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T cells in Cardiac Allograft Vasculopathy Are Skewed to Memory Th-1 Cells in the Presence of a Distinct Th-2 Population

M. C. Hagemeijer[a], M. F. M. van Oosterhout[a], D. F. van Wichen[a], J. van Kuik[a], E. Siera-de Koning[a], F. H. J. Gmelig Meyling[b], M. E. I. Schipper[a], N. de Jonge[c] and R. A. de Weger[a,*]

[a] *Departments of Pathology,* [b] *Immunology and* [c] *Heart and Lung, University Medical Center Utrecht, The Netherlands*
* *Corresponding author: R. A. de Weger, r.deweger@umcutrecht.nl*

Cardiac allograft vasculopathy (CAV) in heart transplantation (HTx) patients remains the major complication for long-term survival, due to concentric neointima hyperplasia induced by infiltrating mononuclear cells (MNC). Previously, we showed that activated memory T-helper-1 (Th-1) cells are the major component of infiltrating MNC in coronary arteries with CAV. In this study, a more detailed characterization of the MNC in human coronary arteries with CAV (n = 5) was performed and compared to coronary arteries without CAV (n = 5), by investigating MNC markers (CD1a, DRC-1, CD3, CD20, CD27, CD28, CD56, CD68, CD69, FOXP3 and HLA-DR), cytokines (IL-1A, 2, 4, 10, 12B, IFN-γ, and TGF-β 1), and chemokine receptors (CCR3, CCR4, CCR5, CCR7, CCR8, CXCR3 and CX3CR1) by immunohistochemical double-labeling and quantitative PCR on mRNA isolated from laser microdissected layers of coronary arteries. T cells in the neointima and adventitia of CAV were skewed toward an activated memory Th-1 phenotype, but in the presence of a distinct Th-2 population. FOXP3 positive T cells were not detected and production of most cytokines was low or absent, except for IFN-γ, and TGF-β. This typical composition of T-helper cells and especially production of IFN-γ and TGF-β may play an important role in the proliferative CAV reaction.

Key words: Allograft arteriopathy, alloreactive T cells, chronic allograft rejection, coronary artery disease, vasculopathy

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Introduction

Cardiovascular diseases are regarded as one of the principal causes of death (1). The only possible therapy for patients suffering from end-stage heart failure is heart transplantation (HTx). Despite increasing survival rates of HTx recipients, long-term survival is complicated by cardiac allograft vasculopathy (CAV) (2,3).

The pathogenesis of CAV is multifactorial and mediated by an incompletely understood (adaptive) immunological process, with a nonimmunological onset (response to endothelial cell injury; (4)). It affects the coronary arteries from the allograft by remodeling the diameter of the vascular wall. The observed diffuse luminal narrowing is mediated by a characteristic mononuclear cell (MNC) infiltrate, composed of lymphocytes and macrophages, vascular smooth muscle cells (SMC) and fibroblasts (5). We (6), and others (7) have recently described that the neointima is composed of two distinct layers: a luminal layer (NI-LL) composed of loose connective tissue and often heavily infiltrated by MNC, and a layer composed of smooth muscle cells (NI-SMC) directly adjacent to the lamina elastica interna and media.

Clinical and experimental studies demonstrated that infiltration and recruitment of inflammatory MNC from the arterial lumen into the neointima and from capillaries into the adventitia is induced by the presence of pro-inflammatory cytokines like interferon (IFN)-γ, transforming growth factor (TGF)-β and chemokines, such as IFN-inducible protein 10 (IP-10), monokine induced by IFN-γ (Mig) and IFN-inducible T-cell α-chemoattractant (I-TAC) (8–11). These are initially produced by injured endothelial cells posttransplantation, but over time mainly by MNC. The presence of infiltrating MNC in the NI-LL and outside the vessel wall in the adventitia, contribute extensively to the progression of CAV and mediates the concentric occlusion of the coronary arteries in the donor heart by (I) proliferation and differentiation of SMC and/or fibroblasts (12), (II) the deposition of extra-cellular matrix (ECM) components in the intimal layer, and (III) the continuous release of immune modulators, which maintain the chronic immune response (2,3,6).

The differential expression of chemokine receptors on T helper-1 (Th-1) and T helper-2 (Th-2)-cells, is

associated with a type I (inflammation and killing of intracellular pathogens) or a type II (allergy, immune regulation, matrix deposition and tissue remodeling) immune response (13–17). Therefore, chemokine receptor expression profiles provide an opportunity to investigate the type of immune response in CAV.

Recently, we demonstrated that in coronary arteries with CAV an abundant infiltrate of activated (memory) Th-1 lymphocytes was present, with only relatively small numbers of B cells and macrophages (6). This infiltrate was mainly localized in the NI-LL and adventitia. The aim of the present study was to obtain a better insight in the type of immune response in the transplanted coronary arteries and the immunological mechanisms resulting in CAV. Therefore, we characterized the infiltrated helper T cells in CAV in more detail, by extending the marker profile of these cells and by characterizing their cytokine and chemokine receptor profile, by immunohistochemical (IHC) studies and quantitative PCR (Q-PCR) on mRNA isolated from distinct layers of human coronary arteries obtained by tissue laser microdissection.

Materials and Methods

Patient population

Coronary arteries of HTx patients were obtained at autopsy. Informed consent of all patients was obtained prior to HTx. All patients were treated with a standard triple immunosuppressive therapy, consisting of cyclosporine A, azathioprine and steroids. Patient characteristics are summarized in Table 1. CAV was assessed histologically, as recently described (6). In this study, a group of five coronary arteries diagnosed with CAV was compared to five coronary arteries without CAV (references). The references consisted of three coronary arteries from patients without HTx and two coronary arteries of donor hearts less than 3 months after HTx. No histological differences were observed between both types of references. Only arteries without atherosclerotic plaques were selected.

Table 1: Patient characteristics

Patient	Gender	Age	Post-HT× (days)	Cause of death
References				
1	Female	20	–	Cardiogenic shock
2	Male	56	1	Lung embolism
3	Male	61	63	Malignancy
4	Male	46	–	Malignancy
5	Male	64	–	Hepatic cirrhosis
CAV				
1	Male	64	365	Acute cellular rejection
2	Male	59	370	CAV, ischemia
3	Female	16	485	CAV, ischemia
4	Male	64	5184	CAV, ischemia
5	Female	16	481	CAV, ischemia

The characteristics of the patients used in the present study. Different coronary arteries were obtained at post-mortem examination and were snap frozen in liquid nitrogen or fixed in formalin and embedded in paraffin and used for immunohistochemistry and/or mRNA isolation.

