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Detection of TCD4$^+$ subsets in human carotid atheroma

Rômulo Tadeu Dias Oliveira [a], Rosiane Maria Silva [a], Fabio Haach Teo [a], Marcela Franco Mineiro [a], Maria Carolina Ferreira [a], Albina Altemani [b], Ronei Luciano Mamoni [a], Fábio Husseman Menezes [c], Maria Heloisa Souza Lima Blotta [a,*]

[a] Department of Clinical Pathology, Faculty of Medical Sciences, State University of Campinas (UNICAMP), Campinas, SP, Brazil
[b] Department of Pathology, Faculty of Medical Sciences, State University of Campinas (UNICAMP), Campinas, SP, Brazil
[c] Department of Surgery, Faculty of Medical Sciences, State University of Campinas (UNICAMP), Campinas, SP, Brazil

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ABSTRACT

Activated TCD4$^+$ cells are detected in human atherosclerotic plaques which indicate their participation in disease progression and destabilization. Among these cells, IFN-γ-producing T cells (T_H1) are recognized as having a pro-atherogenic role. Recently, the IL-17-producing T helper lineage of cells (T_H17) has been identified in atherosclerotic lesions. They have been linked to atheroma development through the production of pro-inflammatory mediators present in these lesions. Furthermore, IL-22 producing TCD4$^+$ cells (T_H22) have been identified in the atheromatous environment, but their presence and function has not been investigated. The aim of this study was to analyze the immune response mediated by pro-inflammatory subtypes of TCD4$^+$ cells in atheromatous lesions. Atherosclerotic plaques of 57 patients with critical stenosis of carotid submitted to endarterectomy were evaluated. Three carotid fragments from organ donors were used as control. mRNA analysis showed expression of T_H1 (IFN-γ, T-bet, IL-2, IL-12p35, TNF-α and IL-18); T_H2 (GATA-3); T_H17 (IL-17A, IL-17RA, Rorγt, TGF-β, IL-6, IL-1β, IL-23p19, CCL20, CCR4 and CCR6) and T_H22 (IL-22 and Ahr) related markers. Asymptomatic patients showed higher expression of mRNA of IL-10, TGF-β, CCR4 and GATA-3 when compared to symptomatic ones. Immunohistochemistry analysis showed higher levels of IL-23, TGF-β, IL-1β and IL-18 in macrophages and foam cells in unstable lesions compared to stable and control ones. *In vitro* stimulation of atheroma cells induced IL-17 and IFN-γ production. Finally we were able to detect, the following subpopulations of TCD3$^+$ cells: TCD4$^+$ IFN-γ$^+$, TCD4$^+$IL-17$^+$, TCD4$^+$IL-4$^+$, TCD4$^+$IL-22$^+$ and double positive cells (IFN-γ/IL-17$^+$, IFN-γ/IL-22$^+$ or IL-17/IL-22$^+$). Our results showed the presence of distinct TCD4$^+$ cells subsets in human carotid lesions and suggest that interactions among them may contribute to the atheroma progression and destabilization.

1. Introduction

Atherosclerosis is an inflammatory disease in which cholesterol accumulation and modification, endothelial injury and inflammation play a combined role in the development of lesions [1]. The recognition of atheroma related-antigens (e.g. modified lipoproteins and cholesterol crystals) by innate immune receptors (Toll like receptors [TLRs], Nod like receptors [NLRs] and scavenger receptors [SRs]) enables macrophages to produce cytokines that activate and dictate the fate of T lymphocytes [2].

Accumulated evidence suggests that TCD4$^+$ lymphocytes constitute the main population of adaptive immune response involved in atheroma progression, since the adoptive transfer of TCD4$^+$ cells to *scid/scid* mice promotes atheroma development [3]. In human lesions, the presence of interleukin (IL)-12 [4] and IL-18 [5] induces naïve TCD4$^+$ cells to develop a T helper 1 (T_H1) phenotype characterized by secretion of the cytokines interferon gamma (IFN-γ, IL-2 and tumor necrosis factor alpha (TNF-α) and the expression of the transcriptional factor T-bet [6]. In fact, IFN-γ is one of the most important factors involved in atheroma destabilization, since the absence of this cytokine or its receptor decreases lesion formation [7,8]. Atheromatous lesions also contain T helper 2 (T_H2) cells [9], which produce IL-4 and express GATA-3 as the main transcriptional

