TRAIL-expressing T cells induce apoptosis of vascular smooth muscle cells in the atherosclerotic plaque

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Acute coronary syndromes (ACS) are precipitated by a rupture of the atherosclerotic plaque, often at the site of T cell and macrophage infiltration. Here, we show that plaque-infiltrating CD4 T cells effectively kill vascular smooth muscle cells (VSMC). VSMCs sensitive to T cell–mediated killing express the death receptor DR5 (TNF-related apoptosis-inducing ligand [TRAIL] receptor 2), and anti-TRAIL and anti-DR5 antibodies block T cell–mediated apoptosis. CD4 T cells that express TRAIL upon stimulation are expanded in patients with ACS and more effectively induce VSMC apoptosis. Adoptive transfer of plaque-derived CD4 T cells into immunodeficient mice that are engrafted with human atherosclerotic plaque results in apoptosis of VSMCs, which was prevented by coadministration of anti-TRAIL antibody. These data identify that the death pathway is triggered by TRAIL-producing CD4 T cells as a direct mechanism of VSMC apoptosis, a process which may lead to plaque destabilization.

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Abbreviations used: ACS, acute coronary syndrome; FADD, Fas-associated death domain; COR-SMC, coronary artery smooth muscle cell; LDH, lactate dehydrogenase; DN, dominant negative; NC, normal control; TRAIL, TNF-related apoptosis-inducing ligand; VSMC, vascular smooth muscle cell.

The rupture of the surface of atherosclerotic plaque, giving rise to superimposed thrombosis and arterial occlusion, is considered the most frequent primary cause for acute coronary syndromes (ACS) such as unstable angina, myocardial infarction, and sudden cardiac death (1, 2). The atheroma's vulnerability to rupture is correlated with a cellular infiltrate of macrophages and activated T cells in the plaque tissue (3–5). Macrophages have been implicated in destroying cells and matrix components and thus weaken the plaque structure (6–9). It is not known which signals trigger tissue-infiltrating macrophages to secrete damaging enzymes. The inflammatory infiltrate also includes lymphocytes, mast cells, and dendritic cells, which may directly or indirectly contribute to plaque instability.

CD4 T cells, the dominant type of plaque-residing T cells, undergo in situ activation in culprit lesions (4, 10, 11) and include expanded clonotypes with shared T cell receptor sequences, suggesting a role for antigen recognition in T cell activation (4). Plaque-infiltrating CD4 T cells are recruited from the $CD4^+CD28^-$ subset that is enriched in the blood of patients with ACS and typically produce high levels of IFN-γ (12, 13). These CD4 T cells have lost the costimulatory molecule CD28 (14) and, instead, communicate with their microenvironment through a new set of immunoreceptors (15), such as killer immunoglobulin-like receptors. KIR2DS2 endows CD4 T cells with the ability to kill target cells, even in the absence of TCR triggering. $CD4^+CD28^-$ T cells isolated from patients with ACS kill endothelial cells in vitro, making them a prime suspect for mediating endothelial injury (16). Apoptotic death of VSMCs has been proposed as a mechanism of plaque destruction, transforming a stable plaque into rupture-prone lesions (17, 18). Here, we have explored whether plaque-infiltrating CD4 T cells directly cause VSMC apoptosis.

T cells kill target cells via two distinct effector pathways: first, by expelling cytotoxic granules containing perforin and granzyme; or, second, by ligating death receptors (19). CD8 T cells typically use granules to lyse target cells, whereas CD4 T cells preferentially mediate cell death through the production of TNF-like molecules, such as the membrane-integrated protein Fas ligand (FasL) and APO2

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ligand/TNF-related apoptosis-inducing ligand (TRAIL) (20). TRAIL binds to type I transmembrane proteins such as TRAIL-R1 (DR4) and TRAIL-R2 (DR5) (21, 22) and triggers the formation of the death-inducing signaling complex that recruits procaspase-8 through the adaptor protein Fas-associated death domain (FADD) (23). Proteolytically activated caspase-8 mediates the activation of terminal effector caspase-3 and causes target cell death. It is believed that TRAIL causes apoptosis of various tumors, but leaves normal cells unharmed and constitutes a physiologic pathway of CD4 T cell–mediated cytotoxicity against tumor cells (24, 25). However, TRAIL can also induce apoptosis of activated human T cells (26), neutrophils (27), and hepatocytes (28).

We show that VSMCs are susceptible to TRAIL-mediated apoptosis, and that patients with ACS bear increased frequencies of CD4 T cells that express TRAIL upon stimulation and kill VSMCs in a TRAIL-dependent manner. Adoptive transfer of such CD4 T cells causes VSMC death in human carotid plaque, demonstrating the in vivo relevance of this mechanism in plaque destabilization.

RESULTS

CD4 T cells induce VSMC apoptosis

To investigate whether plaque-infiltrating CD4 T cells can directly damage VSMCs, T cell lines and VSMC lines were established from inflamed plaque tissue that was collected by carotid endarterectomy. CD4 T cell lines maintained by polyclonal stimulation were incubated on autologous and heterologous VSMC monolayers. As shown in Fig. 1, plaque-derived CD4 T cells effectively induced VSMC apoptosis. At an effector–target ratio of 20:1, 60–70% of autologous VSMCs were killed within 4 h of coculture. Even at a low effector–target ratio of 2.5:1, one third of the VSMCs underwent apoptosis. VSMC apoptosis was absolutely dependent on T cells; VSMC death in control cultures without T cells was minimal (Fig. 1, a and c). Apoptosis rates at low effector–target ratios increased with prolonged incubation for 24–48 h (not depicted). Cytolytic activity was observed with heterologous VSMCs that were not matched for MHC polymorphisms with the responding T cell population; cytolytic activity on autologous VSMCs derived from the T cell donor was only slightly higher (Fig. 1 a). All VSMC lines expressed MHC class I and HLA-DR molecules, as determined by confocal microscopy (unpublished data). Anti–HLA-DR antibodies blocked CD4 T cell–induced VSMC apoptosis in a dose-dependent manner; antibodies specific for MHC class I molecules had no effect (Fig. 1 b). These findings suggested that the VSMC killing involved interaction between MHC class II molecules and the T cell antigen receptor.