Immunohistochemistry (IHC)

Coronary arteries were evaluated using conventional immunoperoxidase staining as previously described (6,18). IHC procedures varied for each antibody used and are summarized in Table 2. Negative control sections were included in which the primary antibody was omitted, or the primary antibody was replaced by an isotype-matched irrelevant antibody. Only results that stand-up to these controls are presented.

Double-immunofluorescent immunohistochemistry

Combinations of antibodies and pretreatments used in double-staining experiments are summarized in Table 2. The following procedure was applied to all incubations. Sections were pre-absorbed using 10% normal serum in PBS (blocking buffer) corresponding to the species of the secondary antibody for 30 min, and incubated with the primary antibody for 1 h in blocking buffer, followed by an incubation with HRP-labeled secondary antibody for 30 min. Subsequently, the staining was amplified by incubation with TRITC-conjugated tyramide diluted 1:50 in amplification diluents (NEL 702, Perkin Elmer Life Sciences, Boston, MA) for 30 min. The residual endogenous peroxidase activity was blocked by incubating the slides for 20 min in block buffer (1% H_2O_2 in PBS). The previous steps were repeated for the second cascade, that was amplified with FITC-conjugated tyramide diluted 1:50 in amplification diluents (NEL 701, Perkin Elmer Life Sciences) for 5 min. In between the different incubations, the slides were washed in PBS/0.05% Tween-20. Finally, slides were either incubated with DAPI or TOPRO-3 Iodide (Invitrogen Europe BV, Leiden, NL), respectively for 3 or 10 min. Slides were analyzed by confocal laser scan microscopy (CLSM).

Laser tissue microdissection, RNA isolation and cDNA synthesis

Frozen tissue sections of 10 μm were mounted on 1 mm RNAse free PEN membrane slides (P.A. L.M. Microlaser Technologies AG, Bernried, Germany), air-dried and fixed in 96% ethanol for 30 s. Subsequently, slides were rinsed with 70% ethanol, washed with RNAse-free water and stained for 30 s with RNAse-free hematoxylin. The slides were rinsed two times with RNAse-free water, followed by dehydration in ethanol (19). Different arterial layers were microdissected using the P.A.L.M. Microbeam and collected in LPC microfugetubes (P.A.L.M.) for total RNA isolation using the PicoPure RNA isolation kit according to the manufacturer's instructions (Arcturus Bioscience, Sunnyvale, CA). Synthesis of cDNA was performed by adding 2 μLoligo dT (15) primers (0.50 μg; Promega Corporation, Phoenix, AZ), 2 μL of random primers (0.50 μg; Promega) and 4 μL dNTP's (25 mM; Invitrogen Corporation, Carlsbad, CA) to 44 μL RNA derived from the RNA isolation procedure. This solution was heated in a closed Eppendorf tube for 5 min at 65°C and subsequently, the tube was cooled to RT. Sixteen microliters 5× RT-buffer (Invitrogen), 8 μL 0.1M DTT (Invitrogen) and 2 μL RNasin (Promega) were added and vigorously mixed and heated for 2 min at 37°C. Finally, 2 μL Superscript RNaseH-Reverse Transcriptase (Promega) was added and heated in a closed Eppendorf tube for 50 min at 37°C, followed by 15 min at 70°C.

Real-time quantitative-PCR (Q-PCR)

The primer/probe combinations (Assays on Demand) used for Q-PCR were from Applied Biosystems (Foster City, CA). The expression levels of the lymphocyte markers and cytokines: CD3, CD20, CD28, CD56, CD68, FOXP3, IL-1A, IL-2, IL-4, IL-10, IL-12B, IFN-γ, TGF-β1 and chemokine receptors CCR3, CCR4, CCR5, CCR7, CCR8, CXCR3 and CX3CR1 were studied. Per sample 12.5 μL master mix (Applied Biosystems) was used and 1.25 μL primer/probe, 6.25 μL milliQ, and 5 μL cDNA was added. Q-PCR was carried out using the Prism 7900-sequence detection system. Thermal cycling included a denaturation step at 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 60 s. To quantify the data, the comparative Ct method was used. As Q-PCR reference, the house keeping gene GAPDH was used. The calibrator (vascular cDNA) is a sample used for normalization.

Table 2: Pretreatment and immunohistochemical procedures for paraffin-embedded and frozen coronary arteries

Antibody	Antigen retrieval	Primary dilution	Secondary antibody	Origin primary antibody
Single-immunofluorescence				
CD1a^{fr} (Mo)	–	1:40	RbαMoPO (1:50) Rabbit PV	Orthodiagnostics
CD27^{fr} (Mo)	–	1:40	Mouse PV	Novocastra
CD28^{fr} (Mo)	–	1:200	RbαMoPO (1:50) Rabbit PV	Immunotech
CD56^{fr} (Mo)	–	1:50	RbαMoPO (1:50) Rabbit PV	Immunotech
CD69^{pa} (Mo)	EDTA	1:5	RbαMoPO (1:50) Thyr-TRITC	Novocastra
DRC-1^{fr} (Mo)	–	1:20	RbαMoPO (1:50) Rabbit PV	Dakopatts, Denmark
FOX-P3^{fr} (Go)	–	1:100	RbαGoPO (1:50) SwαRbPO (1:100)	Abcam
TGF-β1^{pa} (Rb)	EDTA	1:200	Rabbit PV	Santa Cruz
Double-immunofluorescence				
CCR3^{pa1}	EDTA	1:300	DoαGoPO	Alexis Biochem.
CCR4^{pa1}	EDTA	1:400	DoαGoPO	Alexis Biochem.
CCR5^{pa1}	EDTA	1:300	DoαGoPO	Alexis Biochem.
CCR 7^{pa1}	EDTA	1:250	DoαGoPO	Alexis Biochem.
CCR8^{pa1}	EDTA	1:300	DoαGoPO	Alexis Biochem.
CXCR3^{pa1}	Citrate	1:1000	RbαMoPO (1:200)	Alexis Biochem.
CX3CR1^{pa1}	EDTA	1:200	DoαGoPO	Santa Cruz

Immunofluorescent stainings were performed on tissue frozen in liquid nitrogen (fr) or paraffin-embedded tissue (pa). [1] In double staining procedures the indicated antibody was used with TRITC-conjugated amplification. All CCR antibodies were polyclonal goat antibodies. The origin of the primary antibodies was: Alexis Biochemicals, Lausen, Switzerland; Novocastra Laboratories Ltd., Newcastle, UK; Santa Cruz Biotechnology Inc., Santa Cruz, CA; Abcam Ltd., Cambridge, UK; R&D Systems, Minneapolis, MN. Secondary antibodies: combined mouse and rabbit Powervision (ImmunoLogic, Duiven, the Netherlands), horseradish peroxidase labeled donkey anti-goat IgG (Abcam; 1:500). Double-immunofluorescence labeling of CCR were performed in combination with rabbit- or mouse anti-CD3 (Dakopatts, Glostrup, Denmark; 1:200) in combination with FITC-conjugated amplification.
In addition, HLA-DR ^{1}/CD4^{fr}, CD4/CD28^{fr1} and CCR4 ^{1}/α-SMA ^{pa} double-immunofluorescent staining was performed. Antibodies used: FITC-conjugated anti-human CD4 (BD Biosciences, San Jose, CA; 1:50), mouse anti-human HLA-DR (BD Biosciences; 1:400), and mouse anti-SMA (Sigma Aldrich, Zwijndrecht, the Netherlands; 1:8000).

