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ORIGINAL CONTRIBUTION

Functional profile of activated dendritic cells in unstable atherosclerotic plaque

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Abstract *Background* Unstable atherosclerotic plaque typically contains an infiltrate of activated macrophages and activated T cells. This study established a functional profile of plaque-residing dendritic cells (DC) to examine whether they can function as Ag-presenting cells to facilitate in situ T-cell activation. *Methods* Carotid artery plaque tissues were collected from 19 asymptomatic and 38 symptomatic patients undergoing endarterectomy. Matched samples of normal coronary artery wall, stable nonruptured plaque, and eroded unstable plaque were harvested from patients with fatal myocardial infarction. Quantitative PCR and immunohistochemistry were used to analyze the tissues for markers of DC activation (CD83, CD86, CCL19, CCL21) and correlate them with T-cell activation (IFN-γ, TNF-α). *Results* Carotid artery plaques from patients with ischemic symptoms compared to asymptomatic patients were characterized by the presence of high amount of T-cells ($P < 0.01$) and tissue production of high levels of the T-cell cytokines IFN-γ ($P = 0.001$) and TNF-α ($P = 0.006$). Plaque tissues from patients with ischemic complications contained elevated levels of CD83 ($P < 0.001$), a marker of DC activation, and the DC chemokines CCL19 ($P = 0.001$) and CCL21 ($P < 0.02$).

Unstable coronary artery plaques were similarly correlated compared to carotid plaques from symptomatic patients with the accumulation of T cells ($P = 0.001$) and the production of T cell chemokines IFN-γ ($P = 0.001$) and TNF-α ($P = 0.002$).

Immunohistochemistry confirmed the presence of $CD83^+$ DC in the shoulder region of unstable plaques, where they produced the T cell-attracting chemokines CCL19 and CCL21. Mapping of activated DC demonstrated close contact between mature DC and T cells expressing the activation marker CD40 ligand (CD40L). *Conclusion* Activated and fully mature DC are represented in the inflammatory infiltrate characteristic for unstable carotid and coronary atheroma. Such DC produce chemokines, and thus can regulate the cell traffic into the lesion. Through the expression of the costimulatory ligand CD86, plaque-residing DC can augment T-cell stimulation and provide optimal stimulation conditions for T lymphocytes, resembling the microenvironment in organized lymphoid tissues.

Key words Atherosclerosis – dendritic cell – inflammation – T cell

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Introduction

Complications of atherosclerosis, especially acute coronary syndromes (ACS), have been linked to rupture of the atherosclerotic plaque, causing atherothrombosis and vessel occlusion [12, 19]. Rupture-prone atherosclerotic plaque is characterized by a large lipid core, a thin fibrous cap, neovascularization, and the presence of an inflammatory infiltrate, often localized in the shoulder region [3, 11, 13, 15, 41, 42]. Plaque-infiltrating inflammatory cells are composed of macrophages and T cells, predominately CD4 T cells [13, 36, 39, 42]. Evidence has been presented that such T cells are in a state of activation, suggesting that they have been exposed to stimulatory signals in the tissue microenvironment [27, 42]. T cells are thought to contribute to plaque inflammation and plaque instability through two fundamental pathways – by direct effector functions such as cytotoxicity [32] and through release of regulatory cytokines that orchestrate the functional activity of macrophages and vascular smooth muscle cells [25].

Little information is available on how T cells receive stimulatory signals in the atheroma. The classical pathway would include recognition of Ags displayed on the surface of Ag-presenting cells [24]. Recent data suggest that T-cell activity in the plaque may be determined by more than triggering of the Ag receptor. Costimulatory signals provided by accessory cells in the lesion may be able to circumvent the need for Ag recognition. Support for this concept comes from a recent study showing that stimulation of plaque-infiltrating CD4 T cells through killer immunoglobulin-like receptors induces cytotoxicity in the absence of Ag recognition [32].

T cells interact with Ag-presenting cells to receive the appropriate signals that initiate effector functions or modulate the functional activity in situ. While a number of different cell types can serve as Ag-presenting cells, the most potent APCs are dendritic cells (DC) [1, 2, 24, 25]. DC are typically localized at mucosal surfaces, in the skin and in lymphoid organs. As immature cells, DC are highly efficient in uptaking Ags. If triggered appropriately, they become migratory and travel to organized lymphoid tissues. Once they have arrived in the lymph node, DC home to the T-cell zones, become resident, and switch their functional profile away from Ag uptake and migration towards Ag presentation and costimulation. Mature DC placed in lymph nodes also are potent producers of chemokines such as CCL19 and CCL21, which attract CCR7-expressing naïve and central memory T cells [16, 35, 37]. These events are critical in the priming of naïve T cells and for inducing memory T-cell responses against Ags the host had been primed against in the past.

DC have been described as a resident cell population in atherosclerotic plaque [6]. Available data suggest that they may be in an activated state and thus are capable of providing the necessary signals to T lymphocytes to drive them into proliferation, enhance their survival, and trigger effector functions such as the release of regulatory cytokines [41]. The current study was designed to establish a functional profile of plaque-infiltrating DC in order to understand what their precise role is in initiating and maintaining plaque inflammation. Here we report that unstable coronary and carotid plaques are characterized by the accumulation of fully mature DC that are committed to the production of T cell-attracting chemokines. In the lesion, they co-localize with T cells that have recently undergone activation, supporting the concept that DC remain key immunoregulatory cells in the unstable plaque.

