Clinical Research

De Novo Expression of Killer Immunoglobulin-Like Receptors and Signaling Proteins Regulates the Cytotoxic Function of CD4 T Cells in Acute Coronary Syndromes

Takako Nakajima, Ömer Goek, Xiaoyu Zhang, Stephen L. Kopecky, Robert L. Frye, Jörg J. Goronzy, Cornelia M. Weyand

Abstract—The inflammatory infiltrate in atherosclerotic plaque is composed of T cells and macrophages. CD4+ T cells with a unique phenotype, CD4⁺CD28null, are preferentially recruited into culprit lesions. These T cells are distinct from classic CD4⁺CD28⁺ T cells in gene expression and function, including their ability to mediate cytolysis. In this study, we have investigated the regulation of CD4⁺CD28null T-cell cytolytic function. In patients with acute coronary syndromes (ACS), CD4⁺CD28null T cells express killer immunoglobulin-like receptors (KIRs). KIRs encompass a polymorphic family of receptors that recognize HLA class I molecules and have been implicated in self-tolerance. CD4⁺CD28null T-cell clones from patients with ACS and age-matched controls were compared for their KIR-expression profile. T-cell clones derived from the patients expressed a broader spectrum of KIRs (P<0.001) with preference for the stimulatory variant, CD158j. Additionally, CD4⁺ T-cell clones from patients but not those from controls acquired de novo expression of the DAP12 molecule, an adapter chain that transmits CD158j-derived signals. Cumulative expression of CD158j and DAP12 endowed cytolytic competence on CD4⁺CD28null T cells, allowing them to kill in the absence of T-cell receptor triggering. Our data demonstrate that CD4⁺CD28null T cells in ACS are characterized by a unique gene expression profile. Consequently, these T cells acquire cytolytic capability that can bypass the need for T-cell receptor triggering and, thus, impose a threat to self-tolerance. (Circ Res. 2003;93:106-113.)

Key Words: killer immunoglobulin-like receptors □ plaque instability □ inflammation □ unstable angina □ myocardial infarction

Acute coronary syndromes (ACS), including unstable angina, sudden cardiac death, and myocardial infarction (MI), represent abrupt complications of coronary atherosclerosis. The development of a tissue defect on the atheroma surface, which leads to superimposed thrombosis and sudden occlusion of the artery, precipitates such acute events. Atherosclerotic plaque rupture is multifactorial. Histological studies have confirmed that activated T cells and macrophages often accumulate in the shoulder region of the plaque, the point of minor resistance in the lesion. Macrophage-derived mediators, including metalloproteinases, have been implicated in the rupture of the fibrous cap, and plaque-infiltrating T cells have the potential to contribute to tissue damage.

Patients with ACS can be distinguished from age-matched healthy controls and patients with stable angina by the increased frequency of a subset of CD4⁺ T cells that are oligoclonally expanded in the peripheral blood and have lost the expression of the costimulatory molecule CD28. Data on the relationship between plaque-infiltrating T cells and these oligoclonally expanded CD4⁺CD28null T cells are limited. However, phenotypic and T-cell receptor sequence analysis in the patients who have been studied indicate that these T cells accumulate in culprit lesions and are not present in stable lesions (authors’ unpublished data, 2003). CD4⁺CD28null T cells are functionally distinct from classic CD4⁺ helper T cells. Besides their ability to release large amounts of interferon (IFN)-γ, CD4⁺CD28null T cells express perforin and granzyme B. On triggering of the T-cell receptor, they lyse target cells, including endothelial cells. It is possible, therefore, that these T cells directly contribute to plaque instability.

CD4⁺CD28null T cells variably express receptors of the killer immunoglobulin-like receptor (KIR) family. KIRs belong to a multigenic family consisting of >13 genes encoded within the leukocyte receptor complex (LRC) on chromosome 19q13.414–16 and are expressed on NK cells and infrequently on CD8⁺ T cells. Haplotypes differ markedly in the number of genes; in addition, allelic polymorphisms have been described. Their critical immunoregulatory
role stems from their ability to recognize major histocompatibility complex (MHC) class I molecules in the absence of exogenous antigen. The KIR family includes both inhibitory and stimulatory receptors. Engagement of inhibitory KIRs mediates the phosphorylation of cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs). Phosphorylated ITIMs serve as recruitment points for cytosolic protein tyrosine phosphatases, which dephosphorylate substrates critical in cellular activation. Stimulatory KIRs lack ITIMs. Instead, they have a charged residue in their transmembrane domain that facilitates interaction with the signal transduction chain, DAP12. DAP12 activates the mitogen-activated protein kinase cascade and ultimately induces degranulation of cytotoxic granules and the release of cytokines.