This method is semi-quantitative because the absolute amount of RNA was not determined.

Statistical analysis

All data are expressed as mean ± SEM. Statistical analysis was performed using the Mann-Whitney test (CAV vs. references) or Wilcoxon test (comparing various CAV layers). p-values less than 0.05 were considered statistically significant.

Results

Immunohistochemical composition of MNC infiltrate in arterial walls with CAV

Characterization of the MNC cells by CD markers

References (n = 5) and CAV arteries (n = 5) were analyzed by conventional IHC (Figure 1) to characterize the infiltrating MNC population in the arterial walls. In references, individual lymphocytes and small numbers of resident macrophages were observed. The number of lymphocytes was too small to determine a reliable inventory for references.

CAV arteries were divided in several distinct areas: the luminal layer of the neointima composed of loose connective tissue (NI-LL), the neointimal layer composed of smooth muscle cells (NI-SMC), media, adventitia (Figure 1B) and extra vascular accumulations of MNC outside the coronary wall (ExVascMNC; Figure 1A). The largest number of MNC was observed in the NI-LL (mean: 35.5 cells per 10 000 μm^2). In the NI-SMC, the media and adventitia the mean numbers were respectively 21.4, 4.4 and 15.4 cells per 10 000 μm^2. IHC analysis of CAV (Table 3) showed that most MNC were CD3+ T cells in a CD4:CD8 ratio of 1.8–2.1. B-cells (CD20; 6–12% of total MNC) and macrophages (CD68; 8–14%) were present in lower numbers. Interestingly, no FOXP3 (T-regulatory cells: Tregs) positive cells were detected in three of the five CAV vessels and in the other two arteries only few single positive cells were observed (<1%). In none of the CAV arteries CD56 positive NK-cells were present. Dendritic cells (CD1a and anti-DRC-1) were absent in these arteries, except for some positive cells in the adventitia. In the ExVascMNC, a few CD1a+ cells were detected. In these areas the CD4:CD8 ratio was 2.2, and almost 50% of the cells were B cells. In addition, CD56+ NK cells were absent in these ExVascMNC, but small numbers of Tregs (1–6%) and macrophages (3%) were observed.

Activation markers

MNC present in the neointima and adventitia of CAV arteries expressed the activation markers CD27 (56–58%) and CD69 (66–70%) abundantly (Figure 2). A relatively larger number of CD27+ MNC were situated at the inner lining of the endothelium in the NI-LL and in the adventitia