Materials and methods

Specimens

A total of 57 human carotid artery plaques were collected from patients undergoing endarterectomy: 38 patients had ischemic symptoms, such as transient ischemic attack or stroke, while 19 patients had asymptomatic carotid atherosclerosis. Approval of this study was given by the Mayo Clinic Institutional Review Board, and appropriate informed consent was obtained. According CD86, 26 human carotid artery plaques were collected, 16 plaque samples from symptomatic patients and 10 from asymptomatic patients. Approval of this study was given by the University Clinic Heidelberg Institutional Review Board, and appropriate informed consent was obtained.

Coronary artery tissues were derived from postmortem specimens of seven patients. In each patient, a sample of the culprit lesion that had caused the fatal ischemia was collected for further analysis. In addition, a piece of stable, unruptured plaque and a piece of normal, unaffected coronary artery, which served as controls, were harvested from each donor. Approval of this study was given by University of Essen Institutional Review Board, and appropriate informed consent was obtained.

The tissue samples were shock frozen in liquid nitrogen in order to extract cDNA or were embedded in OCT compound (Sikura Fine-Tek, Torrence, CA) immediately after endarterectomy and stored at –80 °C until use. OCT frozen samples were used for immunohistochemistry staining.

Quantitative-polymerase chain reaction (QPCR) in human atherosclerotic plaques

Optimal conditions for all primers used in this study were established by amplifying cDNA samples from human PBMC-derived mature DC, human-activated T cell

clones, human tonsils, and activated human PBMC. Sequences of primers used are listed in Table 1. Total cellular RNA from the plaques was isolated using TRIzol reagent (Invitrogen Life Technologies), reverse transcribed into single-stranded cDNA using AMV reverse transcriptase (Roche Molecular Biochemicals, Indianapolis, IN), and amplified by PCR on a Perkin-Elmer 9600 (Perkin-Elmer, Emeryville, CA) using specific primer pairs. Transcripts for the chemokines were quantified using the Stratagene Mx4000. The method to quantify tissue cytokine mRNA has been described in detail previously [30, 31].

The PCR thermal cycling procedures were as follows: initial denaturation at 95 °C for 30 s, followed by 40 cycles of amplification at 95 °C for 30 s, annealing at primer-specific temperature for 1 min, and 72 °C for 90 s. cDNA concentrations of the measured chemokines were adjusted to 200,000 copies of β-actin; only IFN-γ concentrations were adjusted to 2,000,000 β-actin copies.

To measure CD86, a cohort of carotid artery plaques was collected from patients undergoing a carotid endarterectomy. Total cellular RNA was isolated from carotid artery plaques, using the RNeasy kit by Qiagen (Hilden, Germany). Reverse transcription of 2 µg total RNA was performed using the Boehringer cDNA kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. cDNA was diluted to 1:10 and 2 µl were used for PCR. A Roche real-time PCR kit with SYBR Green (Roche Diagnostics, Mannheim, Germany) was used for quantitative PCR with the conditions being: 55 cycles total, each with 95 °C for 5 s, 57 °C for 10 s and 72 °C for 12 s. The sequence of the CD86 primer is shown in Table 1. Data were analyzed on the basis of the relative expression method with the formula relative expression $2^{-\Delta CT}$, where ΔC_T is the difference in threshold cycle between the gene of interest and the housekeeping gene (β-actin) as a control.

Table 1 Primer sequences, sense and antisense, and the optimal temperatures are shown. Specificity of primers and probes used for quantitative-PCR

CD83		
sense	5′-GTTATTGGAGGGTGGTGAAGAGAGG-3′	55 °C
antisense	5′-GTGAGGAGTCACTAGCCCTAAATGC-3′	
CCL19		
sense	5′-CACCAATGATGCTGAAGACTGC-3′	60 °C
antisense	5′-CGGCGCTTCATCTTGGC-3′	
CCL21		
sense	5′-CAGAGAGACCGAGGAGGGAGAG-3′	60 °C
antisense	5′-GCGGGTGGGAAGACAGAACAGC-3′	
TNF-α		
sense	5′-CTTTGGGATCATTGCCCTGTG-3′	60 °C
antisense	5′-CGAAGTGGTGGTCTTGTTGCT-3′	
TCR-α		
sense	5′-CCTTCAACAACAGCATTATTCCAG-3′	55 °C
antisense	5′-CGAGGGAGCACAGGCTGTCTTA-3′	
IFN-γ		
sense	5′-ACCTTAAGAAATATTTTAATGC-3′	55 °C
antisense	5′-ACCGAATAATTAGTCAGCTT-3′	
β-actin		
sense	5′-ATGGCCACGGCTGCTTCCAGC-3′	55 °C
antisense	5′-CATGGTGGTGCCGCCAGACAG-3′	
CD86		
sense	5′-GTAGTATTTTGGCAGGACCAGG-3′	60 °C
antisense	5′-ATTCCTGTGGGCTTTTTGTG-3′	

