Pathogen-Sensing Plasmacytoid Dendritic Cells Stimulate Cytotoxic T-Cell Function in the Atherosclerotic Plaque Through Interferon-α

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Background—Unstable atherosclerotic plaque is characterized by an infiltrate of inflammatory cells. Both macrophages and T cells have been implicated in mediating the tissue injury leading to plaque rupture; however, signals regulating their activation remain unidentified. Infectious episodes have been suspected to render plaques vulnerable to rupture. We therefore explored whether plasmacytoid dendritic cells (pDCs) that specialize in sensing bacterial and viral products can regulate effector functions of plaque-residing T cells and thus connect host infection and plaque instability.

Methods and Results—pDCs were identified in 53% of carotid atheromas (n = 30) in which they localized to the shoulder region and produced the potent immunoregulatory cytokine interferon (INF)-α. IFN-α transcript concentrations in atheroma tissues correlated strongly with plaque instability (P < 0.0001). Plaque-residing pDCs responded to pathogen-derived motifs, Cpg-containing oligodeoxynucleotides binding to toll-like receptor 9, with enhanced IFN-α transcription (P = 0.03) and secretion (P = 0.007). IFN-α emerged as a potent regulator of T-cell function, even in the absence of antigen recognition. Specifically, IFN-α induced a 10-fold increase of tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) on the surface of CD4 T cells (P < 0.0001) and enabled them to effectively kill vascular smooth muscle cells (P = 0.0003).

Conclusions—pDCs in atherosclerotic plaque sense microbial motifs and amplify cytolytic T-cell functions, thus providing a link between host-infectious episodes and acute immune-mediated complications of atherosclerosis. (Circulation. 2006;114:2482-2489.)

Key Words: immune system • inflammation • lymphocytes • muscle, smooth • plaque rupture • plasmacytoid dendritic cell • toll-like receptor

Atherosclerotic plaque inflammation is a well-established risk factor for rupture of the protective cap, subsequent thrombotic vessel occlusion, and acute coronary syndrome (ACS). Accordingly, recruitment of lymphocytes and antigen-presenting cells to the atheroma is a critical event in the transition of a stable atherosclerotic lesion to a vulnerable plaque. Although plaque-residing macrophages have been recognized as participants in the tissue injury leading to plaque destabilization, it has recently become clear that T cells also have tissue-injurious effector functions. In particular, plaque-infiltrating CD4 T cells have cytotoxic capability, harming endothelial cells (ECs) as well as vascular smooth muscle cells (VSMCs). It is unclear how such T cells are activated in the plaque environment.

Dendritic cells (DCs) residing in the plaque may facilitate in situ T-cell activation. DCs activate T cells by presenting antigen and providing costimulatory signals. DCs also affect T cells in an antigen-independent way by secreting cytokines, such as interleukin (IL)-12, which regulate the recruitment of plaque-infiltrating CD4 T cells. DCs colocalize with T cells in the plaque, emphasizing their potential role in modulating T-cell function in vivo. The presence of DCs in rupture-prone areas of the atheroma raises the possibility that they interfere directly with tissue-damaging effector pathways. The capacity of DCs to stimulate T cells depends crucially on their stage of maturation. Toll-like receptors (TLR) provide a powerful mechanism of DC maturation by recognizing pathogen-associated molecular patterns (PAMPs) that signal “danger” imposed by infection or tissue damage. Overexpression of TLR4 on circulating antigen-presenting cells in ACS and expression of TLR1, TLR2, and TLR4 in...
the atherosclerotic plaque have been described.13–16 Candidate TLRs in the atherosclerotic plaque include microbodies and (modified) autoantigens such as human heat shock protein 60 and oxidized low-density lipoprotein.17–19 To date, studies have focused on CD11c+ myeloid DCs in atherosclerotic plaques. However, DCs also include another major subtype, CD11c+CD123high plasmacytoid DCs (pDCs), which express TLR7 and TLR9.20 Oligodeoxynucleotides containing particular CpG motifs (CpG ODN) typically found in microbial DNA are recognized by TLR9 and facilitate abundant interferon (IFN)-α production.20