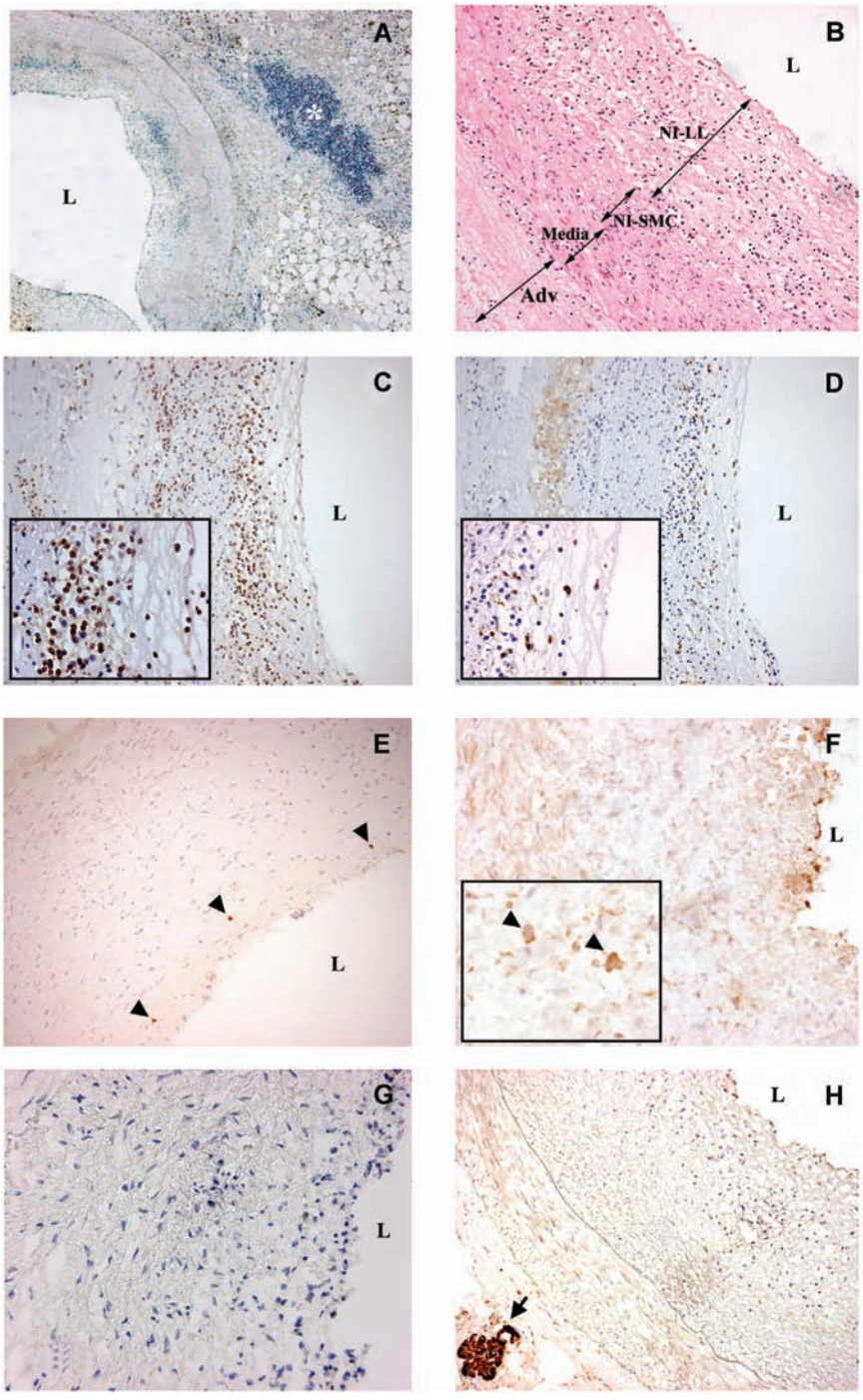

Figure 1: Immunohistochemistry of coronary arteries with CAV. A: CAV artery with extra vascular infiltrate of mononuclear cells outside the vessel wall (* ExVascMNC). IHC staining for CD68 showing an even distribution of macrophages inside the vessel wall (×50). B: HE staining of an arterial wall with CAV. The different layers are indicated: the luminal layer of the intima composed of loose connective tissue with MNC (NI-LL), the neointimal layer composed of SMC (NI-SMC), the tunica media containing normal SMC and the adventitia (Adv) (×100). C: IHC for CD3 in a CAV vessel. Insert shows many CD3 positive T cells in the neointima (×100; insert ×400). D: IHC for CD68 in a CAV vessel. Insert shows distribution of CD68 positive macrophages in the neointima (×100; insert ×400). E: IHC for CD3 in the neointima of reference arteries, showing three T cells indicated by the arrowheads (×100). F: IHC for CD68 in the neointima of reference arteries, showing macrophages indicated by the arrowheads (×100; insert ×600). G: IHC for FOX-P3 in CAV arteries demonstrating the absence of FOXP3 positive cells (×100). H: IHC for CD56 in CAV: no CD56 positive MNC were detected. The arrow indicates a CD56 positive nerve fiber (×100). In all the pictures, L indicates the lumen of the coronary vessel.

adjacent to the capillaries. Similar percentages of these activation marker positive lymphocytes were detected in the ExVascMNC, but not in references. CD28 was only expressed on 9% of MNC in the NI-LL (Figure 3A) and 17% in the adventitia. In the ExVascMNC, 52% of MNC was CD28 positive (Figure 3B). In the intima only few CD4+ cells were CD28 positive (Figure 3C). Most CD markers (CD3, CD20, CD27, CD28 and CD69) showed a stronger IHC expression in the ExVascMNC compared to the MNC in CAV.

To characterize the activation status of the T cells in more detail, HLA-DR expression on T cells was analyzed in double immunofluorescent studies. HLA-DR expression, almost absent in references, was detected on >50% of the MNC, and on approximately 20% of CD4+ T-lymphocytes

Table 3: Immunohistochemical results

Antibody	Neointima[1] %	Tunica Media %	Adventitia %	ExVascMNC %
CD1a	–	–	<1	<5
CD3	64	41	58	53
CD20	12	7	6	47
CD27	56	25	58	68
CD28	9	<1	17	52
CD56	–	–	–	–
CD68	14	8	10	3
CD69	66	57	70	60
DRC-1	–	–	–	<1
FOXP3	<1	<1	<1	4
TGF-β1	47	39	47	50

Immunoperoxidase staining of CD-markers on coronary arteries with CAV. Only CD69 was evaluated using immunofluorescence (TRITC-conjugated amplification). Results were scored for the indicated layers (n = 5). Mean percentages of cells positive for specific antibodies are indicated as percentage of total number of MNC.

[1]Percentage in NI-LL and NI-SMC did not differ and are therefore grouped as neointima.

<1 : in some slides a single cell or some cells were detected, but not in all sections; <5 : some CAV tissue slides showed a small number of positive cells, but not all; – : no positive cells were detected.

in the neointima and adventitia of CAV. In the ExVascMNC, HLA-DR+/CD4+ T cells were present, but in these areas HLA-DR expression was mainly detected on large numbers of B cells and macrophages (data not shown).

T-cell characterization by chemokine receptors

The expression of chemokine receptors associated with Th-1 (CCR5, CXCR3 and CX3CR1), Th-2 (CCR3, CCR4 and CCR8) or T-memory cells (CCR7 and CX3CR1) was analyzed by double-immunofluorescence with CD3 (Table 4 and Figure 4). Only the data of chemokine receptor expression in the neointima and adventitia are depicted, as most MNC were detected in these areas, but the composition in the other layers of CAV did not differ significantly. A high percentage of T-lymphocytes expressed the Th-1 associated receptors CXCR3 (>90%) and CCR5 (58–66%). Markers associated with a T-memory phenotype (CCR7 and CX3CR1) were expressed on 25–32% of T cells. A smaller population of T-lymphocytes expressed the Th-2 associated receptors CCR3 (40–47%) and CCR8 (38–41%).

CCR4 was also investigated as a Th-2 marker. A considerable number of T cells were positive for CCR4. However, this receptor was also expressed on SMC (both in references and CAV; Figure 5), which made an accurate detection of CCR4 on MNC unreliable. Other chemokine receptors tested were not expressed on SMC.

In double immunofluorescence studies the expression of the chemokine receptors was also studied on CD68+ macrophages in CAV. CCR5 (57–63%) and CCR3 (50–55%) were also expressed on macrophages (Figure 5). A small percentage of macrophages were positive for CX3CR1 (25%) and CCR7 (27%), but CCR8 was not expressed on macrophages.

Cytokine and chemokine receptor expression in CAV and references by Q-PCR

To analyze gene expression, mRNA was isolated from microdissected layers of coronary arteries: intima and media of references, and NI-LL, NI-SMC, media and adventitia of CAV. For comparison, mRNA was isolated from microdissected ExVascMNC. Gene expression was determined by Q-PCR.

MNC markers

As expected CD3, CD20, and CD68 expression was strong in the NI-LL and adventitia (not statistically different; Figure 6). The expression of CD3 and CD20 was absent in references. The expression level of CD3, CD20, CD27, CD28 and CD69 was stronger in the ExVascMNC than in the NI-LL (shown in part in Figure 6). CD68 was expressed in references although less pronounced ($p < 0.05$) compared to CAV arteries. No or very low expression of FOXP3 and CD56 was detected in references and CAV. FOXP3, but hardly any CD56 expression was detected in the ExVascMNC.