Immunohistochemical analysis

OCT-embedded sections of all 57 carotid artery plaque samples were cut into 8-µm sections. Tissue sections were fixed with acetone for 10 min, dried for 30 min, and soaked for 5 min in 1 % paraformaldehyde solution, pH 7.4. The sections were incubated in 5 % goat or rabbit serum (Invitrogen Life Technologies, Carlsbad, CA). Slides were stained with primary Ab anti-CD83 (Research Diagnostics, Flanders, NJ) with a working dilution of 1:300; anti-CD3 (DAKO, Carpinteria, CA) 1:300, anti-Fascin (DAKO, Carpinteria, CA), 1:600; anti-CD86 (Santa Cruz Biotechnology, Santa Cruz, CA), 1:400; anti-CD40 ligand (CD40L) (R&D Systems, Minneapolis, MN), 1:50; anti-CCL21 (R&D Systems, Minneapolis, MN), 1:3000; or anti-CCL19 (R&D Systems, Minneapolis, MN), 1:300 at room temperature for 1 h at 4 °C in a humidified chamber. The sections were then washed with tap water and incubated at room temperature with biotin-conjugated goat anti-rabbit Ig (DAKO, Carpinteria, CA) or rabbit anti-mouse Ig (DAKO, Carpinteria, CA) secondary Ab for 30 min. After washing with tap water, avidin-biotinylated enzyme solution (ABC-AP kit; Vector Laboratories, Burlingame, CA; or ABC-IP kit, Vector Laboratories, Burlingame, CA) was added for 30 min at room temperature. Blue staining was produced using Vector Blue (Vector Laboratories, Burlingame, CA) as the chromagen for 20 to 30 min. Sections were counterstained with hematoxylin solution (Surgipath, Richmond, IL) for 2 to 5 min. The procedure for single staining has been previously published [31]. Tissue sections were viewed by light microscopy and were photographed using an LSM-510 microscopic system (Carl Zeiss Instruments, Thornwood, NY).

Overlays were performed using the image analyzer KS400 from Kontron/Zeiss, Germany. The program uses two succeeding stains of serial sections. A minimum of three identical parts of the two stains are needed for the program in order to present colocalization of antibody-marked cells.

Statistical analysis

Data were analyzed with the use of SPSS (10.0) for Windows. Group comparisons are based on Student's *t*-tests.

Continuous data are presented as mean and SD or median and interquartile range. Statistical significance was assigned when $p \leq 0.05$.

Results

Production of chemokines and cytokines in the atheroma

One of the major functions of activated DC is the production of chemokines through which they regulate the recruitment of T lymphocytes. To determine whether DC in the inflamed atherosclerotic plaque are activated and are producing chemokines, we examined tissue concentrations of chemokine transcripts in carotid endarterectomy samples. To correlate DC activation and chemokine production with the intensity of inflammatory responses ongoing in the plaque, we also measured expressions of the proinflammatory cytokines IFN-γ and TNF-α. Carotid plaque tissue was collected from 57 patients, of which 19 had asymptomatic disease, and 38 presented with ischemic complications prior to the revascularization procedure. As shown in Fig. 1, tissues obtained from symptomatic patients contained markedly higher concentrations of T-cell receptor α-chain transcripts ($P = 0.01$) than did tissues derived from asymptomatic patients. This demonstrated the presence of a T-lymphocyte infiltrate in the lesions from the symptomatic patients and the lack of such an infiltrate in the atheromas of the asymptomatic patients. Not only did T cells accumulate in such plaques, they also produced IFN-γ [42]. Median levels of IFN-γ-specific sequences were fourfold higher in the material collected from symptomatic patients compared to asymptomatic patients (Fig. 1, median level of symptomatic 154 and asymptomatic 37, $P = 0.001$). Further support for ongoing inflammatory responses in the symptomatic patients' carotid atheroma came from the measurement of TNF-α. Adjusted cDNA copy numbers for TNF-α were fivefold higher in plaque tissue from the clinically symptomatic patient (median level of symptomatic 2601 and asymptomatic 467, $P = 0.006$).

The immuno-inflammatory response in the carotid atheromas was associated with the accumulation of CD83-expressing DC. Median CD83 copy numbers reached 988 in the tissue extracts from the symptomatic patients and 356 in the tissues from the asymptomatic patients ($P < 0.001$). Tissue levels of transcripts for the T cell-attracting chemokines CCL19 and CCL21 were both highly enriched in carotid lesions from the symptomatic patients. CCL19-specific sequences were distinctly low in tissues from the asymptomatic patients but reached a median value of 2,132 in plaque tissue from patients with ischemic complications. CCL21-specific sequences were highly abundant and were present even in tissues that lacked inflammation. Baseline levels of 8,397copies were detected in plaques from asymptomatic patients. In the atheromas from the symptomatic patients, transcript concentrations were as high as 15,708 copies with median levels found at 6,639. To address the question of whether DC in the inflamed atheroma express costimulatory molecules and thus are able to support T-cell activation, the level of the costimulatory ligand CD86 was measured in tissue extracts. Significantly higher expression of CD86 was seen in samples from symptomatic patients ($P = 0.02$, Fig. 2).

These data demonstrated that the clinical presentation of the patient correlated with the composition of the inflammatory infiltrate in the atheroma. Patients who came to clinical attention with CNS ischemia had high numbers of T cells and activated DC in the tissue lesion. In such patients, IFN-γ and TNF-α were produced in the carotid atheroma, and abundant message for chemokines was present.