Abbreviations: IFN-γ, interferon-gamma; IL, interleukin; T_H, T helper cell; T-bet, T-box expressed in T cells; TNF-α, tumor necrosis factor-α; GATA-3, GATA binding protein 3; Rorγt, related orphan receptor-γt; TGF-β, transforming growth factor beta; CCL20, CC chemokine ligand 20; CCR, CC chemokine receptor; Ahr, aryl hydrocarbon receptor; Scid, severe combined immunodeficiency; LDL, low density lipoprotein; CE, carotid endarterectomy.

* Corresponding author. Address: Department of Clinical Pathology, Faculty of Medical Sciences, State University of Campinas (UNICAMP), PO Box 6111, 13083-970 Campinas, SP, Brazil. Tel.: +55 19 3521 9453; fax: +55 19 3521 9434.
E-mail address: heblotta@fcm.unicamp.br (Maria Heloisa Souza Lima Blotta).

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factor [6]. The role of T_H2 cells is still under investigation and contradictory results have been recently reported. It has been shown that the absence of IL-4 in $LDLr^{-/-}$ mice promotes small lesions [10], while animals which are resistant to atheroma development are protected against fatty streak development by Th2 cells [11].

Recent studies have pointed out the participation of T helper 17 (T_H17) cells in the immune response that takes place in atherosclerotic lesions [12]. The polarization to T_H17 subtype depends on the presence of tumoral growth factor beta (TGF-β and the pro-inflammatory cytokines IL-6 [13] or IL-21 [14], while its expansion is promoted by IL-23 [15]. In addition, the combination of interleukin 1 beta (IL-1β and IL-6 promotes T_H17 differentiation [16]. This lineage of T cells express the specific master transcription factor Rorγt [17] and the surface chemokine receptors CCR4 and CCR6 [18], being characterized by the production of IL-17A, IL-17F, IL-21, IL-22 [12]. Although $TCD4^+IL\text{-}17^+$ cells have been detected in human lesions [19], their role remain unclear. Interleukin 17 has an anti-inflammatory role in $LDLR^{-/-}$ $SOCS3^{-/-}$ mice [20]. On the other hand, the treatment of $ApoE^{-/-}$ mice with neutralizing antibody against IL-17 or transplantation of IL-17A deficient bone marrow in a $LDLr^{-/-}$ background inhibit lesion growth [21].

A new population of $TCD4^+$ cells that produce IL-22 (T_H22), a cytokine belonging to the IL-10 family [22], was recently described. These cells are generated in the presence of TNF-α and IL-6 [23] and express the aryl hydrocarbon receptor (Ahr) as the major transcriptional factor [24]. They have been associated with chronic inflammatory diseases like rheumatoid arthritis, Crohn's disease and psoriasis [25]. More recently, this T helper subtype was detected in atherosclerotic lesions [26], but its role in atherosclerosis pathogenesis have not yet been studied.

This cross-sectional study sought to investigate the concurrence of distinct subtypes of pro-inflammatory $TCD4^+$ cells in human carotid atheromatous lesions. The main endpoint was to quantify the different cell subtypes and their products. We hypothesized that Th17 as well as Th1 could be the major lymphocyte inflammatory populations in the plaque setting. The local dominance of one subset of $TCD4^+$ cells could influence the course of lesion progression and stability.

2. Materials and methods

2.1. Samples

A total of 57 human artery samples were collected from patients undergoing carotid endarterectomy (CE). The samples removed contained the endothelial and intima layers of the artery. Three samples of carotid arteries from organ donors were used as controls. This study was approved by the Ethics Committee of the State University of Campinas Medical School and written consent was obtained from each participant.

2.2. Quantitative real-time RT-PCR

In order to determine the subpopulations of $TCD4^+$ cells present in atheromatous lesions, we evaluated the genetic expression of molecules related to different T helper (T_H) subtypes. Total cellular RNA from 34 carotid plaques were extracted after dissociation of samples in TRIzol® reagent (Invitrogen, Carlsbad, CA) using a Power Gen 125 equipment (Fisher Scientific) according to manufacturer's protocol. Three-hundred micrograms of cDNA were amplified with specific primers (Table 1) using SYBR® Green PCR Master Mix (Applied Biosystems) methodology. Real-time reverse transcription polymerase chain reaction was performed using a StepOne equipment (Applied Byosistems). Each sample was run in duplicate. YWHAZ was used as the housekeeping gene to calculate relative expression of target genes using the method described by Pfaffl [27].