In this study, we describe that CD4+ T cells from patients with ACS express multiple inhibitory and stimulatory KIRs whereas KIR gene transcription is infrequent in CD4+CD28null T cells from healthy controls. More importantly, CD4+CD28null T cells from patients with ACS frequently coexpress stimulatory KIRs with the adapter molecule DAP12. DAP12 is also encoded in the LRC, suggesting a unique role for this gene complex in CD4+CD28null T cells. CD4+ T cells coexpressing KIR2DS2 and DAP12 can access cellular activation pathways and circumvent the need for T-cell receptor signaling.

Materials and Methods

Study Population

Seventy-three patients admitted to the Mayo Clinic Cardiology Unit with the diagnosis of recent-onset unstable angina or acute ST-elevation MI were enrolled. Inclusion and exclusion criteria have been described. Coronary atherosclerotic plaques were obtained at autopsy from patients with fatal MI within 2 hours after death. Half of the plaque was used for histological examination, and the other half was used for RNA extraction. Plaques without inflammation or with RNA degradation were excluded. Control coronary artery samples were obtained from patients who died from nonvascular causes. The protocol was approved by the Mayo Clinic Institutional Review Board.

Flow Cytometry

Peripheral blood mononuclear cells (PBMCs) and T-cell clones were stained with PE-labeled anti-CD158b/j (Beckman Coulter, Miami, Fla). PerCP-labeled anti-CD4, and FITC-labeled anti-CD28 mAbs (both Becton Dickinson, San Jose, Calif). The cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson), and the surface expression of these molecules was calculated using WinMDI software (Joseph Trotter, Scripps Research Institute, La Jolla, Calif).

T-Cell Cloning

CD4+CD28null T-cell clones were established from PBMCs of three patients with ACS and three healthy age-matched controls known to have an expansion of CD4+CD28null T cells. CD4+CD28null cells were sorted from PBMCs on a FACSVantage flow cytometer (Becton Dickinson), stimulated with anti-CD3 mAb (OrthoClone OKT3; Ortho Diagnostics, Raritan, NJ) in the presence of irradiated feeder cells, and then cloned by limiting dilution in the presence of 50 U/mL recombinant human interleukin (IL)-2 (Proleukin; Chiron, Emeryville, Calif) as described. CD4+ T cells that lacked CD28 expression and, thus, resembled patients with unstable angina, were analyzed from all patients were analyzed by flow cytometry with an antibody that stained 3 of the 13 known KIR-encoded molecules and the release of cytokines.
isoforms, CD158b1, CD158b2, and CD158j (online Table 1). CD158b/j expression was typically found on CD4^+CD28^{null} T cells (Figure 1A). In some patients, KIRs were present on most CD4^+CD28^{null} T cells. Consistent with a close association between the loss of CD28 and a gain of CD158, CD4^+CD158b/j^{+} T cells were rare in patients lacking CD4^+CD28^{null} T cells. Healthy individuals had very low frequencies of CD4^+CD28^{null} T cells, and we have failed to detect KIR molecules on CD4^+ T cells in healthy donors (Figure 1B).\textsuperscript{13,24} Results for all patients who had \textasciitilde5% CD4^+CD28^{null} T cells are summarized in Figure 1C. CD4^+CD28^{+} T cells were essentially negative for CD158b/j. Conversely, the CD4^+CD28^{null} subset regularly included T cells that expressed KIRs.