To examine whether the ability to induce VSMC apoptosis was restricted to plaque-infiltrating T cells, CD4 T cells were isolated from the peripheral blood of patients with ACS and added to VSMC monolayers. CD4 T cells induced apoptosis in 40–50% of VSMCs (Fig. 1 c). Peripheral blood

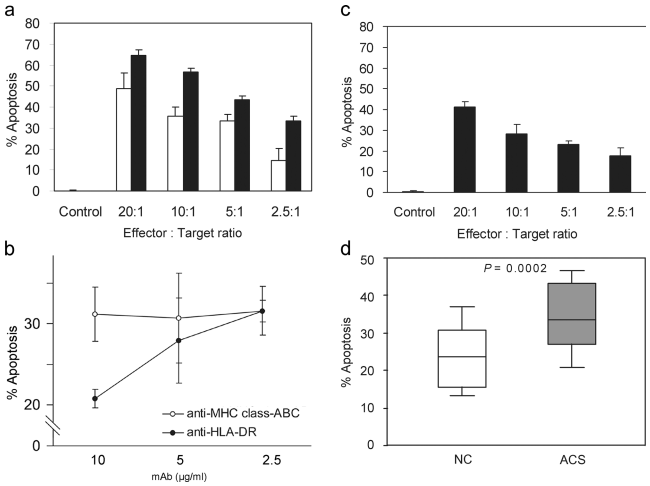

Figure 1. CD4 T cells induce VSMC apoptosis. (a) CD4 T cell lines and VSMC lines were established from carotid endarterectomy samples of 4 patients. CD4 T cells were expanded and incubated on heterologous (open bar) or autologous (shaded bar) SMC monolayers at indicated effector–target cell ratios. VSMC apoptosis was assessed by DAPI staining after 4 h of coculture. Results from a representative experiment are shown as mean ± SD of triplicates. VSMC apoptosis in cultures without added T cells (indicated as control) was minimal. (b) Coronary SMCs were pretreated with increasing concentrations of anti-HLA class I mAb (W6/32) or anti–HLA-DR mAb (L243) for 1 h, and then cocultured with CD4 T cells at an effector–target ratio of 10:1 for 4 h before apoptosis analysis. Results from one of seven experiments are shown as mean ± SD of triplicate cultures. (c) CD4 T cells were isolated from PBMC by negative selection and added to heterologous VSMC monolayers at indicated effector–target cell ratios. Frequencies of apoptotic VSMCs were determined after 4 h. Results are shown as mean ± SD of triplicates and are representative of eight patients with acute coronary syndrome (ACS). Controls indicate VSMCs without added T cells. (d) PBMC-derived CD4 T cells from 50 patients with ACS and from 33 normal controls (NC) were incubated on coronary-SMC monolayers for 4 h at an effector–target ratio of 10:1. Frequencies of apoptotic cells are shown as box plots displaying medians: 25th and 75th percentiles as boxes; and 10th and 90th percentiles as whiskers. Patient-derived CD4 T cells induced VSMC death at significantly higher frequencies ($P = 0.0002$ by Mann-Whitney U test) than those from controls.

CD4 T cells were also able to kill both autologous and allogeneic VSMCs.

To assess whether CD4 T cells from patients with ACS had a higher ability to kill VSMCs, a cohort of 50 patients with either myocardial infarction (72% male, 60.9 ± 12.1 yr old) or unstable angina (72% male, 66.9 ± 12.4 yr old) was compared with 33 age-matched controls (58% male, 57.3 ± 16.8 yr old). The VSMCs used for these experiments were derived from the coronary artery of a single donor (coronary artery smooth muscle cell [COR-SMC]). 20–25% of the VSMCs underwent apoptosis when exposed to PBMC-derived CD4 T cells isolated from controls at an effector–target ratioof 10:1. The rate of VSMC apoptosis was sub-stantially increased with T cells collected from peripheral blood of ACS patients ($P = 0.0002$) (Fig. 1 d). A similar increase in VSMC apoptotic activity was not observed with CD4 T cells

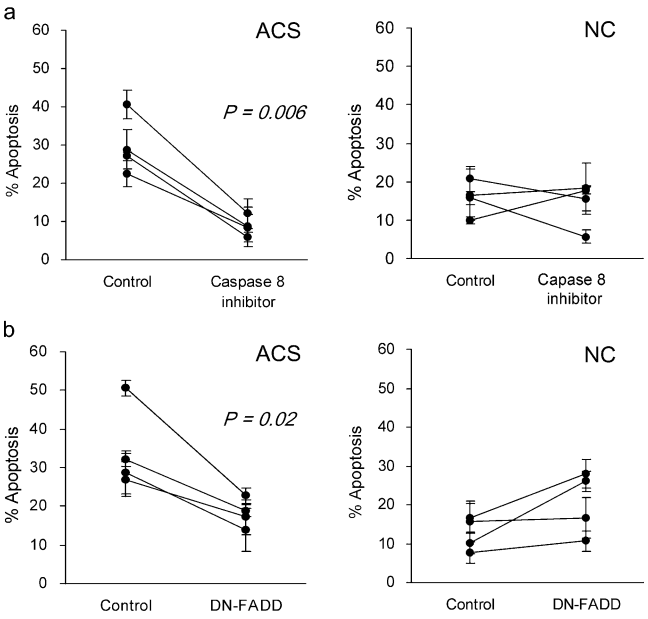

Figure 2. Death pathway dependence of CD4 T cell–mediated VSMC apoptosis. (a) CD4 T cells from four patients with ACS (left) and four control subjects (NC, right) were added to coronary SMC monolayers that had been pretreated with medium (control) or caspase-8/granzyme B Inhibitor I (25 μg/ml) for 1 h. The frequencies of apoptotic VSMCs are shown as mean ± SD of triplicate cultures. (b) VSMCs propagated in collagen-coated 96-well plates were transfected with either GFP (control) or DN-FADD-GFP (0.05 μg/well). CD4 T cells from patients with ACS ($n = 4$, left panel) and NC ($n = 4$, right panel) were added at an effector–target cell ratio of 10:1. Frequencies of apoptotic VSMCs are shown as mean ± SD of triplicates. Results of control and treated samples were compared by Wilcoxon sign rank test.