IFN-α is best known for its vital function in host immune responses that protect from infections. Specifically, IFN-α enhances cytotoxicity of CD8 and natural killer cells and induces the expansion and activation of Th1-polarized CD4 T cells.22,23 IFN-α amplifies the ability of CD4 T cells to kill tumor cells.24 Proapoptotic effects of CD4 T cells have recently been implicated in tissue-injurious responses in atherosclerotic plaques.25–27 TNF-related apoptosis-inducing ligand (TRAIL) is expressed on CD4 T cells and binds to death receptor (DR)5, which is upregulated on stressed VSMCs.8 DR5 ligation initiates the death pathway leading to VSMC apoptosis, a mechanism proposed to contribute to weakening of the fibrous cap.26–28

We examined whether pDCs have immunoregulatory functions in the atherosclerotic plaque and respond to PAMPs. We further investigated the role of IFN-α in regulating T-cell function through the induction of TRAIL, a powerful mediator of apoptosis implicated in cell death associated with plaque vulnerability.

Methods

Study Population

Blood was drawn from 31 patients (61% male, aged 55 ± 10 years) with ACS (32% ST-elevation myocardial infarction) at admission. Patients with infectious, autoimmune, or neoplastic disease were ineligible. Thirty-one sex- and age-matched individuals without cardiovascular disease served as controls. Thirty carotid artery specimens were collected from patients undergoing endarterectomy procedures (Table I in the online-only Data Supplement). We determined atherosclerotic plaque vulnerability using a score modified from Depre et al29 and Naghavi et al.4 The size of the lipid core (0 to 2 points), the density of the inflammatory infiltrate (0 to 2 points), and the presence of residual thrombus (0 to 1 point) were determined in each plaque by macroscopic and microscopic analysis (Table I in the online-only Data Supplement). We determined atherosclerotic plaque vulnerability using a score modified from Depre et al29 and Naghavi et al.4

Immunohistochemistry

Frozen carotid plaque tissues were cut into 5-μm sections, fixed in acetone for 10 minutes, and dried. Endogenous peroxidase was blocked, and 5% of the appropriate animal serum was added to inhibit nonspecific staining. Slides were stained with the following antibodies for 1 hour at room temperature: mouse anti-human IFN-α (1:400; BD Pharmingen, San Jose, Calif), mouse anti-human CD123 for pDCs (1:100; BD Pharmingen), mouse anti-human TLR9 (1:100; Imgenex, San Diego, Calif), mouse anti-human CD3 for T cells (1:200; Dako, Carpinteria, Calif), mouse anti-human CD11c for myeloid DCs (1:200; Dako), and rabbit anti-human von Willebrand factor (vWF) for endothelial cells (1:1000; Dako). A biotin-conjugated goat anti-mouse (Dako) or sheep anti-rabbit (Abcam, Cambridge, Mass) antibody served as secondary antibody (1:125 to 1:600, 30 minutes at room temperature). Brown color was developed with the use of a peroxidase solution (ABC-peroxidase kit; Vector Laboratories, Burlingame, Calif) and 3,3′-diaminobenzidine (DAB) (Dako) as chromogen. For double staining, red color was developed with the use of an alkaline phosphatase solution (ABC-AP kit; Vector Laboratories) and Vector Red (Vector Laboratories). Levamisole was used to inhibit endogenous alkaline phosphatase activity. Slides were counterstained with hematoxylin (Vector Laboratories) for 2 minutes.