### KIR Expression Profiles on CD4^+ T-Cell Clones Isolated From Patients With ACS

KIR genes display allelic sequence polymorphisms,\textsuperscript{14–16} but available monoclonal antibodies incompletely detect this diversity. To decipher the profile of KIR molecules, we established a panel of 76 CD4^+CD28^{null} T-cell clones from three patients with acute plaque instability. Cell-surface phenotypes of the clonal T-cell cultures were confirmed by flow cytometry. Clones expressed the T-cell markers, CD3 and CD4, and were free of CD158b/j-expressing NK cells (Figure 1D). All T-cell clones were examined by RT-PCR for the presence of 11 different KIR gene transcripts, including six inhibitory isoforms (KIR2DL1, KIR2DL2, KIR2DL3, KIR2DL4, KIR3DL1, and KIR3DL2) and five stimulatory isoforms (KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, and KIR3DS1). Gene expression was confirmed by cell-surface staining for CD158b/j, which recognizes gene products of KIR2DS2, KIR2DL2, and KIR2DL3 (Figure 1D). To ascertain whether the KIR expression profile in patients with ACS is qualitatively different from healthy controls, we identified three healthy individuals from a large population screening who carried CD4^+CD28^{null} T cells. The KIR genotypes of the three patients with ACS and the three healthy individuals were similar. All of them carried the long KIR haplotype, encompassing gene loci for stimulatory and inhibitory receptors. A set of 84 CD4^+CD28^{null} T-cell clones was isolated from these healthy donors and was analyzed in parallel with the samples from the patients with ACS.

Overall, KIR genes were frequently transcribed in CD4^+CD28^{null} T cells (Figure 2). However, the patterns of KIR expression were markedly different between clones derived from controls and patients (\(P<0.001\)). Thirty-five percent of the control clones lacked all inhibitory KIRs, and 45% expressed a single member of the inhibitory KIR types. Less than 20% of the control clones had more than one inhibitory KIR, 82% of the control clones were negative for

### Demographic and Clinical Characteristics of the Study Population

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**Figure 1.** CD4^+ T cells from patients with ACS express MHC class I-recognizing receptors. PBMCs from 73 patients with ACS were analyzed by 3-color flow cytometry for the expression of CD4, CD28, and CD158b/j (encoded by KIR2DS2, KIR2DL2, and KIR2DL3). Representative analyses from a patient with ACS (A) and from a healthy control (B) are shown. The patient had an expanded population of CD4^+CD28^{null} T cells (9.2%; A, left). CD158b/j expression was rare on gated CD4^+CD28^{+} T cells (A, center) and common on CD4^+CD28^{null} T cells (A, right). CD4^+ T cells from the control individual did not include an appreciable number of CD28^{null} (B, left) or CD158b/j^{+} T cells (B, right). Frequencies of CD158b/j-expressing CD4^+CD28^{+} and CD4^+CD28^{null} T cells from the patients are shown as box plots displaying medians and 25th and 75th percentiles as boxes. CD4^+CD28^{null} T cells had a higher proportion of CD158b/j^{+} CD4^+ T cells (\(P=0.001\)) (C). CD4^+ T-cell clones isolated from patients with ACS showed high surface expression of CD158b/j. A representative staining is shown (D).
all stimulatory receptors tested, and 10% expressed a single activating KIR. On average, control clones expressed 1.1 KIR isoforms/cell (composed of 0.9 inhibitory and 0.2 stimulatory isoforms). T-cell clones from patients had an average of 3.5 KIR isoforms per cell, with 2.4 inhibitory and 1.1 stimulatory members. Most patient-derived clones expressed combinations of KIRs.

KIR profiles on patient-derived and control clones were also qualitatively different (Figure 3). In the control clones, only KIR2DL4 and KIR3DL2 were consistently frequent, whereas clones from patients with ACS expressed one or more additional inhibitory receptors, such as KIR2DL2, KIR2DL3, and KIR3DL1. The difference in KIR profiles was most striking for two stimulatory receptors, KIR2DS2 and KIR2DS4. Only 4 of the 84 control clones expressed the KIR2DS2 gene compared with 33 of the 76 patient-derived T-cell clones.