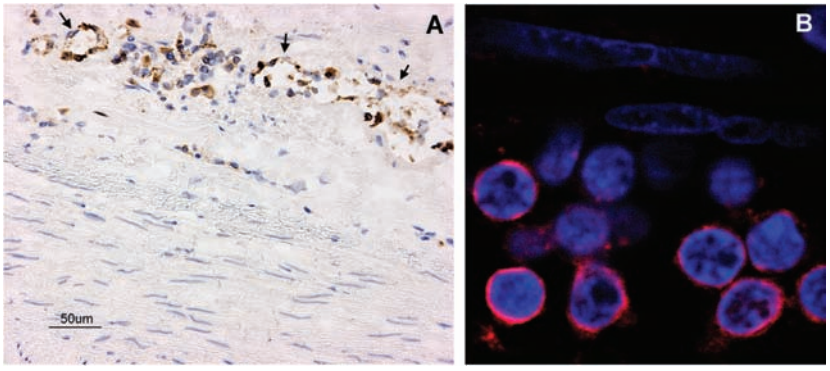

Figure 2: Immunohistochemistry of lymphocyte activation markers in CAV. A: Activation marker CD27 was preferentially expressed on inflammatory cells situated in the NI-LL near the endothelial layer (not shown here) and in small capillaries situated in the adventitia, indicated by the arrows (×200). B: Infiltrating cells in CAV vessels expressed CD69 (TRITC-conjugated amplification, red), underscoring the activation status of inflammatory cells present in the NI-LL (shown here) and adventitia. Nuclear staining visualized with DAPI (blue; ×800).

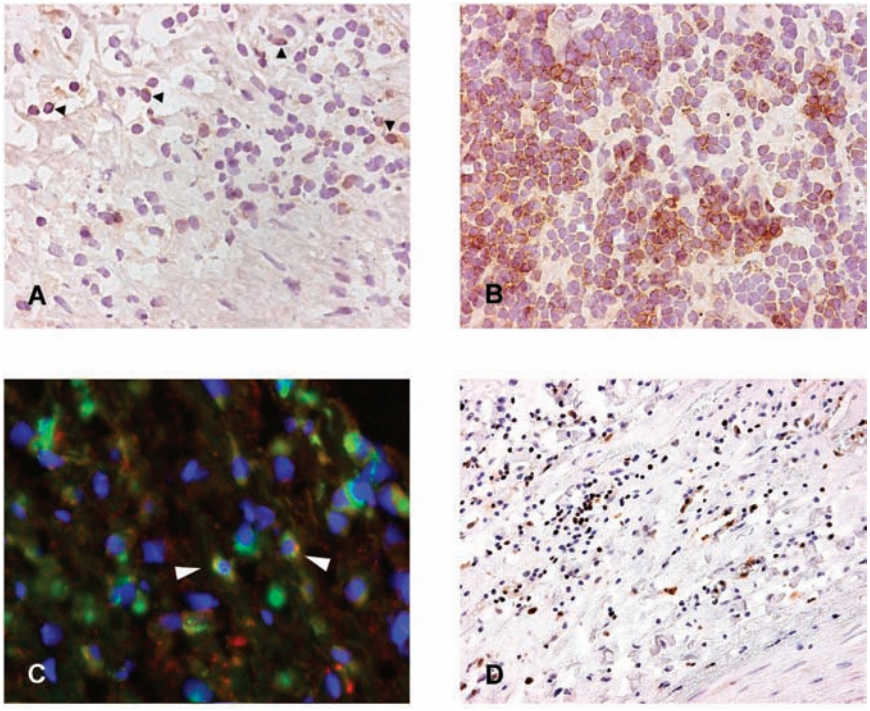

Figure 3: Immunohistochemical detection of CD28 and TGF-β1 in CAV. A: IHC of CD28 showing few positive lymphocytes in the NI-LL (arrowheads; ×400). B: IHC of CD28 showing large numbers of positive lymphocytes in the ExVascMNC (×400). C: Double CD4/CD28 immunofluorescence in the NI-LL, showing only few double positive lymphocytes (arrowheads; ×600). D: IHC of TGF-β1 showing expression in mononuclear cells in the adventitia of a coronary artery with CAV (×200).

Cytokines

Interleukin 1A, 2 and 4 were not detected in either of the layers of reference or CAV vessels. IL-2 expression was only detected in the ExVascMNC (Figure 7). In CAV only a low expression of IL-10 and IL-12B in the intima and adventitia was detected. These cytokines were hardly or not expressed in other CAV layers and references. Like IL-2, IL-10 was expressed at a low level in ExVascMNC, but IL-12B was negative.

IFN-γ showed a strong expression in the NI-LL, adventitia and ExVascMNC. An intermediate and weak expression of IFN-γ was detected in the media and NI-SMC, respectively (the expression between layers was not statistically different). In contrast to IFN-γ, TGF-β1 was not only detected at high levels in NI-LL and adventitia, but also in the intima of references. Expression of TGF-β1 in the ExVascMNC was low compared to the IFN-γ in this area.

Table 4: Double-immunofluorescence IHC results of chemokine receptor expression on CD3+ T-lymphocytes

	Chemokine receptor	Neointima %	Adventitia %
Th-1	CCR5	58	66
	CXCR3	>90	>90
Th-2	CCR3	40	47
	CCR8	38	41
T-memory	CCR7	29	32
	CX3CR1	25	28

Mean percentages of chemokine receptor positive cells, expressed as percentage of total number of T-lymphocytes (CD3) scored for the indicated areas (n = 5).

Chemokine receptors

Gene expression in CAV of Th-1 receptors CCR5 and CXCR3 was strong and intermediate, respectively in the NI-LL and adventitia ($p > 0.05$), and intermediate/weak in NI-SMC ($p < 0.05$; vs. NI-LL) and media ($p > 0.05$; Figure 8A). Both receptors were also expressed in the ExVascMNC, but in references only a low expression of CCR5 was detected. CX3CR1 was strongly expressed in NI-LL and adventitia, but only marginally (although not significantly different) expressed in all other CAV layers and references. CCR7 showed a distribution pattern as CX3CR1, but was particularly strong in ExVascMNC.

Th-2 receptor CCR3 showed a strong expression in NI-LL and adventitia of CAV, but an intermediate expression ($p < 0.05$; Figure 8B) in NI-SMC, media, ExVascMNC and references. CCR8 showed a similar but somewhat weaker distribution in CAV compared to CCR3 (not significantly different between CAV layers), but higher in the ExVascMNC, and absent in references. CCR4 was expressed in all layers of CAV, and in references (data not shown).

Localization of TGF-β1 in CAV vessels

TGF-β1 gene expression was detected by Q-PCR in all layers of CAV and reference arteries (Figure 7). As shown in Figure 3D, TGF-β1 was localized by IHC in MNC and in fibroblasts throughout the arteries.