Quantitative Reverse Transcriptase Polymerase Chain Reaction

Total RNA was isolated from shock-frozen endarterectomy and cell culture samples with the use of TRizol (InviGen Life Technologies, Grand Island, NY) and transformed into cDNA with avian myeloblastosis virus reverse transcription (RT) (Roche Molecular Biochemicals). cDNA was amplified with primers specific for IFN-α (5′-A T G C G G A C T C A C T G T G T 3′-5′-G T G T G T A T G A G -3′), TLR9 (5′-T G A G A C T C T C A G C C C A C T G -3′), TRAIL (5′-A C C A A C C A G C T C G A G A C A G T -3′ and 5′-C A A G T G C A G T G C A G -3′), and 5′-C A T G G T G T G C C C A C A C G A -3′, as described elsewhere.20 Amplifications were performed in a Mx3000 polymerase chain reaction (PCR) machine (Stratagene, Cedar Creek, Tex) under the following cycling conditions: denaturation at 94°C for 10 minutes, followed by 40 cycles at 95°C for 30 seconds, 55°C for 60 seconds, and 72°C for 60 seconds. For each sample, PCR reactions were done in triplicate. The level of gene expression was determined by interpolation with a standard curve. cDNA copy numbers are expressed relative to 2×106 β-actin copies.

Stimulation of Explanted Atherosclerotic Plaque Tissue

Fresh tissue from 6 soft lipid-rich carotid plaques was cut into small pieces. For each plaque, equal numbers of nonadjacent plaque pieces were randomly distributed into wells of a 48-well plate filled with RPMI medium that contained 10% fetal calf serum. Tissue fragments were stimulated with 100 μg/mL synthetic CpG ODN (2006, TCCTCGTTTTGGCTTGGTGCTG) for 24 hours. Stimulations were done in duplicate. After stimulation, tissue was shock-frozen for RNA isolation. In addition, IFN-α was measured in supernatants with an enzyme-linked immunosorbent assay (PBL Biomedical Laboratories, Piscataway, NJ).

Cells

Peripheral blood mononuclear cells were isolated from fresh blood with the use of Ficoll-Hypaque (Amershams Biosciences, Piscataway, NJ). CD4 T cells were isolated by negative selection (RosetteSep, StemCell Technologies, purity ≥94%). T-cell lines were isolated from carotid artery plaques by culturing tissue fragments with 50 U/mL recombinant IL-2 (Proleukin Chiron, Emeryville, Calif). Every 7 days, tissue-derived T cells were stimulated with 1×106/mL irradiated peripheral blood mononuclear cells, 1.5×106/mL irradiated Epstein-Barr virus–transformed B cells, 30 ng/mL anti-CD3 monoclonal antibody (Ortho Diagnostics), and 50 U/mL recombinant human IL-2. Tissue-derived CD4 T cells were memory T cells and lacked CD28 (data not shown). T cells were stimulated for 2 hours with IFN-α (200 U/mL, PBL, Biomedical Laboratories) or with 250 ng/mL anti-CD3 monoclonal antibody in the presence of FcRII F815 cells. Human coronary smooth muscle cells (SMCs) (Cambrex, Walkersville, Md) were grown in SmGM-2 smooth muscle medium (Cambrex).

Flow Cytometry

Multicolor flow cytometry analysis was performed by staining peripheral blood mononuclear cells and T cells for 30 minutes with
peridinin chlorophyll protein–conjugated anti-CD4 monoclonal antibody, phycoerythrin-conjugated anti-TRAIL monoclonal antibody, and respective isotype control antibodies (all Becton Dickinson, Franklin Lakes, NJ). Cells were analyzed with a FACSCalibur flow cytometer (Becton Dickinson) and WinMDI software (Scripps Research Institute).

Apoptosis Assays
Confluent coronary SMCs grown in collagen-coated 96-well plates were pretreated for 1 hour with the nuclear binding dye 6′-diamidino-2-phenylindole (DAPI) (1 μg/mL; Sigma-Aldrich, St Louis, Mo). Freshly isolated T cells or resting T-cell lines pretreated (Figure 1A) or at the base of the plaque (Figure 1C). In addition, IFN-α–producing cells were negative for CD11c (data not shown).

Results
IFN-α–Producing pDCs in the Atherosclerotic Plaque
Sixteen of 30 atherosclerotic plaques (53%) contained cells staining positive for CD123, a marker typically expressed on pDCs. CD123+ pDCs were located in the shoulder region (Figure 1A) or at the base of the plaque (Figure 1C). In addition, IFN-α–producing cells were restricted to these atheroma regions (Figure 1B, 1D). Double staining demonstrated that pDCs were the main source of IFN-α (>90%) (Figure 1C, 1D). The number of pDCs was 10-fold higher in unstable than in stable plaques (P<0.0001; Figure 1E). ECs, which can express low CD123 transcripts, were identified through the vWF marker and remained negative for IFN-α (data not shown).