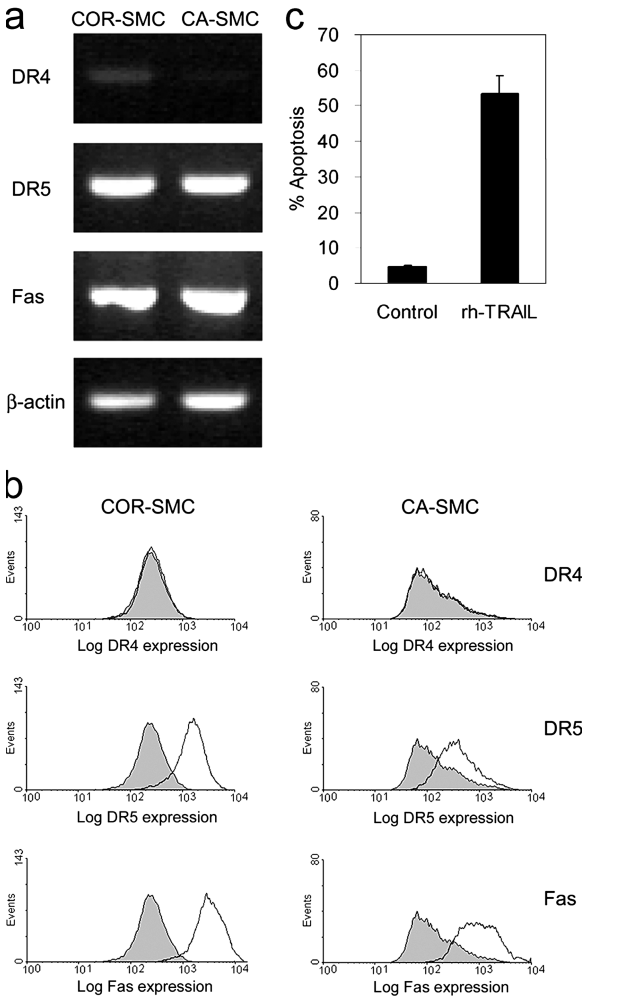

Figure 3. VSMCs express death receptors and are susceptible to TRAIL-mediated apoptosis. (a) Expressions of DR4, DR5, and Fas mRNA in VSMC were tested by RT-PCR. Results representative of experiments with 10 coronary (COR-SMC) and carotid artery-derived cell lines (CA-SMC) are shown. There was consistent expression of Fas and DR5 in all lines; in particular, there was no difference between COR-SMC and CA-SMC. (b) Surface death receptor expression on COR-SMCs and CA-SMCs was evaluated by FACS analysis. Histograms are representative of five experiments. Results from staining with antibodies specific for DR4, DR5, and Fas are shown as open histograms; background fluorescence with isotype-matched control antibody is indicated by shaded histograms. (c) Sensitivity of VSMC to TRAIL-mediated apoptosis was assessed by culturing VSMC without (control) or with 400 ng/ml of recombinant human (rh)-soluble Flag-tagged TRAIL (rh-TRAIL) along with cross-linking 2 μg/ml of anti-FLAG antibody M2 for 12 h. Apoptotic cells were identified by DAPI staining. Results from one of four experiments are shown as mean ± SD of triplicate cultures.

from patients with stable coronary artery disease (unpublished data). These data demonstrated that VSMCs were susceptible to T cell–mediated death, and that CD4 T cells from patients with ACS had higher VSMC cytotoxicity.

CD4 T cell–mediated VSMC apoptosis depends on the death pathway

T cells can kill target cells by different pathways; e.g., by releasing lytic enzymes or by triggering of the death pathway (19, 29, 30). Clustering of cell surface death receptors initiates death pathway–induced apoptosis via FADD recruitment and eventual caspase-8 activation. As shown in Fig. 2 a, caspase-8/granzyme B blocking in VSMCs resulted in a marked inhibition of VSMC apoptosis ($P = 0.006$ in ACS patients). Inhibition of apoptosis induced by CD4 T cells from normal donors was more variable. To lend further support to the assertion that VSMCs were killed via death pathway triggering, CD4 T cells were incubated on VSMCs transfected with dominant-negative (DN)–FADD–GFP or control GFP plasmid (Fig. 2 b). Blocking the function of FADD led to a marked decrease in the apoptosis rate ($P = 0.02$). These experiments established that CD4 T cells induced VSMC apoptosis by triggering the death pathway.

VSMCs express death receptors and are susceptible to TRAIL-mediated apoptosis

The expression of death receptors on VSMCs was examined by RT-PCR and FACS analysis. RT-PCR yielded positive results for three death receptors, DR4, DR5, and Fas. The transcripts for DR5 and Fas were abundant, whereas the signal for DR4 was only faint (Fig. 3 a). To assess surface expression

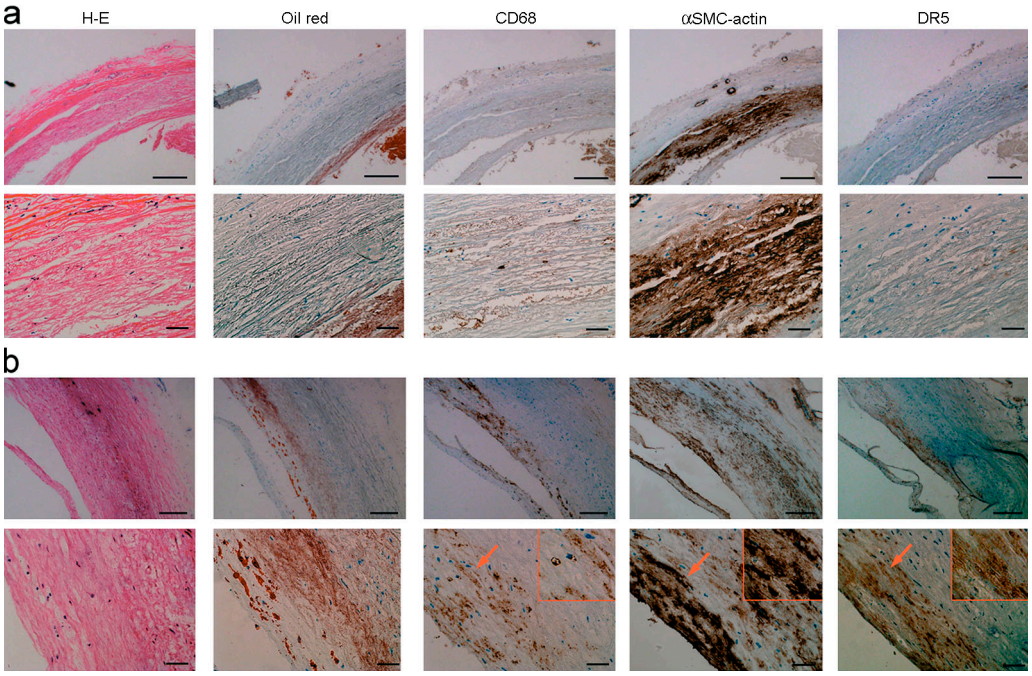

Figure 4. VSMCs in atherosclerotic plaque express TRAIL-R2/DR5. Serial frozen sections from normal carotid artery wall (a) and carotid artery plaque (b) were stained with hematoxylin-eosin, oil-red, anti-CD68 mAb, anti–α-smooth muscle cell actin Ab,and anti-DR5 Ab. Bound primary antibodies were visualized with DAB (brown).Original magnifications of × 40 (top) and × 200 (bottom) are shown. Bars; (top) 500; (bottom) 100 μm. Insets represent higher magnification of areas indicated by arrows. Results are representative for immunohistochemistries from 11 carotid artery plaques.

of these receptors, VSMCs were stained with antibody and analyzed by FACS. DR5 and Fas were present at high membrane density, whereas DR4 was not detected (Fig. 3 b).

To examine whether VSMCs are sensitive to death receptor–mediated killing, VSMCs were incubated with rh-TRAIL. Apoptotic cells were identified according to morphology and DAPI nuclear staining. Cells that were spread out on the surface and displaying normal nuclei were considered viable, whereas cells that had acquired a round shape and fragmented nuclei were considered apoptotic. Rh-TRAIL was highly effective in inducing apoptosis. After 12 h of exposure, >50% of cells had typical nuclear disintegration and lost adherence to the surface (Fig. 3c).

To examine whether DR5 expression in VSMC lines appropriately reflected tissue resident cell phenotypes in the atherosclerotic plaque, serial frozen sections from carotid arteries were stained with anti-CD68 mAb, to identify tissue-infiltrating macrophages; with anti–α-smooth muscle cell actin, to identify VSMCs; and with anti-DR5 antibody (Fig. 4). Atherosclerotic plaques were identified by oil-red staining. The plaque-free carotid artery wall served as the control. DR5 was expressed on VSMCs in the plaque cap region, but not on VSMCs in normal carotid artery walls.