Instability of coronary artery plaque is associated with tissue production of proinflammatory cytokines and chemokines

To investigate whether instability of coronary artery plaque was similarly correlated with the accumulation

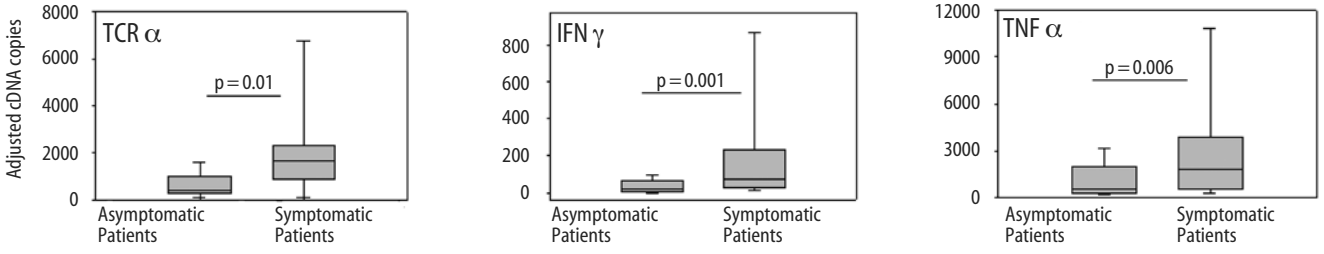

Fig. 1 Expression of chemokines and cytokines in carotid plaque. Carotid artery plaque samples of patients, undergoing an endarterectomy were collected from 19 asymptomatic and 38 symptomatic patients. cDNA was generated from tissue extracts, and cytokine and chemokine transcripts were measured by quantitative-PCR. Copy numbers were adjusted for copies of the housekeeping gene β actin. Concentrations of TCR-α transcripts were used to estimate the density of the lymphocyte infiltrate. Results are shown as box plots displaying medians and 25th and 75th percentiles as boxes and 10th and 90th percentiles as whiskers

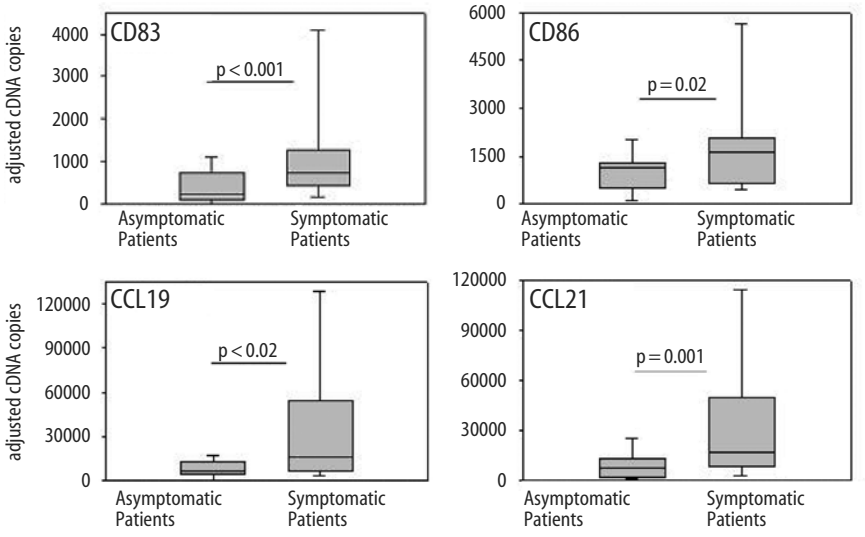

Fig. 2 Expression of chemokines and cytokines in carotid plaque. CD83 mRNA was used to identify the presence of activated DC. Results are shown as box plots displaying medians and 25th and 75th percentiles as boxes and 10th and 90th percentiles as whiskers

of immuno-inflammatory cells and the production of chemokines, we collected a set of coronary artery samples from patients with fatal myocardial infarction. Seven patients were enrolled into this study. In each patient, a part of the culprit lesion that showed typical features of plaque rupture and superimposed atherothrombosis was harvested. For comparison, a stable plaque with intact surface was collected, and a piece of morphologically normal coronary artery was secured. Tissue expression in these matched samples for the proinflammatory cytokines IFN-γ and TNF-α were determined by real-time PCR (Fig. 3). Possible involvement of activated $CD83^+$ DC was tested by quantifying CD83 transcripts and by measuring tissue concentrations of sequences specific for the chemokine CCL19. For most markers, the stable plaque tissue was indistinguishable from the normal coronary artery. Very low levels of T-cell receptor α-chain sequences, TNF-α sequences, IFN-γ sequences, and CD83 sequences were expressed in normal coronary artery wall as well as in stable atheroma. Normal coronary arteries contained baseline levels of CCL19-specific transcripts, and these