Table 1
Primers used for quantitative RT-PCR.

IL-17	
Sense	5′-AATCTCCACCGCAATGAGGA-3′
Antisense	5′-ACGTTCCCATCAGCGTTGA-3′
IL-17RA	
Sense	5′-CTACTATGTGGCGGGCATTT-3′
Antisense	5′-TCGGCACTAGCGGTTAAGTT-3′
CCL20	
Sense	5′-CTGGCTGCTTTGATGTCAGT-3′
Antisense	5′-CGTGTGAAGCCCACAATAAA-3′
CCR6	
Sense	5′-TGGTGAGCTGGAGTCATCAG-3
Antisense	5′-CACTCCCTTCAGCCTCACTC-3′
CCR4	
Sense	5′-CCATCTCGGATCTGCTCTTT-3′
Antisense	5′-AGCCCACCAAGTACATCCAG-3′
IFN-γ	
Sense	5′-CTAATTATTCGGTAACTGACTTGA-3′
Antisense	5′-ACAGTTCAGCCATCACTTGGA-3′
IL-2	
Sense	5′-AGTCCCTGGGTCTTAAGTGAA AG-3′
Antisense	5′-CAAGAAGGCCACAGAACTGAA-3′
IL-23p19	
Sense	5′-CTCAGTGCCAGCAGCTTTCAC-3′
Antisense	5′-TCTCTTAGATCCATGTGTCCCACTAG -3′
IL-1β	
Sense	5′-CACGATGCACCTGTACGATCA-3′
Antisense	5′-AGACATCACCAAGCTTTTTTGCT-3′
TNF-α	
Sense	5′-TGGCCCAGGCAGTCAGA-3′
Antisense	5′-GGTTTGCTACAACATGGGCTACA-3′
IL-6	
Sense	5′-GGTACATCCTCGACGGCATCT-3′
Antisense	5′-GTGCCTCTTTGCTGCTTTCAC-3′
IL-12p35	
Sense	5′-CCTGGACCACCTCAGTTTGG-3′
Antisense	5′-TGAAGGCATGGGAACATTCC-3′
IL-22	
Sense	5′-GCAGGCTTGACAAGTCCAACT-3′
Antisense	5′-GCCTCCTTAGCCAGCATGAA -3′
IL-18	
Sense	5′-CAGACCTTCCAGATCGCTTC-3′
Antisense	5′-GGGTGCATTATCTCTACAGTCAGAA-3′
IL-10	
Sense	5′-GGCCAGGGCACCCAGTCT -3′
Antisense	5′-TCGAAGCATGTTAGGCAGGTT-3′
TGF-β	
Sense	5′-TGAGGGCTTTCGCCTTAGC-3′
Antisense	5′-CGGTAGTGAACCCGTTGATGT-3′
GATA-3	
Sense	5′-AAGACATCCAGACCAGAAAC-3′
Antisense	5′-GTTAAACGAGCTGTTCTTGGG-3′
T-bet	
Sense	5′-GCGCCAGGAAGTTTCATTT-3′
Antisense	5′-CATTCTGGTAGGCAGTCACG-3′
Rorγt	
Sense	5′-AGAGGGACTCCTTGCCTCTC-3′
Antisense	5′-CAGCATCTGCTCACTTCCAA-3′
Ahr	
Sense	5′-CAGTTTATTCATGCAGCTGATATGCT-3′
Antisense	5′-CCGGAAAACTATCATGCCACTT-3′
YWHAZ	
Sense	5′-ACTTTTGGTACATTGTGGCTTCAA-3′
Antisense	5′-CCGCCAGGACAAACCAGTAT-3′

2.3. Histological analysis and Immunohistochemistry

Fourteen of 57 carotid samples were fixed in 4% formaldehyde and included in paraffin. Serial tissue sections were used and each of seven different antibodies [CD68 (DAKO), IL-1β (SantaCruzBiotechnology), IL-17 (CloneH-132 – SantaCruz); IL-18 (SantaCruz);