CD4 T cells induce VSMC apoptosis through TRAIL

To determine whether TRAIL or FasL were involved in CD4 T cell–mediated VSMC injury, anti-TRAIL and anti-FasL antibodies were added to the T cell–VSMC cultures. As shown in Fig. 5 a, TRAIL-reactive antibody inhibited VSMC apoptosis. In the presence of control antibody, ∼45% of VSMCs were killed at an effector–target ratio of 5:1. To reach similar levels of apoptosis in the presence of anti-TRAIL mAb, four times as many T cells (effector–target ratio of 20:1) were necessary, suggesting that TRAIL was responsible for >75% of the cytotoxic activity. Antibodies blocking Fas–FasL interaction had no effect on the level of VSMC apoptosis. Inhibition by anti-TRAIL antibody was only seen for the CD4 T cells deriving from ACS patients. Apoptosis levels in control T cell cultures were unaffected by TRAIL blockade. Similar results were obtained in experiments using anti-DR5 antibodies. The rate of VSMC apoptosis induced by CD4 T cells from ACS patients was reduced by ∼50% when the TRAIL–DR5 interaction was inhibited by anti-DR5 Ab (Fig. 5 b).

To examine whether TRAIL-expressing CD4 T cells were increased in frequency in patients with ACS, PBMC from patients and age-matched controls were analyzed by FACS for the membrane expression of TRAIL. TRAIL expression on CD4 T cells required cell activation; spontaneous expression was absent in ACS patients and controls. This result was consistent with the observation that cytotoxic activity was dependent on the recognition of HLA-DR molecules (Fig. 1 b). After TCR triggering, TRAIL expression was significantly increased ($P = 0.002$) in ACS patients compared with controls (Fig. 5 c).

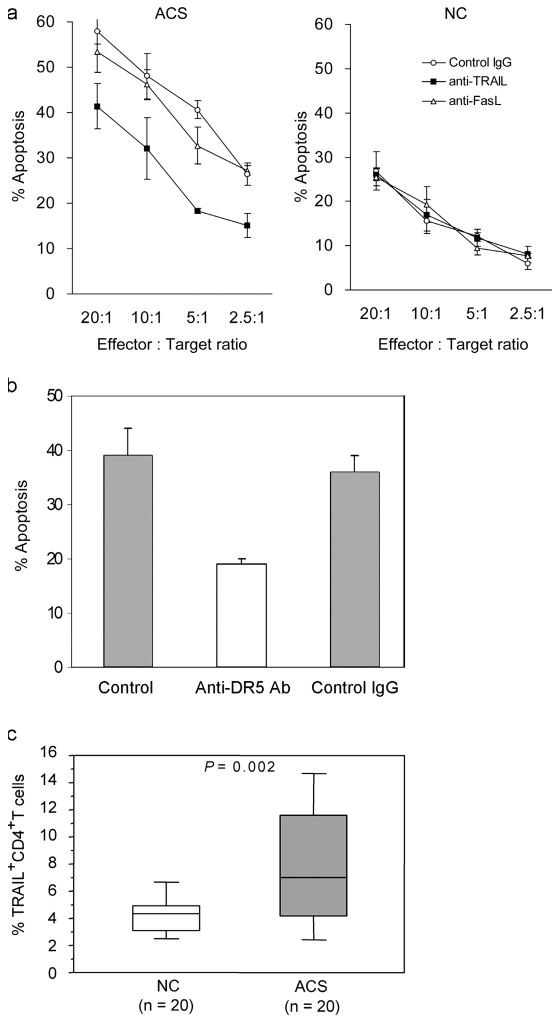

Figure 5. CD4 T cell–induced VSMC apoptosis is TRAIL-mediated. (a) CD4 T cells from patients with ACS and normal controls (NC) were pretreated with anti-Fas ligand, anti-TRAIL, or isotype control IgG_{2a} antibody. After 1 h, T cells were added to VSMC monolayers at the indicated effector–target ratios. Frequencies of apoptotic VSMCs were assessed after 4 h. Apoptosis rates are given as mean ± SD of triplicate cocultures. Results are representative of five ACS patients. (b) CD4 T cells from an ACS patient were cocultured with VSMCs in the presence of a soluble-anti-DR5 mAb or isotype-matched control IgG at an effector to target ratio of 20:1. Apoptotic rates were determined after 4 h and are given as mean ± SD of triplicate cultures. (c) Peripheral blood lymphocytes were stimulated with cross-linked anti-CD3 for 2 h, and TRAIL expression on CD4 T cells was examined by flow cytometry. Results from 20 patients with ACS and 20 age-matched controls are shown as box plots.

CD4 T cells induce VSMC apoptosis in atherosclerotic plaque

To test the relevance of CD4 T cell–mediated killing of VSMCs in vivo, we implanted carotid plaque tissue into immunodeficient mice and adoptively transferred CD4 T cells into the chimeras. Mice engrafted with carotid artery specimens lacking atherosclerotic plaques served as controls. T cell lines were established from a plaque of the same artery that was implanted into the mice. 7 d after implantation, full engraftment was achieved, and 6×10^6 CD4 T cells were injected intravenously into the chimeras; 24 h later, the human tissue grafts were explanted. In addition to the adoptive transfer experiments in which the T cells and atheroma tissue derived from the same donor, CD4 T cell lines were also injected into chimeras engrafted with heterologous tissue. Infiltration of adoptively transferred T cells into the graft tissue was assessed by quantifying TCR-specific transcripts. TRAIL expression in the tissue, before and after T cell transfer, was measured by real-time PCR. As shown in Fig. 6 a, T cell–derived TCR mRNA was low in control carotid wall grafts that lacked plaque formation. In contrast, TCR sequences in the tissue increased more than fourfold, from a median of 1,800 copies to a median of 7,508 copies when CD4 T cells were injected into chimeras implanted with actively inflamed carotid artery plaque. In parallel, the tissue expression of TRAIL in the plaque tissue increased 2.5-fold, whereas levels remained unchanged in control tissue. Results were similar, irrespective of whether adoptively transferred CD4 T cells and plaque tissues derived from the same or different patients. These data suggested that the inflamed plaque tissue facilitated CD4 T cell recruitment and retention and that the infiltration of CD4 T cells into the tissue site was associated with enhanced expression of TRAIL.

To determine whether adoptive transfer of CD4 T cells induced VSMC apoptosis, tissue sections of the explanted grafts were analyzed for the frequency of $TUNEL^+$ cells. As expected, the frequency of $TUNEL^+$ cells was higher in the plaques than in noninflamed control wall sections (Fig. 6 b). Areas with apoptotic cells were patchy and cosegregated with areas containing inflammatory infiltrates. After CD4 T cell transfer, distribution patterns of apoptotic cells continued to be discontinuous. Immunohistochemical stains showed that VSMCs accounted for the majority of apoptotic cells in the lesion (Fig. 6 b). The percentage of $TUNEL^+$ cells in these patchy areas with inflammatory infiltrates increased from 20% to >60% in the adoptively transferred mice (Fig. 6 c), demonstrating that infusion of CD4 T cells resulted in massive VSMC injury.