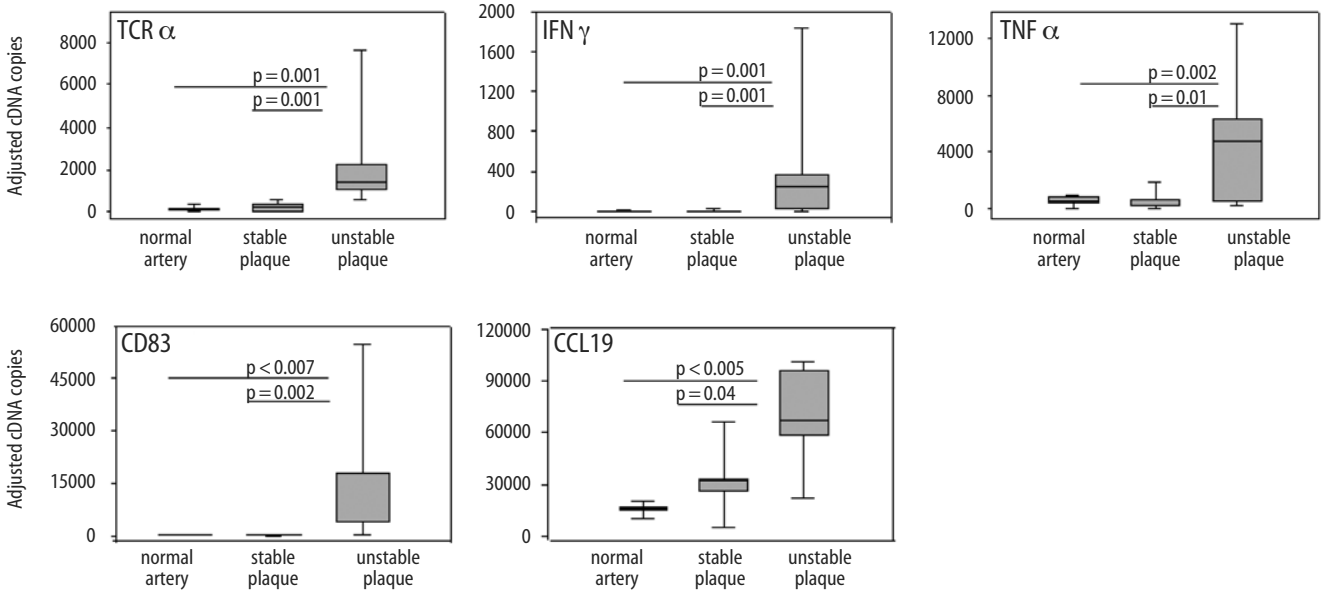

Fig. 3 Expression of chemokines and cytokines in coronary artery plaque tissue. Coronary artery samples were harvested from 7 patients. In each patient, a part of an unstable plaque, a part of a stable plaque, and a piece of coronary artery without atherosclerosis were harvested. cDNA was generated from tissue extracts, and real-time PCR was used to quantify chemokine- and cytokine-specific transcripts. Results are shown as box plots displaying medians and 25th and 75th percentiles as boxes and 10th and 90th percentiles as whiskers. Expression of CD83 was used to estimate the load of activated DC. T-cell infiltration was determined by the level of T-cell receptor sequences

levels were higher in stable plaque tissue. Significantly elevated concentrations of TCR-α copies indicated accumulation of T cells in the unstable plaques ($P = 0.001$). This was associated with the tissue expression of IFN-γ transcripts ($P = 0.001$). Unstable plaque tissue had high levels of TNF-α-specific sequences (median at 4,750, $P = 0.002$). Accumulation of activated DC was indicated by the expression of CD83-specific sequences, which was exclusively the case for unstable plaque ($P < 0.007$). Ruptured plaque tissue also showed a significant increase in the concentration of mRNA specific for the chemokine CCL19 ($P < 0.005$).

Activated DC are the cellular sources of the chemokines CCL19 and CCL21 in atherosclerotic plaque

To identify the cellular source of the chemokines detected in both carotid and coronary artery plaques, we utilized immunohistochemical staining. Frozen tissue sections from all 57 carotid endarterectomy material samples were stained with the pan-DC marker fascin and the DC marker CD83. Fascin-positive cells could be found in plaque tissue that lacked an inflammatory infiltrate as well as in inflamed lesions. Such cells had considerable cell size and were often stretched out. Also, stretched-out cells were characteristically arranged between parallel layers of vascular smooth muscle cells. Typical DC stains are presented in Fig. 4.

To address the question of whether such DC had undergone activation, we searched for the surface expression of the costimulatory ligand CD86. In consecutive tissue sections, essentially all $CD83^+$ activated DC stained positive for CD86. These findings supported the concept that DC populating the atheroma were in a state of differentiation that allowed them to effectively activate T cells and provide the necessary costimulatory signals for optimal triggering.

To identify the cellular origin of the chemokines CCL19 and CCL21, serial tissue sections were prepared from plaques with an inflammatory infiltrate, and immunohistochemical staining was performed with appropriate antibodies. Cells secreting CCL21 protein were dispersed throughout the tissue but were clearly more frequent in the shoulder region associated with the inflammatory infiltrate. Comparison of serial tissue sections demonstrated that the major cellular source for CCL21 were $CD83^+$ DC. CCL19 had a similar distribution pattern. Cells with the morphology of DC stained positive for CCL19.