Expression of KIR2DS2 in Atherosclerotic Plaque
To estimate the biological significance of KIR2DS2 expression in CD4+CD28null T cells for plaque vulnerability, we examined unstable coronary plaques from 16 patients with fatal MI and 17 coronary arteries from control patients for KIR2DS2 transcripts. In 12 of the 16 samples from patients with MI, KIR2DS2 transcripts were detected. Figure 4 shows a representative selection of six arterial tissues that contained KIR2DS2 transcripts. These KIR2DS2+ tissues also contained T-cell receptor–specific sequences, demonstrating the presence of a T-cell infiltrate. None of the tissues contained CD16 transcripts, a marker for NK cells. Control tissues did not have any detectable T-cell receptor or KIR2DS2 mRNA. The data demonstrate the close association between KIR2DS2 and T cells in the plaque. The frequency of KIR2DS2 expression in the tissues was higher than expected. In a random population, only 40% of individuals inherit a
KIR haplotype that includes KIR2DS2.25–27 The more frequent KIR2DS2 expression (75%) in the coronary artery tissues raises the possibility that the gene is enriched in patients with coronary artery disease.

**Cytotoxic Potential of CD4⁺ CD158j⁺ T Cells**

The frequent expression of the KIR2DS2 gene in CD4⁺ T cells from patients with ACS and in the atherosclerotic plaque tissue raised the question of whether CD158j regulated the activation of these T cells. CD158j triggers cytolysis by inducing the degranulation of perforin/granzyme-containing granules. In contrast to CD4⁺CD28null T cells, CD4⁺CD28⁺ T cells possess perforin/granzyme granules and, on T-cell receptor triggering, lyse target cells. To test the role of CD158j in regulating the functional activity of CD4⁺CD28null T cells, we used endothelial cell layers formed by HUVECs as target cells. A biotin matrix was applied to the surface of the HUVECs, permitting effective crosslinking of avidin-conjugated antibody molecules at the T-cell/target-cell interface.

CD158j⁺ T-cell clones isolated from patients with ACS effectively lysed endothelial targets on anti-CD3 mAb-mediated crosslinking of the T-cell receptor (Figure 5). More importantly, triggering with anti-CD158b/j mAb was sufficient to induce killing by some CD158j⁺ T-cell clones (top). In CD158j⁺ T-cell clones, cytotoxicity could only be induced through a T-cell receptor–mediated signal (Figure 5, bottom).

After screening all available CD4⁺CD158j⁺ T-cell clones, it became apparent that CD158j was functional in some T-cell clones (Figure 5, top) but not in others (Figure 5, middle). Coexpression of potentially inhibitory KIR2DL2 and KIR2DL3 genes could not explain the lack of cytotoxicity; CD158j⁺CD158b⁺CD158b² T-cell clones also failed to kill (data not shown). The observation of functional and nonfunctional CD158j molecules raised the question of whether other molecular components were necessary to complement the cell-surface receptor expression. CD158j does not have a cytoplasmic signaling domain but binds to DAP12 adapter dimers.19 DAP12 activates the ZAP70/Syk pathway, leading to phospholipase C-γ phosphorylation, calcium release, and cytotoxicity.28

DAP12 is usually not transcribed in CD4⁺ T cells.13 All CD4⁺ T-cell clones were therefore screened for the expression of DAP12 by RT-PCR. DAP12 was distinctly infrequent in control clones—none of the KIR2DS2-expressing clones from healthy individuals transcribed the DAP12 gene (Figure 6A). However, DAP12 was frequently transcribed by the patient-derived clones, 48 of the 75 T-cell clones were positive, and 21% of the KIR2DS2⁺CD4⁺ T-cell clones coexpressed DAP12. CD158j-mediated cytotoxicity was an exclusive feature of T-cell clones that transcribed both KIR2DS2 and DAP12 (Figure 5, top). T-cell clones that expressed KIR2DS2 in the absence of DAP12 did not kill via CD158j-triggering (Figure 5, middle).