IFN-α in the Vulnerable Atheroma
IFN-α production in the atherosclerotic plaque was confirmed at the transcription level by quantitative RT-PCR. Tissue extracts were prepared from 30 plaques and 12 unaffected parts of carotid arteries. Plaques showed higher concentrations of IFN-α transcripts than normal vessel walls (P<0.0001; Figure 2). To assess the relation between plaque vulnerability and IFN-α tissue levels, endarterectomy samples were graded according to the presence of thrombus, lipid content, and density of the inflammatory infiltrate (TLI score). IFN-α mRNA levels were markedly higher in plaques with high TLI scores than in noninflamed, nonthrombosed plaques (P<0.0001; Figure 2).

The TLR9 Ligand CpG ODN Induces IFN-α in Atherosclerotic Plaques
Immunohistochemical staining revealed TLR9 expression in the atherosclerotic plaque (Figure 3A). TLR9 transcripts correlated closely with IFN-α production in atheroma tissue,
IFN-α Induces TRAIL on CD4 T Cells

CD4 T cells are the dominant lymphocyte population in the atheroma, and IFN-α may modulate their function by affecting TRAIL surface expression. IFN-α-producing cells and T cells were typically positioned in close vicinity (Figure 4A, 4B). Examining 30 endarterectomy samples for TRAIL and IFN-α transcript levels revealed a close correlation of both markers ($r=0.67$, $P<0.0001$; Figure 4C). In addition, plaque tissue activation with CpG ODN was associated with a significant increase in TRAIL mRNA compared with unstimulated controls ($n=12$ per group) ($P=0.057$).

Immunomodulatory effects of IFN-α in CD4 T cells were examined in peripheral blood CD4 T cells collected from ACS patients. Two hours of stimulation was sufficient to upregulate TRAIL surface expression on CD4 T cells by 10-fold ($P<0.0001$; Figure 4E and 4F). Remarkably, IFN-α much more efficiently brought TRAIL to the T-cell surface than did triggering of the T-cell receptor through anti-CD3 antibody (Figure 4E). IFN-α exposure not only enhanced TRAIL surface expression, but it also markedly increased transcription of the TNF-like molecule ($P=0.009$; Figure 4G).

IFN-α–mediated TRAIL upregulation on CD4 T cells was not unique for ACS patients. Rather, peripheral blood CD4 T cells collected from age- and sex-matched controls responded similarly. However, IFN-α–induced TRAIL upregulation in plaque-derived CD4 T cells was more pronounced, with >90% of CD4 T cells positive for TRAIL (data not shown).

IFN-α Induces TRAIL-Mediated SMC Apoptosis

To explore the functional relevance of IFN-α–induced TRAIL expression on CD4 T cells, we investigated the death-inducing functions of such CD4 T cells. Coronary SMCs, which express the TRAIL receptor DR5, were chosen as targets. Plaque-derived CD4 T cells were added to coronary SMC monolayers for 4 hours at different effector to target ratios. CD4 T cells induced coronary SMC apoptosis very effectively (Figure 5A). Coronary SMC apoptosis was quantified by assessing the frequency of cells with typical nuclear changes of apoptosis through DAPI staining and by staining with the apoptosis mem-

Figure 2. Production of IFN-α in the atherosclerotic plaque. Carotid atheromas were collected by endarterectomy. mRNA was isolated from 30 plaque tissues and 12 carotid wall pieces free of atheroma. IFN-α cDNA copies were measured by quantitative RT-PCR and adjusted to $2 \times 10^5 \beta$-actin copies. Plaques were categorized on the basis of the presence of residual thrombus, the size of the lipid core, and the density of the cellular infiltrate, as described in Methods. Box plots show median (bold black lines), interquartile range (boxes), and values within 1.5 interquartile ranges from the upper or lower edge of the box (whiskers).