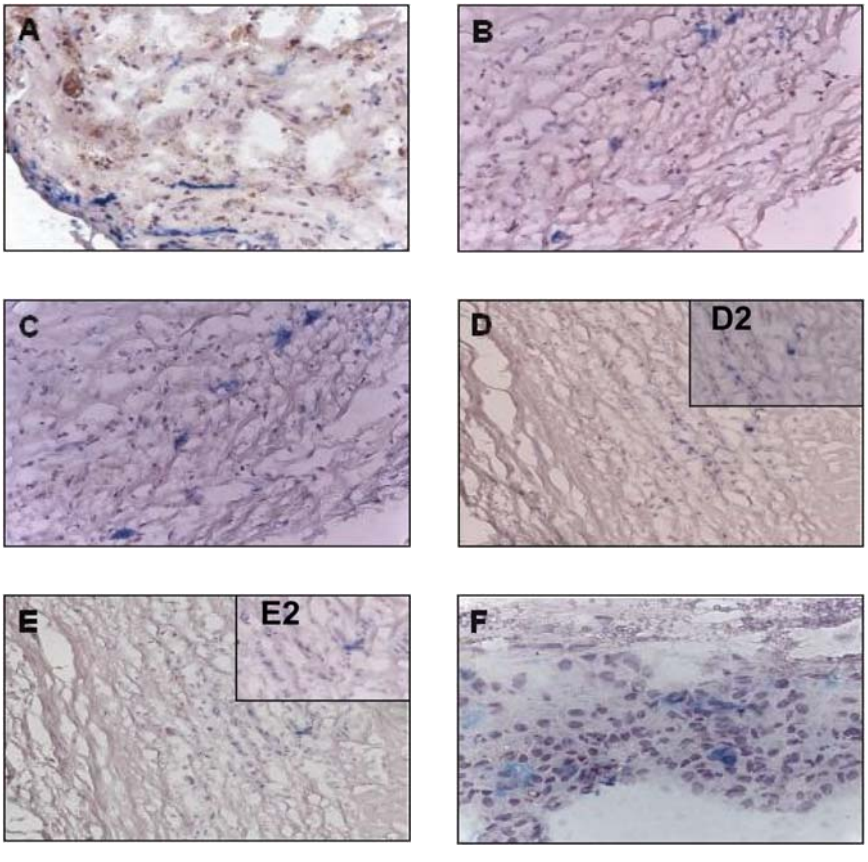

Fig. 4 DC in inflamed atherosclerotic lesions are activated and produce chemokines. Frozen tissue sections were prepared from inflamed carotid artery plaques and used for immunohistochemical stains. **A** DC were identified by staining with the pan-DC marker fascin (blue). Consecutive tissue sections were stained with (**B**) anti-CD83 (blue) and (**C**) anti-CD86 (blue). Staining of consecutive tissue sections with (**D**) anti-CD83 (blue) and (**E**) anti-CCL21 demonstrated that activated DC produced the chemokine CCL21. **F** Cells with the morphology of DC also stained positive for CCL19 (blue). Original magnification × 40 (**A–E**) and × 60 (**D2**, **E2**, **F**)

Activated DC in the atheroma co-localize with activated T lymphocytes

Expression of CD86 by tissue-infiltrating DC suggested that they were capable of serving as Ag-presenting cells for T lymphocytes. The release of CCL19 and CCL21 by such DC could also facilitate the recruitment of selected T-cell populations, particularly central memory T cells. To examine whether DC had a role in interacting with T cells, we analyzed the spatial relationship between the two cell populations. DC accumulated within the infiltrate of mononuclear cells. Pseudoimages generated from serial tissue sections allowed for the projection of $CD83^+$ DC onto a map of CD3-expressing T cells. Activated DC always resided in close vicinity to $CD3^+$ T cells (Fig. 5).

The co-localization of DC and T cells was associated with functional selection of the involved T cells. Upon activation through T-cell receptors, T cells upregulate the surface expression of CD40 ligand (CD40L). We therefore utilized this marker to identify recently activated T cells. As shown in Fig. 6, such CD40L-positive T cells were in close contact with activated $CD83^+$ DC. In summary, these data suggest that DC contributing to the inflammatory infiltrate in the unstable atherosclerotic plaque play a role in regulating T-cell function in the lesion.

Discussion

The process of plaque destabilization and rupture has been closely associated with the presence of inflammatory cells including activated macrophages and activated T lymphocytes. In the current study, we attempted to understand which signals are relevant in activating T lymphocytes in the tissue environment of the inflamed plaque. Here we describe that inflamed plaques, as opposed to stable plaques, harbor a population of fully matured and activated DC. We have found that such DC produce T cell-attracting chemokines and express costimulatory molecules, thereby identifying them as ideal partners in T-cell recruitment and T-cell activation. Data reported here support the model that DC, which are known for their superb ability to facilitate and fine tune T-cell function, play an important role in the immune responses occurring in the atheroma.

Our study confirms prior reports that atherosclerotic plaque with an inflammatory infiltrate contains DC and that these DC are in a state of activation [6, 41]. In a cohort of carotid endarterectomy samples, accumulation of T cells in the atheroma clearly correlated with the clinical status of the patient. Endarterectomy tissues from asymptomatic patients contained only low levels of T-cell receptor transcripts, but essentially all tissue samples from symptomatic patients had high concentrations of T cell-specific sequences. This rule held for plaques in coronary arteries as well. Notably, only the unstable coronary plaques which had caused fatal myocardial infarction were a site of T-cell accumulation. Normal-appearing coronary artery walls were distinctly free of T-lymphocyte infiltration, and stable plaque tissues did not contain tissue-residing T cells. Evidence for functional activity of the accumulated T cells came from the demonstration that T-cell infiltrates were closely correlated with active transcription of the IFN-γ gene [17, 27, 36, 37]. IFN-γ is a quintessential effector molecule produced by activated T cells and is highly effective in regulating the functional activity of macrophages.