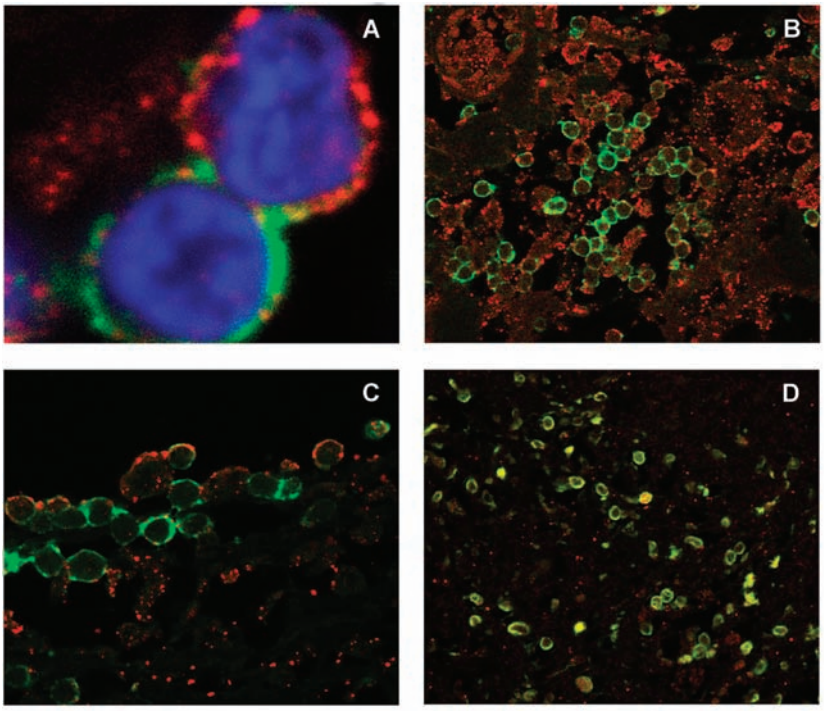

Figure 4: Double-immunofluorescent immunohistochemistry of infiltrating CD3+ T cells in CAV vessels. In all the pictures, chemokine receptor expression was visualized by TRITC-conjugated amplification (red) and CD3+ cells by FITC-conjugated amplification (green). A: Granular CCR8 expression was detected on T-lymphocytes. Nuclear staining was obtained with DAPI (blue) (×800). B: CCR3 expression was detected on T-lymphocytes present in the NI-LL and adventitia (shown here) (×200). C: CX3CR1 expression was detected on T cells present in the NI-LL near the lumen (×200). D: CXCR3 expression was detected almost exclusively on T cells present in the NI-LL. Double staining appears as yellow staining (×200).

Discussion

Previously, we demonstrated that the neointima in CAV was composed of two distinctive layers: a layer composed of loose connective tissue adjacent to the lumen (NI-LL), containing numerous infiltrating MNC, and a layer next to the internal elastic lamina composed of SMC (NI-SMC). We speculated that this concentric hyperplasia is due to MNC infiltration, and ECM deposition and not due to SMC proliferation as suggested in most studies on CAV (2). Our results demonstrated that especially within the NI-LL and the adventitia of CAV, an infiltrate of MNC is present (6). To get a better understanding of the type of immune response inducing CAV in human HTx patients, a more detailed characterization of the infiltrating T cells was performed in the present study.

Most infiltrating MNC in CAV were T-lymphocytes (CD3) with minor populations of macrophages (CD68) and B-lymphocytes (CD20) localized predominantly in the NI-LL

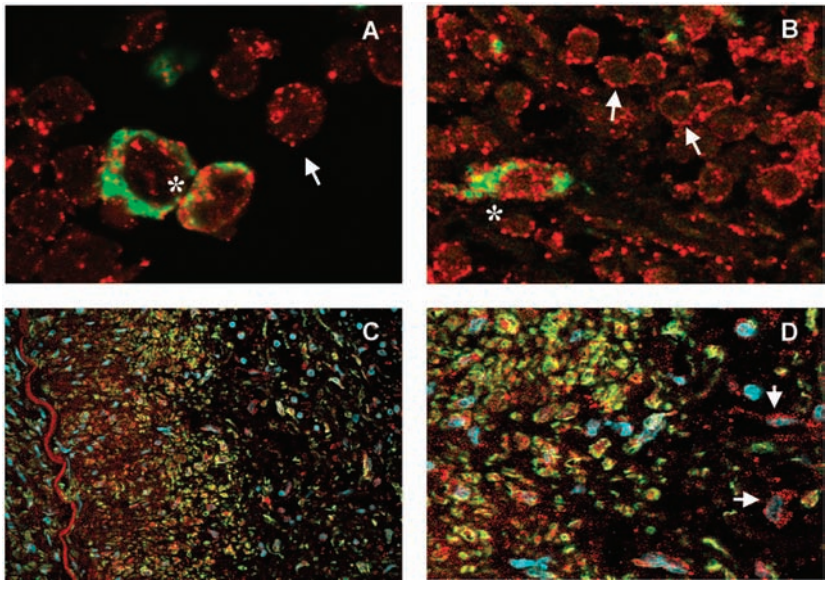

Figure 5: Chemokine receptor expression detected by double-immunofluorescent immunohistochemistry in CAV. A: CCR5 expression (TRITC-conjugated amplification, red) was detected on T-lymphocytes (single red positive: arrow) and macrophages (CD68, FITC-conjugated amplification, green) (×400). B: CCR3 expression (TRITC-conjugated amplification, red) was detected on T-lymphocytes (single red positive: arrows) and macrophages (CD68, FITC-conjugated amplification, green) present in the NI-LL and adventitia (×400). C and D: CCR4 expression (TRITC-conjugated amplification, red) was detected on T cells (single red positive: arrow) and smooth muscle cells (anti-smooth muscle actin, FITC-conjugated amplification, green). SMC are double stained, which presents a yellow staining (C: ×100; D: ×400).