CD4 T cells mediate VSMC apoptosis in vivo through the TRAIL pathway

To examine whether the TRAIL pathway was involved in CD4 T cell–mediated apoptosis in vivo, TRAIL-blocking antibodies were included in the adoptive transfer experiments. 6×10^6 CD4 T cells were coadministered with anti-TRAIL mAb into chimeras implanted with autologous carotid tissue. As controls, mice implanted with the same tissue were either treated with 6×10^6 CD4 T cells combined with isotype-matched control antibody or injected with control antibody only. Results shown in Fig. 7 demonstrated that 10–20% of the cells in the inflamed plaque tissues recovered from mice treated with only control IgG antibody were apoptotic. Adoptive transfer of CD4 T cells in combination with control IgG antibody resulted in massive apoptosis in the lesion. After injecting

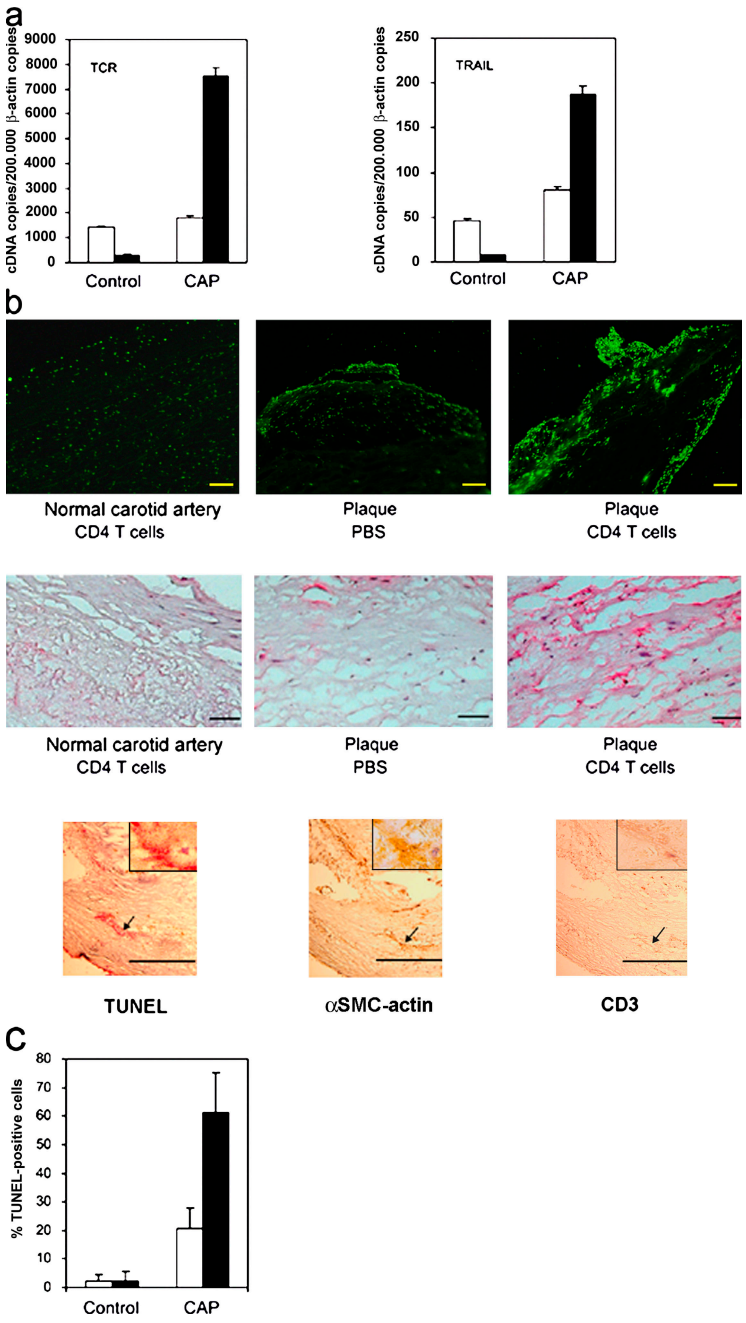

Figure 6. Adoptively transferred CD4 T cells infiltrate into atherosclerotic plaque and induce apoptosis. (a) Carotid artery plaque tissue (CAP) and control carotid artery wall lacking plaque were implanted into SCID mice. 7 d after implantation, the chimeras received 6×10^6 CD4 T cells (shaded bars) or PBS (open bars) by intravenous injection. 24 h later, the human tissue grafts were explanted, and TCR α-chain mRNA and TRAIL mRNA were measured by real-time PCR. cDNA concentrations were adjusted to 200,000 copies β-actin. Results from one of eight experiments are shown as mean ± SD of triplicate measurements. (b) Control carotid artery tissue and carotid artery plaque tissue were engrafted into mice. 6×10^6 CD4 T cells were adoptively transferred into the mice; control animals received PBS. Apoptotic cells were detected by staining for $TUNEL^+$ cells (Vector red). Representative immunofluorescence images (top, original magnification, 100) and immunohistochemistry images (middle, original magnification, 200) are shown. Bars: (top) 200 μm; (middle) 100 μm. Staining of serial sections showed that $TUNEL^+$ cells are α-smooth muscle actin-expressing cells and not $CD3^+$ cells (bottom, original magnification, 100; insets, 400). Bars, 500 μm. (c) Quantitative assessment of apoptotic cells in arterial control and plaque tissue (CAP) that had been engrafted into immunodeficient mice is shown. Mice were given PBS (open bars) or were adoptively transferred with CD4 T cells (shaded bars). Data are expressed as the mean ± SD percentage of $TUNEL^+$ cells in five visual fields and are representative of eight adoptive transfer experiments.

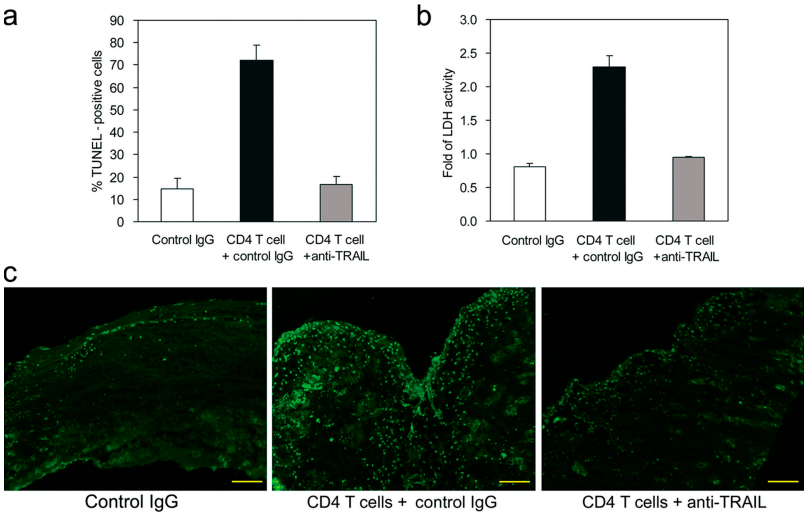

Figure 7. TRAIL-expressing CD4 T cells mediate VSMC apoptosis in vivo. CD4 T cells were isolated from carotid artery plaque and expanded in vitro. SCID mice were implanted with pieces of the same plaque tissue. Engrafted mice received an intravenous administration of control IgG_{2a} (300 μg/mouse; open bars), 6×10^6 CD4 T cells and control IgG_{2a} (300 μg/mouse; shaded bar), or 6×10^6 CD4 T cells and anti-TRAIL antibody (300 μg/mouse; shaded bar). Blood was drawn from the mice before and 24 h after T cell transfer. Human artery grafts were harvested 24 h after treatment and processed for immunohistochemistry. (a) Apoptosis rates in tissue-residing cells were measured by the TUNEL assay. Results from one of eight experiments are shown as mean ± SD of triplicate visual fields. (b) CD4 T cell–mediated injury of VSMCs in the tissue was estimated by determining the activity of LDH in the serum of the chimeras. Results are expressed as the mean ± SD fold-increase of LDH activity after T cell transfer and are representative of eight experiments. (c) Immunohistochemistry stains from a representative adoptive experiment are shown. Compared with the spontaneous rate of $TUNEL^+$ cells (green fluorescence, left), injection of autologous CD4 T cells caused a marked increase of apoptotic cells (middle). Anti-TRAIL mAb coadministration prevented the increased apoptosis after CD4 T cell transfer (right). Original magnification, 100. Bar, 200 μm.