The current study was not designed to allow for a direct comparison of immune events in carotid plaque and coronary plaque; however, data presented here suggest that very similar events may occur in these two different vascular sites. There was a trend for immune

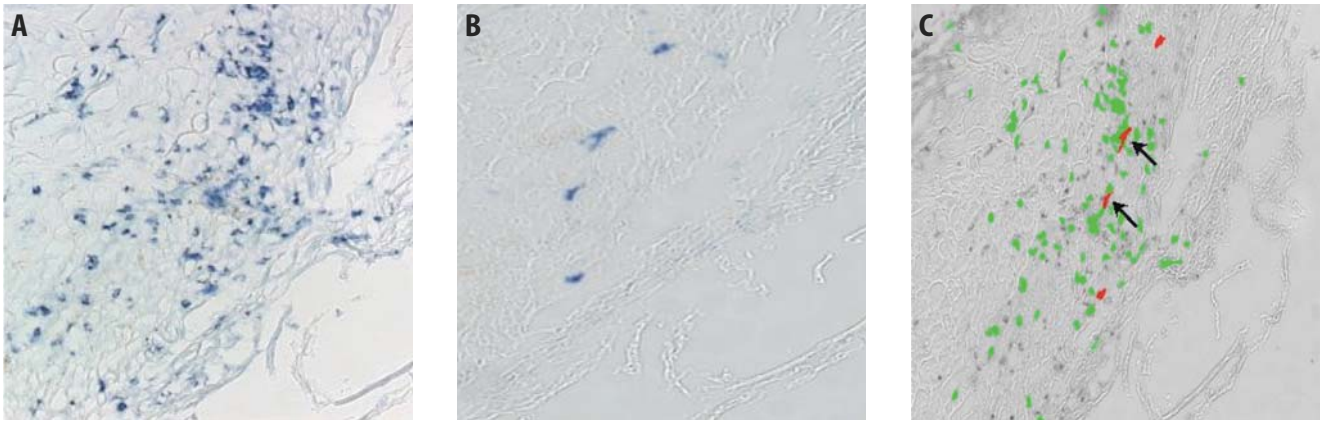

Fig. 5 Spatial relationship of T cells and DC in the inflamed plaque. **A** Frozen sections were stained with anti-CD3 (blue) antibodies to detect T cells. **B** DC were identified with antibodies to CD83 (blue). **C** Serial tissue sections were overlaid. Pseudoimages were generated to show the spatial relationship of T cells (green) and DC (red). Original magnification × 20 ⟶ shoes the close vicinity of activated DC to $CD3^+$ T cells

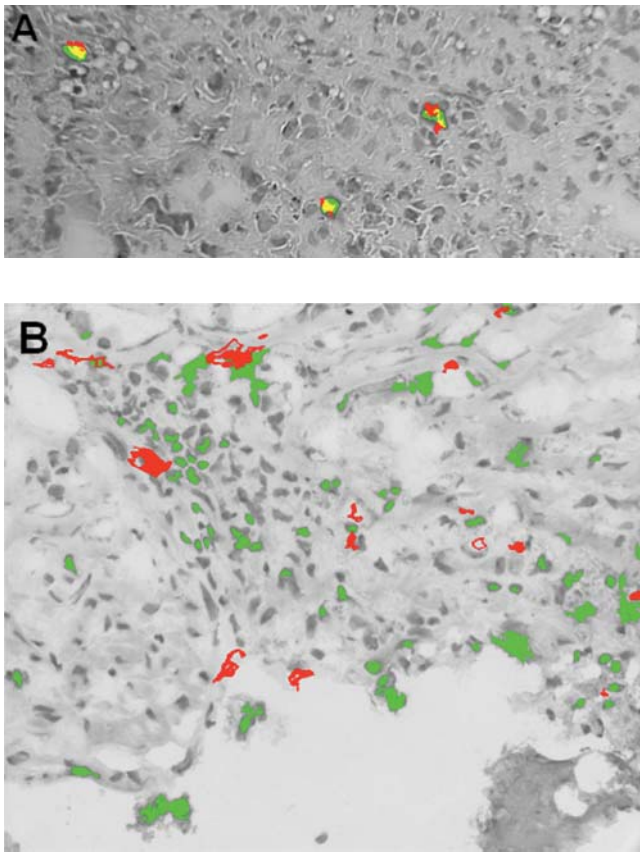

Fig. 6 In situ activated T cells co-localize with DC. Frozen tissue sections from inflamed carotid plaques were stained with either anti-CD3 or anti-CD40L. Pseudoimages were overlaid (**A**) with CD3 shown in green and CD40L in red. **B** Pseudoimages generated from consecutive tissue sections were combined to show the colocalization of activated DC (stained with anti-CD83, red) and activated T cells (stained with anti-CD40L, green). Original magnification × 60

markers examined here to be more abundant in the coronary lesions. Further studies will be necessary to address this issue and to verify that a higher degree of immune stimulation occurs in coronary lesions versus carotid lesions.

Data presented here provide strong support for the notion that DC regulate T-cell recruitment and T-cell activation in the atheroma. For T lymphocytes to undergo activation, they need Ag-presenting cells that not only present protein peptides on their surface but also provide ligands for costimulatory molecules. A number of different cell types have been suspected to possess Ag-presenting capabilities in inflammatory lesions [7, 20, 21, 26, 34]. Surprisingly, the only cell type actively expressing the CD86 costimulatory ligand was $CD83^+$ DC. Immature DC are negative for CD83 and require multiple signals to enter the maturation process that finally leads to the acquisition of CD83 on the cell surface. CD83 is, therefore, an excellent marker for fully maturated human DC. $CD83^+$ DC have been described in another model of vascular inflammation, giant cell vasculitis, as critical Ag-presenting cells. Indeed, in that disease model, only $CD83^+$ DC were capable of sustaining T-cell activation in the vessel wall [30, 31].