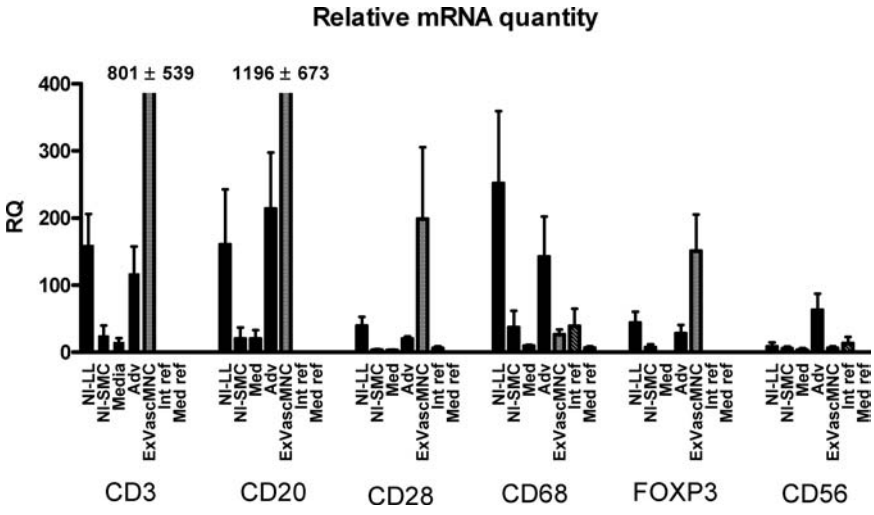

Figure 6: Gene expression profile of lymphocyte markers in CAV and reference vessels. Q-PCR data of MNC markers determined as relative mRNA quantity (RQ) in various layers of CAV and reference vessels as indicated in the figure (mean ± SEM). NI-LL is the luminal layer of the neointima, NI-SMC is the SMC layer of neointima adjacent of the media, media, adv is the adventitia, Int Ref is intima of references and Med Ref is media of references. ExVascMNC indicates mRNA isolated from MNC accumulated in the surrounding of the coronary arteries in CAV.

and the adventitia. Although T cells and B cells were almost absent in references, macrophages showed a similar distribution in CAV and references (although less in number in references than in CAV). CD56 positive NK-cells, and CD1a or DRC-1 positive dendritic cells were not detected in CAV and references.

In CAV many T-lymphocytes expressed CD27, CD69 and HLA-DR and can therefore be regarded as activated T cells. This is in agreement with experimental models that have reported an important causative role of activated T-lymphocytes in the onset of CAV (20,21). Besides, stimulation of CD27 by its ligand CD70 results in activation of IFN-γ producing Th1 cells (22), which is in accordance with the importance of these cells in CAV development (8,23). CD69 is considered to be an activation marker of T cells, selectively expressed in chronic inflammatory infiltrates (24). In the present study, CD27 and CD69 expressing T-lymphocytes were observed in the NI-LL and adventitia, suggesting an active infiltration of these cells in CAV. Tregs express FOXP3 and are CD4+CD25+ (25). FOXP3 was not or only in very small numbers detected in CAV vessels by IHC and by Q-PCR. As previously shown, IL-10 mRNA levels were relatively abundant in coronary arteries of transplanted hearts without CAV and low in HTx+CAV (6). The lack of FOXP3 and relative low amounts of IL-10 gene expression indicated a virtual absence of Tregs in CAV. Similar findings were recently published during chronic rejection in kidney transplantation. In that study a lack of FOXP3+ CD4+CD25+ lymphocytes was observed in kidney recipients with chronic rejection, whereas these cells were detected in patients without chronic rejection (26).

Chemokines and chemokine receptors are potential targets for CAV therapy (27–31). Moreover, chemokine receptor expression profiles on MNC allow to distinguish between different types of immune responses (15). As no NK-cells or dendritic cells were detected in CAV, a

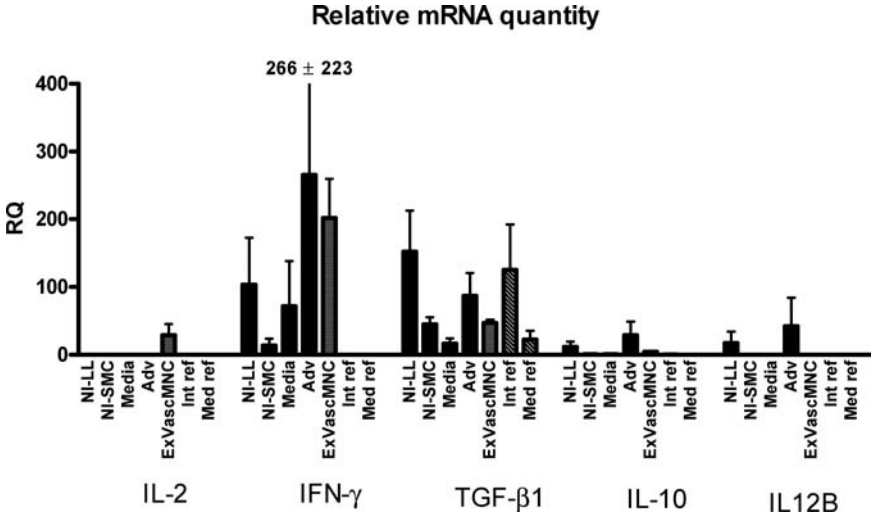

Figure 7: Gene expression profile of cytokines in CAV and reference vessels. Q-PCR data of cytokines determined as relative mRNA quantity (RQ) in various layers of CAV and reference vessels (mean ± SEM). See Figure 6 for explanation of the abbreviations.