Figure 3. The TLR9 ligand CpG ODN induces IFN-α in the atherosclerotic plaque. Frozen sections from carotid atherosclerotic plaques were stained with anti-TLR9 antibody (A; DAB, brown, magnification $\times 200$, bar=200 μm). IFN-α and TLR9 transcript levels were determined by quantitative RT-PCR in extracts from 26 carotid endarterectomy samples (B). Tissue pieces from soft lipid-rich carotid plaques were cultured in the absence or presence of 100 μg/mL synthetic CpG ODN for 24 hours ($n=12$ per group). IFN-α mRNA production was measured in tissue extracts by quantitative RT-PCR (C). IFN-α release was determined in culture supernatants by enzyme-linked immunosorbent assay (D). Box plots were used for data presentation as described in Figure 2.
brane marker annexin. As shown in Figure 5B, coronary SMCs with intense DAPI staining of the nucleus double stained for annexin. IFN-α amplified CD4 T-cell killing ability at all effector to target ratios \((P=0.002; \text{Figure 5A})\). Incubating coronary SMCs with IFN-α alone did not induce apoptosis. Using peripheral CD4 T cells from 10 ACS patients, we found a consistent increase of coronary SMC apoptosis after pretreating CD4 T cells with IFN-α \((P=0.0003; \text{Figure 5C})\). Detection of apoptotic coronary SMCs by annexin confirmed enhanced coronary SMC apoptosis with IFN-α–pretreated CD4 T cells compared with untreated CD4 T cells \((23\pm3.5\% \text{ versus } 15\pm2.6\%; P=0.005)\). Apoptosis could be inhibited by blocking with a neutralizing antibody specific for TRAIL in a dose-dependent manner \((P=0.0002; \text{Figure 5D})\), documenting that CD4 T cells utilize TRAIL to trigger the SMC death pathway.

Discussion

CD4 T cells, the dominant type of lymphocytes in the atherosclerotic plaque,\(^{30}\) have powerful tissue-damaging functions. They are able to activate macrophages via IFN-\(\gamma\),\(^{31,32}\) inducing the release of matrix-degrading proteases. CD4 effector cells also have the ability to directly kill plaque-residing cells, including ECs and VSMCs.\(^{7,8}\) T cells are generally controlled through TCRs by recognizing antigen, yet a unique subset of CD4 T cells characterized by CD28 loss can be activated independently from TCRs.\(^{5}\) In this report we provide data that IFN-α released in the plaque controls CD4 T-cell cytotoxic effector functions. The efficacy of IFN-α in rapidly modulating CD4 T-cell function is not only independent from antigen recognition; it also outperforms signals initiated by TCR cross-linking. Indeed, plaque-residing CD4 T cells are remarkably sensitive to the IFN-α action. IFN-α biases CD4 T cells toward harming matrix-
after stimulation of plaque explants with the TLR9 ligand CpG ODN confirmed the functional relevance of TLR9 triggering for activation of IFN-α-producing cells in the intact tissue. With regard to human pathogens, viruses such as herpes simplex virus type 1, cytomegalovirus, and influenza virus stimulate pDCs to produce IFN-α via well-understood signaling pathways involving myeloid differentiation factor 88 and IFN regulatory factor 7.34,37,38 IFN-α production also plays a critical role in response to bacterial infections.39 The sensitivity of IFN-α-producing cells to multiple pathogens correlates well with the concept of the infectious burden, emphasizing the importance of numerous infections rather than the role of a single and specific pathogen.40 Multiple suspects have been found in the plaque,41–43 but data presented here would also allow systemic disease to modulate plaque inflammation. Circulating “danger” signals may trigger pDCs to produce IFN-α, potentially explaining the link between acute infections and inflammatory activation of vulnerable plaques.44