CD4 T cells, >70% of VSMCs in the lesion stained $TUNEL^+$ (Fig. 7 a). Apoptosis rates returned to baseline when CD4 T cells were coadministered with TRAIL-blocking antibody. In contrast, TCR mRNA in the tissues was equal, irrespective of whether anti-TRAIL or control Ab was administered, indicating that anti-TRAIL Ab did not influence T cell infiltration and survival.

To use a different measure of VSMC death, we analyzed the leakage of lactate dehydrogenase (LDH) from the arterial tissue. As shown in Fig. 7 b, the activity of LDH in the chimeras' blood remained stable for >24 h in mice treated with control antibody. Infusion of 6×10^6 CD4 T cells resulted in a twofold increase in LDH activity, supporting the interpretation that the T cells had a direct role in damaging VSMCs. This T cell–mediated enhancement of LDH activity could be blocked by coadministration of anti-TRAIL antibody. These data demonstrated that CD4 T cells have a role in inducing VSMC apoptosis in vivo and that they use TRAIL to trigger VSMC death.

DISCUSSION

The loss of VSMCs in the fibrous cap and accelerated degradation of the collagen matrix have been postulated as critical mechanisms in transforming stable plaque into rupture-prone lesions (31, 32). We have demonstrated that CD4 T cells kill cultured VSMCs by expressing the TNF-like ligand TRAIL and by triggering death receptors on the surface of VSMCs. Patients with ACS have higher frequencies of apoptosis-inducing CD4 T cells in circulation, suggesting an abnormality in T cell differentiation that predisposes these patients to T cell–mediated VSMC damage, plaque rupture, and ischemic manifestations of atherosclerosis.

Recent evidence suggests that the thin-cap fibroatheroma is the major precursor lesion to ACS (31). Plaque-infiltrating macrophages have been implicated in plaque destabilization through the production of metalloproteinases and, possibly, other tissue-injurious mediators (7–9, 33). Macrophages cultured with VSMCs for 7 d kill VSMCs in a Fas–Fas ligand and TNF-dependent manner (8, 9). Also, unstable lesions contain a high frequency of activated T cells, which could directly promote the death of plaque-residing cells (10, 11, 34). Apoptotic cells can be identified in the unstable plaque in vivo and include cells of the inflammatory infiltrate but also VSMCs (18, 35, 36); the absolute number of apoptotic VSMCs in these studies is likely an underestimate given that apoptotic cells are rapidly cleared by macrophages in vivo. In the present study, CD4 T cells equipped to induce apoptosis of cultured VSMCs not only accumulated in the plaque, but also circulated in the blood of patients with ACS. In vivo studies with human carotid plaque–SCID chimeras confirmed the ability of CD4 T cells to infiltrate into the lesion and mediate VSMC killing. The human–mouse chimera model likely is a more sensitive system to assess apoptosis than the natural host because the chimeric mice do not have circulating human monocytes, and

murine monocytes hardly enter the human tissue. The inhibitory effect of anti-TRAIL, both in vitro and in vivo, established that this TNF-like molecule was the major inducer of VSMC apoptosis.

T cells have been implicated in the pathogenesis of atherosclerosis and probably contribute to multiple aspects of the disease process (37). They consistently accumulate in complex lesions; and T cell deficiency reduces lesion size and cellular infiltration (38, 39). Also, multiple cytokines intimately involved in T cell function (such as IL-15, IL-12 and IL-18), are expressed in the lesions and directly regulate plaque inflammation and lesion size (40–43). We focused on T cells as effector cells and not on their role as key regulators of in situ immunity. CD4 T cells, the dominant lymphocyte population in human atheromas, are usually not considered to lyse neighboring cells. However, CD4 T cells isolated from lesions that cause fatal myocardial infarction have a unique phenotype and functional profile (4, 13). They lack expression of CD28 and, instead, have acquired a new set of immunoregulatory receptors. Stimulation of killer immunoglobulin-like receptors, selectively expressed on CD4 T cells in ACS, facilitates cytolysis and has been shown to lyse endothelial cells (15, 16).

TCR triggering induces TRAIL expression on T cells, but cytokines, such as IFNs, can modulate this response (44). TRAIL is stored in cytoplasmic vesicles and can be rapidly brought to the cell surface. Spontaneous expression of surface TRAIL on CD4 T cells from patients with ACS is not increased, suggesting that cross-linking of the TCR is necessary to initiate the cascade leading to T cell–mediated cytotoxicity. This is further supported by the finding that the activity of CD4 T cells is dependent on the recognition of MHC class II molecules. Different antigens have been implicated in driving the response of T cells in the atherosclerotic lesion, including oxidized low density lipoprotein (45, 46) and chlamydial heat shock proteins (47). $CD4^+CD28^-$ T cells proliferate when activated with HSP60 (48). The possibility remains that a spectrum of antigens, not just a single antigen, is responsible for the stimulation of tissue-infiltrating T cells. T cells from patients with ACS are prematurely aged and have characteristics of senescent cells (49). Senescent T cells are less stringent in antigen-specific responses to enter activation. Cumulative gene expression of regulatory molecules can partially or sometimes even completely substitute for TCR-mediated signals, rendering T cells responsive to antigen-nonspecific cues in their microenvironment (15, 50). This may also explain why CD4 T cell–induced apoptosis, although dependent on the recognition of MHC class II molecules, was not restricted by MHC polymorphisms.

TRAIL is best known for its tumoricidal activity. Recombinant soluble forms of TRAIL have been found to induce cell death predominantly in transformed cells while not affecting normal tissues. However, TRAIL receptors are widely expressed on many cell types, and the physiologic functions of this TNF family member appear to be rather broad (21, 51). Besides playing a suspected role in T cell and NK cell tumor immunosurveillance, TRAIL has been implicated in the thymic clonal deletion of autoreactive T cells and may be involved in peripheral homeostatic T cell compartment control (26, 52). Data presented here reinforce previous papers asserting that TRAIL causes tissue injury in untransformed cells. In cholestatic hepatocyte injury, bile acids facilitate TRAIL receptor oligomerization and activation (53). Human endothelial cells were found to express DR4 and DR5, and ~30% were susceptible to TRAIL-induced apoptosis. When TRAIL was injected into human skin xenografts, it caused focal injury to tissue-residing endothelial cells (54). Other cell types susceptible to TRAIL-mediated apoptosis include developing erythrocytes (55), prostate cells, and T lymphocytes. Controversial results have been reported regarding TRAIL's effect on normal T lymphocytes, ranging from apoptosis resistance (56) and induced cell cycle arrest without cell death (57) to TRAIL-stimulated apoptosis (58). Also, studies on TRAIL's critical role in establishing and maintaining immunologic tolerance by regulating negative selection of thymocytes have not been confirmed by other investigators (59). Insensitivity to TRAIL's apoptotic effects has been recently associated with the high expression of FLICE inhibitory protein, which provides a mechanism through which TRAIL-producing T cells and NK cells protect themselves while using TRAIL to kill DR5-expressing target cells (56).