**Reconstitution of Cytotoxicity by Transducing DAP12**

To confirm that complementation of KIR2DS2 and DAP12 gene expression was required for functional activity of the stimulatory receptor, we selected CD158j⁺DAP12⁺ T-cell clones and transduced them with a vaccinia virus/DAP12 construct. Transduction with the vaccinia virus wild-type construct did not affect the ability of CD4⁺ T-cell clones to lyse target cells on crosslinking of the T-cell receptor (Figure 6B). In wild-type transduced T-cell clones, crosslinking with anti-CD158b/j mAb was insufficient to induce cytotoxicity. However, vaccinia virus-mediated transfer of the DAP12 gene reconstituted cytolytic activity in T-cell clones triggered by crosslinking of CD158j. These experiments established the functional significance of complementing KIR2DS2-expressing CD4⁺ T cells with DAP12. They also confirmed that deficiency of DAP12 expression and not expression of inhibitory receptors was responsible for the nonfunctionality of CD158j in some of the patient-derived and all of the control T-cell clones.

**Discussion**

In this study, we report that CD4⁺ T cells in patients with ACS have undergone profound changes in gene expression...
T Cells in ACS

Figure 6. DAP12 is differentially expressed in CD4<sup>+</sup>CD28<sup>−</sup>/H11002 T-cell clones and is required for CD158j-mediated cytotoxicity. Seventy-six CD4<sup>+</sup>CD28<sup>−</sup>/H11002 T-cell clones from patients with ACS (filled bars) and 84 T-cell clones from healthy controls (open bars) were analyzed for the expression of DAP12. In the controls, most clones lacked CD158j and DAP12. T-cell clones from patients with ACS frequently expressed DAP12, and 21% of all clones coexpressed CD158j and DAP12 (A). CD4<sup>+</sup>CD28<sup>−</sup>/H11002 T-cell clones were infected with vaccinia virus containing DAP12 cDNA or wild-type (WT) virus. Transfection was confirmed by DAP12-specific PCR (B). Transfected clones were tested in a redirected cytotoxicity assay. DAP12- and WT-infected T-cell clones killed on T-cell receptor (TCR)-specific ligands (C). In the absence of DAP12, T-cell clones were unable to initiate cytolysis after CD158j stimulation (C). DAP12 expression restored this cytotoxic response (C).

and function. Most significantly, these CD4<sup>+</sup> T cells have entered a program of successive de novo expression of genes encoded in the LRC on chromosome 19. The acquisition of KIR2DS2 and its signaling protein, DAP12, endowed CD4<sup>+</sup> T cells with cytolytic capability. CD158j<sup>−</sup>/H11001/CD158<sup>+</sup>/H11001 CD4<sup>+</sup> T cells were exclusively found in patients with ACS, emphasizing an association between such specialized T cells and plaque instability. Preliminary data on T-cell lines established from tissue debris collected by distal embolization protection plaque instability. Preliminary data on T-cell lines established from tissue debris collected by distal embolization protection indicate that CD4<sup>+</sup> T cells have a 3-fold higher mortality risk within the first year (authors’ unpublished data, 2003). T cells, through their release of IFN-γ, are the most powerful regulators of macrophage differentiation. Plaque-infiltrating CD4<sup>+</sup>CD28<sup>−</sup>/H11002 T cells produce excessive amounts of IFN-γ, and there is evidence for in vivo IFN-γ release in patients with plaque instability. The in vivo targets of cytolytic plaque-infiltrating T cells remain undetermined, but endothelial cells are effectively lysed in vitro. Killing of endothelial cells could threaten the structural integrity of the plaque, which depends on neoangiogenesis of the growing lesion. Also, lysis of smooth muscle cells could have profound consequences for plaque stability.

This study demonstrates that in ACS, CD4<sup>+</sup>CD28<sup>−</sup>/H11002 T cells do not require signaling through the T-cell receptor but have an alternative means of stimulation. Through acquisition of stimulatory KIRs and the adapter molecule, DAP12, CD4<sup>+</sup>CD28<sup>−</sup>/H11002 T cells lose their dependence on exogenous antigen and gain responsiveness to endogenous signals in their microenvironment. The aberrant expression of stimulatory KIRs obviously poses a threat to self-tolerance. This receptor family recognizes HLA class I molecules in the absence of foreign antigen and, thus, can respond to classic self-structures.