In the immature state, DC are positive for CD11c and fascin, which are global DC markers. Immunohistochemical studies in inflamed plaque tissues demonstrated that essentially all fascin-positive cells were also positive for CD83. These cells must therefore have received triggers that induced the differentiation process from an immature DC to a fully matured DC. The nature of such triggers is currently unclear. Theoretically, such DC could be tissue-resident cells as has been suggested for medium-sized human arteries [30, 31]. Locally produced cytokines as well as indigenous triggers could initiate the process of DC maturation. We addressed the questions of whether TNF-α is produced in inflamed atheroma and could clearly correlate with tissue production of TNF-α in the culprit lesion of the coronaries and clinical symptoms in the carotid tissues. However, it remains possible that TNF-α production reflects the activity of the ongoing inflammation and did not precede the establishment of the inflammatory infiltrate.

Atherosclerotic plaque harbors a number of indigenous ligands that have all been implicated in DC activation, including oxidized low-density lipoproteins, heat shock proteins, and possibly microbial products that through binding to Toll-like receptors are potent activators of DC [7, 8, 14, 33]. We therefore propose that DC are either indigenous cells of the vessel wall or are recruited in their immature state to receive activating signals, transforming them into fully matured, highly effective Ag-presenting cells.

An important aspect of DC function in the atheroma is obviously the production of chemokines. The current study focused on CCL19 and CCL21, two chemokines binding to CCR7. CCR7-expressing T lymphocytes include naïve cells and central memory cells [16, 35]. The close correlations between CCL19 in carotid plaques and CCL21, production in both carotid artery lesions and inflamed coronary plaques with the accumulation of T cells suggests that the chemokines do indeed play a role in bringing T cells into the vessel wall. The current study did not explore other chemokine-receptor ligands such as CCL5, which have been described to be abundantly produced in atheroma and could also have a role in regulating T-cell influx. If indeed CCL19 and CCL21 facilitate the recruitment of naïve T cells into the inflamed atheroma, then it may be possible that Ag-priming occurs in this setting. With the presence of fully matured $CD83^+$ DC and the attraction of $CCR7^+$ naïve cells, all conditions are met that are usually reserved for lymphoid tissues which are specialized in securing priming of T cells against Ags. The data presented here thus suggest that the microenvironment resembling unique conditions in lymphoid organs is being formed in the

atheroma. With the presence of fully matured $CD83^+$ DC, this microenvironment strongly favors immune activation and thus may play a critical role in sustaining inflammatory responses. It has been proposed that ectopic lymphoid microstructures are powerful enough to substitute for the otherwise absolute need for Ags to initiate and maintain T-cell activation [40].

Inflammation of lymphoid microstructures that lend stability to a chronic inflammatory response was also demonstrated by mapping DC in the inflamed plaque. Typically, $CD83^+$ DC were found in the shoulder region of the plaque, an area known to be a locus minoris resistentiae. T lymphocytes in direct contact with such activated DC frequently expressed the activation marker CD40L. CD40L is upregulated early in T cell receptor-mediated triggering of T lymphocytes [18]. CD40L has been discussed to represent an excellent therapeutic target for novel immunomodulatory therapies of atherosclerosis [28, 29].

The molecule CD86 is known to be a specific marker for activated APC, such as DC. It is a costimulatory ligand on the surface of DC and is needed to interact with T cells and activate them. The findings that essentially all $CD86^+$ cells are also $CD83^+$ and that CD86 is predominantly expressed in plaque samples from symptomatic patients underlines a possible role for DC in atherosclerotic lesions and, even more so, in symptomatic patients.

In this study, the spatial relationship between activated T cells and activated DC resembled conditions typically encountered in the T-cell zones of lymph nodes. It is now becoming clear that multiple subsets of DC exist [1, 38]. These subsets differ in their pattern recognition receptors and their functional profiles [38]. Specific subsets of DC have been implicated in biasing T-cell responses in distinct directions, and the precise functional capability of a DC population is now recognized as a critical factor in shaping ensuing immune responses. Several questions remain regarding the DC we examined in this study. Their origin is not understood, and it remains unclear how the differentiation process is regulated and which stimuli are involved in facilitating their transformation into highly activated potent Ag-presenting cells. It remains to be examined which types of Ags such DC have incorporated and present on their surface. A large number of reports in the literature suggest that infectious agents or their derivatives are captured in the atheroma. DC would provide an ideal site for chronic infectious agents to be stored [9]. Data presented here are in line with the interpretation that activated DC are a critical cell population in the production of chemokines and thus shape the composition of the accumulated inflammatory cells. DC control a number of pathways that have gatekeeper function in immune stimulation, including costimulation, Ag presentation, and chemokine production. Recent evidence suggests that costimulatory signals play a crucial role in amplifying immune recognition events in atherosclerosis [9, 10, 18, 28, 29]. Thus, costimulatory ligands expressed on the surface of DC could represent critical determinants in regulating the degree of immune stimulation.

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