IFN-α released in close vicinity to lymphocytes in the atheroma effectively induced the expression of the apoptosis mediator TRAIL on CD4 T cells. Additional upregulation of TRAIL transcripts indicates that IFN-α not only induces surface expression of vesicle-stored TRAIL but also its transcription. As previously shown, CD4 T cells from ACS patients kill coronary SMCs in a TRAIL-dependent manner.8 In this report we demonstrate that IFN-α stimulation of CD4 T cells from ACS patients translates into even more pronounced coronary SMC apoptosis, a process that could be blocked by TRAIL-specific antibodies. Coronary SMCs abundantly express DR5 and are therefore susceptible to TRAIL.5 VSMC loss may result in structural weakening and rupture of the atheroma cap.26–28 Moreover, apoptotic VSMCs induce thrombin generation,45 accelerating the thrombotic occlusion of the artery.28 TRAIL may also induce apoptosis of other plaque-residing cells such as ECs.46 When the unique and powerful ability of IFN-α to induce TRAIL expression on CD4 T cells is considered, IFN-α–induced, TC cell–mediated apoptosis of plaque-residing cells appears to be an important mechanism in plaque destabilization. The presence of IFN-α–producing pDCs in the plaque shoulder and the association of IFN-α transcript with plaque vulnerability emphasize the potential role of IFN-α in inducing plaque rupture. Immunoregulatory function for IFN-α in coronary artery disease is also suggested by the recent recognition that complications of coronary disease are markedly accelerated in systemic lupus erythematosus, a syndrome characterized by the overexpression of IFN-α.47,48

The association of immune effector functions critically involved in host protection, such as the potent antiviral and antitumor effects of IFN-α via TRAIL-mediated killing of tumor cells or infected cells,24 with tissue injury reemphasizes the ambiguity of immune effector pathways. The coexistence of protective and harmful immune functions raises concerns about the cost-benefit ratio of therapeutic interventions targeting such pathways. Enhancing TRAIL–dependent immune reactions in cancer and hepatitis C may indeed put patients with coexisting advanced atherosclerosis at risk for plaque instability.
Our data suggest a model of plaque injury in which pattern recognition receptors such as TLR9 respond to infectious molecular patterns from both viral and bacterial sources and amplify tissue-damaging immune responses in the inflamed plaque. By releasing IFN-α, pDCs not only enhance host protection, but they also enable tissue-injurious effector functions that threaten the tissue integrity of the plaque. This mechanism of tissue damage requires the presence of IFN-α-responsive CD4 T cells in the plaque and sensitivity by plaque-residing cells, including VSMCs and ECs, to TRAIL, a requirement that is met in unstable lesions. TLR9 ligands initiating a cascade of events leading to cellular loss in the plaque may be derived from local sources in the plaque but also from infections distant from the vessel wall lesion. Transient peaks in circulating PAMPs may be sufficient to trigger plaque inflammation and plaque disruption. Protection from plaque instability may therefore require shielding the host from infectious episodes. Similarly, targeting pathways relevant in the immune-mediated injury of the plaque holds the promise for novel therapeutic interventions.

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Disclosures
None.

References
CLINICAL PERSPECTIVE

The immune system employs dendritic cells (DCs) to sense infections. Binding of microbial DNA motifs, so-called unmethylated CpG-containing oligodeoxynucleotides, by toll-like receptor 9 alerts plasmacytoid DCs (pDCs) and initiates immune responses. This article reports that pDCs populate inflamed atherosclerotic plaques, in which they preferentially sit in the shoulder region. When triggered with CpG-containing oligodeoxynucleotides, plaque-residing pDCs release high amounts of interferon-α (IFN-α), which induces neighboring T lymphocytes to express tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), a member of the tumor necrosis factor superfamily with potent apoptosis-inducing capability. TRAIL-expressing T cells function as killer cells that trigger death receptors on vascular smooth muscle cells and thus can facilitate plaque destabilization. This study connects the function of innate immune cells (pDCs) with those of the adaptive immune system (T cells) in the atherosclerotic plaque and provides a mechanism through which antimicrobial immune responses induce unintended tissue damage and plaque destruction. IFN-α has also emerged as a biomarker in patients with chronic autoimmune syndromes, such as systemic lupus erythematosus, and IFN-α–mediated hyperstimulation of cytotoxic T-cell function may represent an important mechanistic link between immune dysfunction and accelerated atherosclerosis in autoimmune patients.
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