The frequency of CD4<sup>+</sup> T-cell clones expressing CD158j was remarkably high in ACS, but the clones often coexpressed the inhibitory receptors CD158b<sup>+</sup>/H9253 and CD158b<sup>−</sup>/H11002. In NK cells, combined expression of CD158j with inhibitory variants is not unusual. The biological significance of this coexpression is presently unclear, but recent data suggest that the model of inhibitory signals counterbalancing stimulatory signals is too simplified. Stimulatory and inhibitory KIRs may bind to distinct ligands. In support of this hypothesis, murine cytomegalovirus susceptibility genes have been mapped to the Ly49 gene region, a gene cluster similar in function to KIRs. The stimulatory Ly49H molecule, but not the homologous inhibitory Ly49 molecule, recognizes a MHc-like molecule encoded by cytomegalovirus. Also, inhibitory KIRs on CD4<sup>+</sup> T cells may not always suppress T-cell activation by stimulatory KIRs. We have preliminary evidence that cotriggering of the inhibitory CD158b<sup>+</sup>/H9253 and the stimulatory CD158j on CD4<sup>+</sup>CD28<sup>−</sup>/H11002 still induces cytotoxic activity (authors’ unpublished data, 2003).

De novo expression of KIRs on CD4<sup>+</sup> T cells was first demonstrated in patients with rheumatoid arthritis (RA), in which CD4<sup>+</sup>CD158<sup>+</sup>/H11001 T cells contribute to synovial inflammatory infiltrates. KIR<sup>−</sup>CD4<sup>+</sup> T cells in RA and ACS have similar phenotypic and functional characteristics, but they are not identical. In RA, CD158j<sup>−</sup>/H11001 CD4<sup>+</sup> T cells do not exhibit cytotoxicity after CD158j stimulation, and, in line with data presented here, they fail to express the DAP12 gene. The CD158j molecule remains functionally active, but instead of inducing degranulation of cytolytic granules,
CD158j crosslinking costimulates T-cell receptor signaling and amplifies the production of IFN-γ. In DAP12 CD4+ T cells, CD158j uses signaling pathways that selectively target the JNK pathway but that cannot confer independence of T-cell receptor triggering.34

In ACS, CD4+CD28null T cells not only express KIRs but also transcribe DAP12, making them completely independent of T-cell receptor stimulation. CD158j and DAP12 expression were not correlated in individual T-cell clones, suggesting that each gene is independently regulated. However, both are encoded in the LRC, raising the possibility of a region-specific gene activation in CD4+CD28null T cells. If this is correct, the functional phenotypes of CD4+CD28null T cells in healthy individuals, patients with ACS, and patients with RA may represent different stages in a differentiation program, ranging from occasional KIR expression in healthy individuals, frequent KIR expression in RA, and DAP12/KIR coexpression in ACS.

In support of this model, patients with RA have a high risk for vascular disease. In its most severe form, RA can manifest as vasculitis. The KIR2DS2 gene and its HLA-C ligand have been identified as risk factors for rheumatoid vasculitis.35 A more frequent vascular complication of RA is coronary artery disease.36,37 The shortened life expectancy of patients with RA has been attributed to an increased rate and an acceleration of ischemic heart disease. The sharing of immunoregulatory abnormalities in patients with RA and ACS suggests common pathogenic pathways in these two syndromes.38,39

The expansion of CD4+CD28null T cells in RA has been attributed to premature aging of the immune system.32,40 It is not known whether T-cell homeostasis is also altered in patients with ACS as seen in RA. Epidemiological data suggest that acute coronary events are preceded by a decade of increased activity in the innate immune system.41–44 Because activation of the innate and adaptive immune systems go hand in hand, it is to be expected that patients with ACS also have chronic triggering of T cells. We propose that patients with ACS accumulate end-differentiated T cells that have undergone profound changes in gene expression and function. The frequencies of these cells seem to be independent of other risk factors of coronary artery disease but may be related to the activation of the innate immune system, as indicated by elevated C-reactive protein levels. One of the characteristics of these cells is the induction of MHC class I-recognition receptors, such as CD158j, that are functional in the presence of the appropriate signal transduction molecule, DAP12. The natural ligand of CD158j in the atherosclerotic plaque remains to be determined. Candidates include MHC class I molecules on stressed and injured cells that need to be removed as well as molecular patterns derived from infectious agents.

Acknowledgments

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Online Table 1. Nomenclature for killer cell immunoglobulin-like receptors

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