VSMCs, both in the atherosclerotic plaque as well as in vitro, strongly expressed DR5, whereas DR4 was essentially absent. Within hours, recombinant TRAIL induced apoptosis in the majority of cultured VSMCs. Such cultured DR5-expressing VSMCs were highly sensitive to TRAIL-expressing CD4 T cells. In vivo transfer of CD4 T cells resulted in an increase of apoptotic VSMCs in the plaque, which could be blocked by anti-TRAIL Ab. CD4 T cells able to be activated by VSMC and to express TRAIL on the cell surface are abundant in ACS patients. A previous study has shown that these T cell abnormalities are not short-lived, likely precede the acute event, and certainly persist over time (12). Also, the presence of such T cells is not unique for ACS but is also found in other chronic diseases (60). The sole presence of such T cells is therefore not sufficient to precipitate disease manifestations. Rather, rate-limiting steps may be the access of these T cells to the vascular wall compartment and expression of DR5 on VSMCs. The mechanisms of DR5 expression are unknown, but the susceptibility of VSMCs to TRAIL-mediated apoptosis was such that it should raise concerns when considering using TRAIL as an anti-tumor agent in humans. T cells may gain access to the atherosclerotic plaque in patients with ACS via one of two routes: they may enter from the macrolumen; or, more likely, they may enter from the network of microcapillaries that develops in the unstable plaque. The mouse chimera model only partially resembles this situation, because T cells must enter exclusively from the microvasculature. Regardless, recruitment will be driven by chemokines produced in the tissue, and these chemokines are likely responsible for the preferential recruitment of T cells into the plaque compared with normal

artery tissue as shown in Fig. 6. Once in the tissue, CD4 T cells are being activated and exert effector functions through TRAIL (Fig. 7).

Implicating CD4 T cells and their ability to up-regulate TRAIL surface expression in plaque destabilization could have direct consequences for the therapeutic approach to patients with vulnerable lesions. Inhibiting TRAIL or TRAIL-producing CD4 T cells and restoring resistance to DR5 triggering in VSMCs could have therapeutic potential for the acute management of patients with unstable angina and myocardial infarction.

MATERIALS AND METHODS

Study population. 25 consecutive patients admitted with ST elevation myocardial infarction and 25 patients treated for unstable angina were enrolled in this study. Blood samples were drawn at the time of admission and processed immediately. 33 age- and sex-matched control subjects had no history of autoimmune disease and no risk factors for cardiovascular disease. Control patients with stable coronary artery disease were matched for common risk factors such as hyperlipidemia, smoking, diabetes mellitus, and hypertension. Carotid artery specimens were collected from patients undergoing endarterectomy procedures. All specimens were examined for the presence and absence of inflammatory infiltrates and plaque. End fragments of the specimens lacking plaque formation and mononuclear cell infiltrate were used as controls. The protocols were approved by the Mayo Clinic Institutional Review Board and the Emory University Institutional Review Board, and appropriate consent was obtained.

Cells. CD4 T cells were isolated from PBMC by negative selection (RosetteSep; StemCell Technologies, Inc.). Purity was 95–98%. Importantly, purified cells did not include any NK cells. Human COR-SMCs were grown on collagen-coated tissue culture plates in SmGM-2 smooth muscle medium (Cambrex). Human carotid artery smooth muscle cell lines (CA-SMC) were established from carotid artery plaque using previously described methods (61). T cell lines were isolated from carotid endarterectomy samples by culturing tissue fragments with 50 U/ml recombinant human (rh) IL-2 (CHIRON). After 7 d, tissue-derived T cells were stimulated with 10^6/ml irradiated PBMCs, 1.5×10^5/ml irradiated EBV-transformed B cells, 30 ng/ml anti-CD3 mAb (Ortho-Clinical Diagnostics), and 50 U/ml rh-IL-2. CD4 T cells were purified by fluorescence-activated cell sorting. Phenotypically, tissue-derived CD4 T cells were different from peripheral CD4 T cells in that they all expressed memory markers and the frequency of CD28 loss was increased (unpublished data).

Analysis of VSMC apoptosis. Apoptosis assays were performed using nuclear fragmentation and condensation of VSMC stained with 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma-Aldrich) as readout. Pilot studies showed an excellent correlation of the apoptosis rates by DAPI staining and by Annexin V staining (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20051062/DC1). Also, costaining of Annexin-positive VSMCs with PI clearly indicated that cell damage was irreversible. To control for subjective interpretation, the reader was blinded as to whether the T cells derived from a patient or control individual. COR-SMCs and CA-SMCs that reached confluence were seeded into collagen-coated 96-well plates at a cell density of 10^4 SMCs/well and incubated with 1 μg/ml DAPI in RPMI supplemented with 1% FBS for 1 h at 37°C. Cultures were washed and purified. CD4 T cells or resting CD4 T cell lines were added to the VSMC monolayer for 4 h at 37°C. VSMCs were examined with fluorescence microscopy, and apoptosis rates were determined. Parallel assays were performed by staining for 4 h T cell–VSMC cocultures with Annexin V. In selected experiments, COR-SMCs were preincubated for 1 h with 1.25 μg/ml to 10 μg/ml anti-MHC class-ABC mAb (W6/32) or anti–HLA-DR mAb (L243) before coculture with T cells. In other experiments, COR-SMCs were transfected with a DN-FADD-GFP–containing plasmid (0.05 μg/well; pcDNA3-GFP-ΔFADD, a gift from G.J. Gores, Mayo Clinic, Rochester, MN) or a GFP-expressing control plasmid pE-GFP-N1 using Opti-MEM I (100 μl/well; Invitrogen-GIBCO) containing LipofectAMINE (0.4 μg/well; Invitrogen-GIBCO) and LipofectAMINE Plus reagent (0.6 μl/well; Invitrogen) (53). Cells were incubated for 4 h at 37°C in a 5% CO_2, 95% air incubator. 24 h later, transfection efficiency was determined by analyzing GFP expression using fluorescence microscopy. In other experiments, COR-SMCs were treated with a caspase-8/granzyme B inhibitor (25 μg/ml; caspase-8 inhibitor I; Calbiochem) for 1 h before apoptosis analysis.

To investigate the sensitivity of VSMCs to TRAIL-mediated apoptosis, VSMCs were incubated with 400 ng/ml of rh-soluble Flag-tagged TRAIL (Qbiogene) and 2 μg/ml of cross-linking FLAG-tagged antibody M2 (Sigma-Aldrich) for 12 h before being analyzed for typical nuclear fragmentation.

To inhibit Fas–Fas ligand–mediated apoptosis, CD4 T cells were incubated with 25 μg/ml of anti–Fas ligand mAb (NOK-1; Becton Dickinson) for 1 h, and then cocultured with VSMCs. TRAIL–DR5 receptor–ligand interactions were blocked with 25 μg/ml of mouse anti-human TRAIL mAb (RIK-2; Becton Dickinson) or 50 μg/ml anti-DR5 mAb (Diaclone). IgG mouse mAbs were used as isotype control.