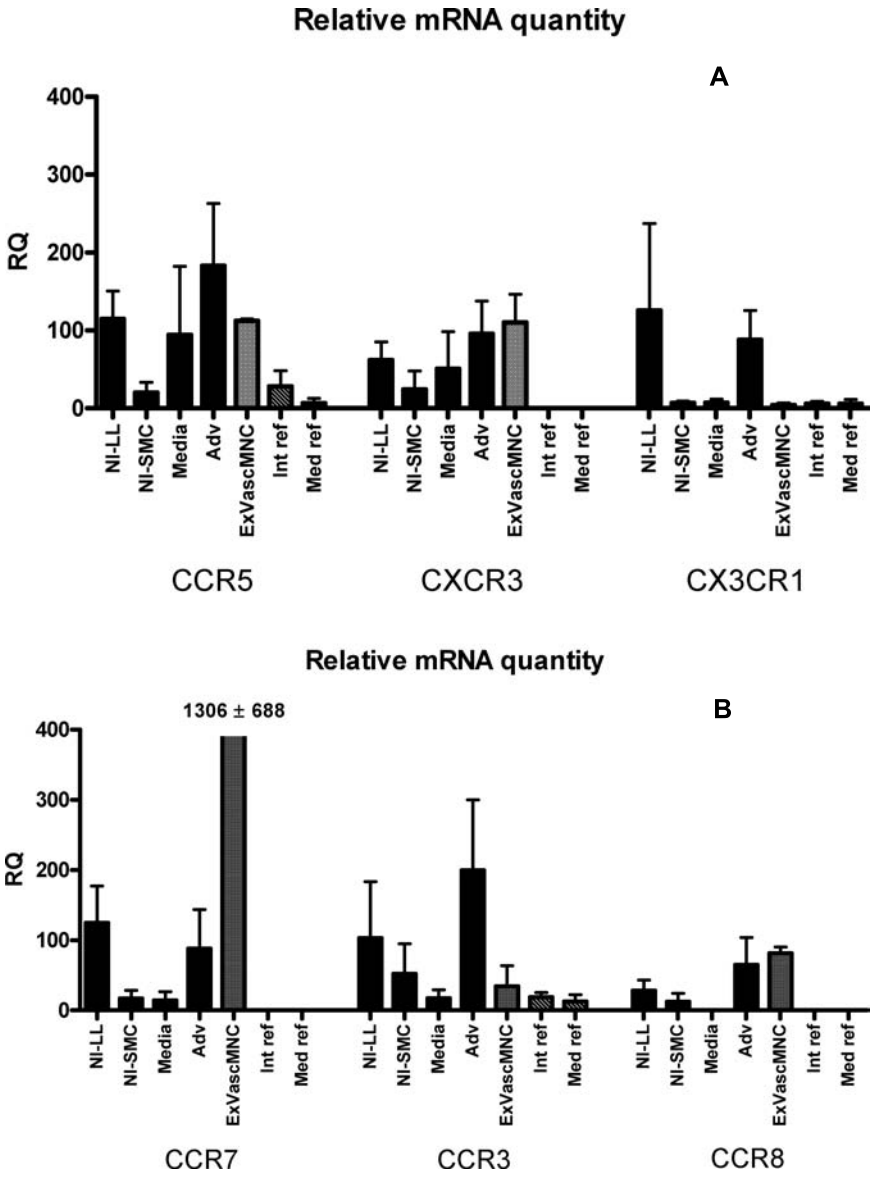

Figure 8: Gene expression profile of chemokine receptors in CAV and reference vessels. A and B Q-PCR data of chemokine receptors determined as relative mRNA quantity (RQ) in various layers of CAV and reference vessels (mean ± SEM). For explanation of the abbreviations see Figure 6.

shared chemokine receptor expression on these cells and T cells, did not influence T-cell chemokine receptor counts. A large number of T-lymphocytes (in the NI-LL and adventitia) expressed Th-1 associated chemokine receptors: CCR5, CXCR3 and CX3CR1. Q-PCR results underscored this protein expression in CAV. CX3CR1 (also expressed on memory T-lymphocytes) is expressed less prominently, but still on a relative high percentage of T-lymphocytes, compared to other infiltrates, for example, ExVascMNC (by IHC and Q-PCR), indicating the presence of a subpopulation of memory Th1-lymphocytes. A subset of memory cells lacking CCR7 are called *effector* memory T cells, whereas T cells expressing CCR7 are called *central* memory T cells, and are lymph-node-homing cells lacking inflammatory functions (32,33). This corresponds with the high level of CCR7 expression in the ExVascMNC (neo-lymphoid tissue). Small numbers of T-lymphocytes in CAV were CCR7 positive (29–32%). The remaining T-lymphocytes were CCR7 negative, suggesting the presence of effector memory T-lymphocytes, which is in concordance with CAV being a chronic inflammatory process (34). Th-1 IFN-γ mRNA expression was strong in CAV. IFN-γ is regarded as one of the most important effector molecules in CAV (8,11,35), as it is activating T-lymphocytes, fibroblasts and SMC, to produce chemokines (2,36,37).

Th-2 chemokine receptors (CCR3 and CCR8) were present on T cells in CAV, but compared to Th-1 receptors in lower numbers. Like Th-1 cells, the CCR3 and CCR8 expressing T cells were detected especially in the NI-LL and adventitia, which was underscored by the Q-PCR data. Q-PCR also showed expression of CCR3 and CCR5 in

references. This indicated that at least some expression of these chemokine receptors is due to macrophages. CCR4 expression was detected throughout all layers of the CAV vessels, including the media. IHC CCR4/αSMA double staining showed CCR4 expression on SMC. Although other chemokine receptors have been described on SMC, these were not detected in the present study (36–38).

Chemokines and cytokines have all been associated with the development of CAV in human and animal studies (9,11,39,40). The chemokines MIG, IP-10, I-TAC and fractalkine are expressed in CAV, as detected by IHC and Q-PCR on mRNA isolated from complete arteries (6). Our present Q-PCR data on isolated layers of CAV confirmed these data. A strong expression of the type I cytokine IFN-γ was detected. However, expression of most other cytokines was very low or lacking. A weak but significant expression of some cytokines was detected in the adventitia and in the ExVascMNC, which may suggest that immunosuppression is strong in the NI-LL and blocks the cytokine production. On the other hand, the typical phenotype of T cells in the NI-LL may be caused by the specific pathophysiological situation in the arterial wall, leading to a relatively weak CD expression and a typical (low) cytokine expression by both Th-1 and Th-2 cells. The observation of Pingiotti et al. (41) that the increase in CD4+/CD28-/CX3CR1+ cells in peripheral blood correlated with accelerated atherosclerosis and rheumatoid arthritis, may well correlate with the small percentage of T cells expressing CD28 and relative high number of CX3CR1 expressing T cells in the NI-LL of CAV. Taken together, the circumstances within the arterial wall of transplanted hearts may lead to a typical form of T-cell activation.

TGF-β is one of the most important stimulators of the recruitment of SMC into the intima (42) but also stimulates a profibrotic response. Although TGF-β1 was expressed in all layers of CAV arteries, a direct link with the proliferation in the intima in CAV could not be made, as TGF-β1 was also expressed in references. This suggests that other factors (alone or in combination with TGF-β1) caused the intima proliferation.

In conclusion, the majority of T-lymphocytes in coronary arteries with CAV are skewed towards an activated memory-Th-1 phenotype, based on CD and chemokine receptor profiles. In general, this type of response is associated with a chronic inflammatory response, instead of a response causing matrix deposition as observed in CAV. However, this study also showed that a significant population of T cells has a Th-2 phenotype. It may be suggested that either these T cells or otherwise macrophages (activated by factors produced by either population of T cells) are responsible for the proliferative response observed in CAV. Another important observation is that both the Th-1 and Th-2 cytokine production was almost absent or weak in CAV. As a result, the T-helper cells will not be able to produce a normal characteristic response. This typical population present in CAV, resulting in a strong IFN-γ and TGF-β1 production, will be an important factor in the proliferative CAV reaction.

Study limitations

This study concentrated on the T-helper cells as they form the major T-cell population in CAV, and we realize that the contribution of CD8+ T cells may be underestimated. Another restriction of this study is that the number of CAV arteries studied was relatively low (n = 5). Besides, the selection of CAV arteries suitable for the laser microdissection studies and without atherosclerosis resulted in 4/5 arteries from hearts of HTx-patients that only lived for 1–1.5 years after transplantation. Nevertheless, the CAV artery from a patient that survived for more than 10 years showed the same results, suggesting that this selection did not influence the data.

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