in controlling T-cell activity. However, a lowered threshold removes stringency and may allow T cells to undergo activation when exposed to low doses of antigen or when recognizing antigens that have structural
similarities to the original antigen. Neoantigens formed in the proinflammatory microenvironment of the plaque, with ongoing oxidative stress modifying host antigenic determinants may thus be sufficient to elicit immune responses that would otherwise be prevented. Modified low-density lipoprotein has been shown to stimulate plaque-invading T cells. Plaque-residing APCs are equipped with a variety of pathogen-sensing receptors and may function as a trap to enrich circulating immune stimulators. With sufficient density of T cells that respond even to low antigen concentrations, conditions are set to break self-tolerance and initiate long-lasting adaptive immune responses. Understanding the molecular defect leading to the rewiring of the TCR signaling cascade may ultimately allow for resetting T-cell response thresholds and avoiding illegitimate immune responses in ACS.

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Disclosures
None.

References


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**Novelty and Significance**

**What Is Known?**

- Vulnerable atherosclerotic plaque is an inflamed lesion, with accumulation of macrophages and T cells.
- T cells are activated in the plaque.
- T-cell activation involves recognition of antigen, followed by a cascade of biochemical modification of intracellular signaling events.

**What New Information Does This Article Contribute?**

- In patients with ACS, T cells respond abnormally, accumulating higher amounts of phosphorylated signaling molecules to a constant stimulus.
- The abnormality occurs early in the activation process and is caused by insufficient deactivation of the enzyme Lck.
- Hyperresponsive T cells have the potential to be tissue-damaging; they may increase the risk of excessive inflammatory responses.

Tissue-damaging immunoinflammatory cells mediate instability of the atherosclerotic plaque with T cells contributing to plaque destabilization through cytokine release or direct cytotoxicity. T cells are activated when binding specific antigen; the intensity of the response, however, depends on complex biochemical events phosphorylating or dephosphorylating a cascade of signaling molecules. The present study examines whether T cells from patients with acute coronary syndrome are hyperresponsive rendering them potentially tissue-injurious. Reactivity of human T cells was quantified in a novel assay using superantigen-loaded antigen-presenting cells and confocal microscopy. T cells from ACS patients accumulated higher amounts of activated signaling molecules. The defect was localized to the Src family kinase Lck, an enzyme regulating very early steps in the signaling cascade. T cells from ACS patients failed to deactivate Lck, prolonging and intensifying the activation process. This is the first study implicating miscalibration of T-cell responses in ACS. The data indicate that not only access to antigen but also the internal “wiring” of the T cell determines when, where, and how T-cell responses occur. Defective Lck inactivation will modulate protective and pathogenic immune responses in ACS. Further research needs to discern how patients acquire this defect and how it affects their immunity inside and outside of the atherosclerotic plaque.
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### Supplemental Table I

Antibodies and Reagents

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**Supplemental Table II**

Demographic and clinical characteristics of study populations

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<td>6</td>
<td>7</td>
<td>0.17</td>
</tr>
</tbody>
</table>
Apoptotic range of target cells during superantigen stimulation

Targets cells were incubated with superantigens at indicated concentrations (0-10ng/ml) for 60 min at 37°C. Cells were labeled with Annexin V (FITC) for 15 min. Apoptosis of target were identified by flow cytometry. Data are presented as means ± SD from three independent experiments.
Supplemental Figure II

Naïve and Memory subpopulations of CD4 T cells

CD4 T cells were isolated from the peripheral blood of ACS patients and age-matched controls (n=10 each) and analyzed for naive (CD45RO⁻) and memory (CD45RO⁺) subpopulations by flow cytometry. Data from five independent experiments are presented as means ± SD.
Expression of CD3 in CD4 T cells from ACS patients and age-matched controls. CD4 T cells were isolated from the peripheral blood of ACS patients (n=8) and age-matched controls (n=8). Surface expression of CD3 was quantified by flow cytometry after staining with anti-CD3 antibodies. A representative histogram is shown in (left) with the black line showing results from an ACS patient and the filled curve from an age-matched control. Mean fluorescence intensities ± SD are given in bars (right).
Repertoire of T cell receptor V\(\beta\) elements in CD4 T cells from ACS patients and age-matched controls.

CD4 T cells were isolated from the peripheral blood of ACS patients (n=8) and age-matched controls (n=8). After staining with specific antibodies, frequencies of CD4 T cells utilizing the TCR V\(\beta\) elements V\(\beta\)2, V\(\beta\)5.2, V\(\beta\)8, and V\(\beta\)12 were determined by flow cytometric analysis. V\(\beta\)5.2, V\(\beta\)8 and V\(\beta\)12 have been implicated in binding SEB; V\(\beta\)2 served as a control. Data are presented as means ± SD.