RT-PCR and real-time PCR. Total RNA was purified from VSMCs using TRIzol (Life Technologies) and reverse-transcribed using AMV reverse transcriptase (Roche Molecular Biochemicals). The liver tumor cell line HepG2 served as positive control. cDNA was amplified with the following primers: DR4, 5′-CAGAACGTCCTGGAGCCTGTAAC-3′ and 5′-ATGTCCATTGCCTGATTCTTTGTG-3′; DR5, 5′-TGCAGCCGTAGTCTTGATTG-3′ and 5′-GCACCAAGTCTGCAAAGTCA-3′; Fas, 5′-TCGTCCAAAAGTGTTAATGCC-3′ and 5′-AAGAAGAAGACAAAGCCACCC-3′; and β-actin, 5′-CATGGTGGTGCCGCCAGACAG-3′ and 5′-ATGGCCACGGCTGCTTCCAGC-3′. RT-PCR products were separated by electrophoresis in 2.0% agarose containing ethidium bromide.

Tissue expression of TCR and TRAIL mRNA was analyzed by real-time PCR. The following primers were used in these experiments: TCR α-chain, 5′-CCTTCAACAACAGCATTATTCCAG -3′ and 5′-CGAGGGAGCACAGGCTGTCTTA -3′; and TRAIL, 5′-ACCAACGAGCTGAAGCAGAT -3′ and 5′-CAAGTGCAAGTTGCTCAGGA -3′. Transcripts for each gene were quantified using PCR instruments (Mx 3000 and Mx 4000; Stratagene). 1 μl cDNA was mixed with a total volume of 50 μl SYBR green master mix (5 μl 10 × PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM each dNTPs, 0.025% BSA, 0.2 μl 5 U/μl Plt. Taq, 1:20,000 SYBR green, and 0.1 μM each primer). PCR amplification protocol involved 40 cycles of denaturation at 95°C for 30 s, primer annealing at 55°C, and primer extension at 72°C for 60 s. cDNA concentrations were adjusted to 2×10^5 copies of β-actin. All PCR reactions were performed in triplicate.

Flow cytometry. For flow cytometric analysis of surface death receptors, VSMC lines were stained with mouse anti–human DR4-PE, mouse anti–human DR5-PE (both eBioscience), and mouse anti–human Fas-PE mAb (BD Biosciences). Cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson). Data were analyzed with WinMDI software (Joseph Trotter, Scripps Research Institute).

TRAIL expression on CD4 T cells was determined by two-color staining with anti-CD4-PerCP mAb and anti-TRAIL-PE mAb (both Becton Dickinson). PBMC were stimulated with 250 ng/ml anti-CD3 mAb (Ortho Biotech) or soluble IgG_{2a} mAb for 2h in the presence of $FcRII^+$ P815 cells. Again, liver tumor cells were used as positive controls.

Immunohistochemistry. Frozen tissues embedded in OCT compound were cut into 5-μm sections, fixed in cold acetone for 10 min, dried, and soaked in 1% paraformaldehyde solution for 5 min. After blocking endogenous peroxidase and incubating with 5% normal goat serum (Invitrogen), the sections were stained for 1 h with affinity-purified rabbit anti–human DR5 mAb (1:200; R&D Systems, Inc.) followed by biotin-conjugated goat

anti–rabbit Ig antibody (1:400; DAKO) for 30 min at room temperature. The color reaction was developed by ABC-peroxidase using 3, 3′-diaminobenzidine (DAB). The tissue sections were washed with tap water, and the slides were counterstained with hematoxylin (Vector Laboratories, Inc.) for 2 min. Serial sections were stained with anti α-SMC actin mAb (1:50) and anti-CD68 mAb; stains were developed with the DAKO ChemMate Envision kit (all DAKO Japan). Hematoxylin-eosin staining was performed on 95% ethanol fixed sections; oil red O staining was performed on 10% formalin fixed sections.

The TUNEL technique was used to quantify apoptotic cells in the tissue. The plaque tissues were cut into 5-μm sections and analyzed by the In Situ Cell Death Detection Kit, AP (Roche). The percentage of $TUNEL^+$ cells was calculated by counting $TUNEL^+$ cells per total nuclei. Serial sections were stained with anti-CD3 (1:200; DAKO) and anti–α-SMC actin mAb (1A4, 1:200; DAKO) followed by secondary biotinylated goat anti–mouse Ig antibody (1:400), ABC-peroxidase, and DAB.

CD4 T cells transfer into human artery SCID chimeras. 6–8-wk-old NOD.CB17-Prkdc^{scid}/J mice (NOD-SCID) or NOD-129S7 (B6)-Rag1 (*tm1Mom*) J mice (Jackson ImmunoResearch Laboratories) were engrafted with human carotid artery plaque tissue placed into a subcutaneous pocket on the midback. Engraftment of small tissue fragments (50–100 mm^3) is rapid, and the graft is vascularized by day 7 with or without minimal murine inflammatory infiltrate within the implanted human tissue. A previous study has shown that the microvasculature within the graft is of human origin connecting to murine granulation tissue encapsulating the graft (62). All animal procedures were approved by the Animal Care and Use Committee. Noninflamed sections of carotid artery walls lacking plaque formation were used as controls. On day 7 after implantation, the mice were injected intravenously with 6×10^6 CD4 T cells or PBS. 24 h after the cell transfer, the carotid artery grafts were explanted and shock frozen in liquid nitrogen for mRNA analysis or embedded in OCT compound (Sakura Fine-Tek) for immunohistochemical analysis.

In selected adoptive transfer experiments, the carotid artery chimera mice were injected intravenously with control IgG_{2a} mAb (300 μg/mouse), CD4 T cells (6×10^6 cells/mouse) combined with control IgG_{2a} mAb (300 μg/mouse), or CD4 T cells combined with anti-human TRAIL mAb (300 μg/mouse; Becton Dickinson) on day 7 after tissue implantation. After 24 h, the carotid artery grafts were explanted, and tissue apoptosis rates were analyzed by TUNEL staining.

To quantify the damage of human VSMCs, blood was drawn from the mice before and 24 h after T cell transfer. LDH activity was measured using a Toxico Assay kit (Sigma-Aldrich). All LDH determinations were performed in triplicate, background absorbance rates were subtracted, and changes in LDH activity were expressed as the ratio of LDH levels before and after T cell transfers.

Statistical analysis. Data were analyzed by Student's *t* test or Mann-Whitney U test, when appropriate. Results are shown as mean ± SD, when parametric, and as box plots with medians and percentiles, when nonparametric testing was used.

Online supplemental material. Fig. S1 shows apoptosis rates assessed by nuclear fragmentation of DAPI-stained VSMCs and by Annexin V staining that were highly correlated (r = 0.95). CD4 T cells were cocultured with VSMCs, and induction of apoptosis was assessed by fluorescence microscopy. Intermediate stages of apoptosis were determined by staining with Alexa Fluor-labeled Annexin V. Nuclear fragmentation was detected by DAPI staining. Membrane damage was measured by permeability to propidium iodide. The online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20051062/DC1.

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