Relationships Between Phenotype and Function of Blood CD4⁺ T-Cells and Ascending Thoracic Aortic Aneurysm: an Experimental Study

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Abstract
Introduction: Non-familial ascending thoracic aorta dilation and aneurysms (TAAAs) are silent diseases in elderly patients. Histopathology revealed that functionally polarized infiltrating CD4⁺ T-cells play a key role in aortic wall weakening.

Objective: To evaluate the possible associations between phenotype and cytokine production of circulating CD4⁺ T-lymphocytes and the presence of TAA in patients with aortic valve disease (AVD).

Methods: We studied blood samples from 10 patients with TAA and 10 patients with AVD. Flow cytometry was used to quantify: a) CD4⁺ T-lymphocytes surface expression of CD25, CD28, and chemokine receptors (CCR5, CXCR3, CX3CR1); b) fractions of *in vitro* stimulated CD4⁺ T-cells producing cytokines (interferon gamma [IFN-γ], interleukin [IL]-17A, IL-21, IL-10); c) CD4⁺CD25⁺FoxP3⁺ regulatory T-cells (Treg) fraction. Enzyme-linked immunosorbent assays (ELISA) were performed for cytokines (IFN-γ, IL-6, IL-10, IL-17A, IL-23, transforming growth factor beta [TGF-β]) and chemokines (RANTES, CX3CL1).

Results: The total CD4⁺CD28⁻CD4⁺/CX3CR1⁺ T-cells fraction was higher (P=0.0323) in AVD (20.452±4.673) than in TAA patients (8.633±2.030). The frequency ratio of CD4⁺ T-lymphocytes producing IFN-γ vs. IL-17A+IL-21 cytokine-producing CD4⁺ T-cells was higher (P=0.0239) in AVD (2.102±0.272) than in TAA (1.365±0.123) patients. The sum of CD4⁺CD28⁻CD4⁺/CX3CR1⁺ T-cells correlated positively with values of the previous cytokine ratio (P=0.0002, R=0.732). The ratio of CD4⁺CD28⁺CD4⁺/CX3CR1⁺ T-cells vs. Treg was higher (P=0.0008) in AVD (20.859±3.393) than in TAA (6.367±1.277) patients.

Conclusion: Our results show that the presence of TAA in subjects with AVD is associated with imbalance between phenotypic and cytokine-producing subsets of circulating CD4⁺ T-lymphocytes, prevalently oriented towards a pro-fibrotic and IFN-γ counteracting effect to functional polarization.

Keywords: Aortic Aneurysm, Thoracic. CD4-Positive T-Lymphocytes. Flow Cytometry.

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Fast Track
INTRODUCTION

The non-familial ascending thoracic aortic dilatations and thoracic aortic aneurysms (TAA) are frequent in individuals older than 65 years of age (approximately 6-9%), with a risk of rupture or dissection ranging from 2 to 3.5 cases per 100,000 patients/year[1].

The progression from aortic dilatation to aneurysm is a multifactorial process partially undiscovered. Besides other well-known mechanisms[2], the role of chronic immune-mediated inflammations in defining biomechanical properties of the aortic wall is still to be determined. In particular, the study of cellular and molecular mechanisms leading to aortic fibrosis, considered the histopathological marker of an altered vascular remodeling process[3], might be an important target in understanding the individual’s susceptibility to non-syndromic ascending thoracic aortic dilatation and TAA formation. Recent findings in humans confirmed the key role of myofibroblasts (MF) in the extracellular matrix (ECM) proteolysis and deposition (fibrosis)[5]. MF activity is modulated by a wide array of pro- and anti-fibrotic cytokines and growth factors released by mononuclear immune cells infiltrating the inflamed tissue[5]. Especially, the cytokines produced by CD4+ T-lymphocytes play a causative role in the initiation and progression of fibrosis associated with pathological conditions such as systemic sclerosis, atherosclerosis, and the use of silicone mammary implants[6]. Given the crucial role of distinct CD4+ T-lymphocyte subsets in normal immune-regulation[7], the evaluation of their presence and function of peripheral blood CD4+ T-lymphocytes with the presence of TAA. Therefore, we have evaluated the presence of possible relationships between this pathological condition and: a) the expression of chemokine receptors and activation markers (CCR5, CXCR3, CX3CR1, CD25) on total blood CD4+ T-cell and on the pro-inflammatory/cytotoxic subset CD4+CD28−, known to be involved in vascular inflammation[10], b) the cytokine production (interferon gamma (IFN-γ), interleukin (IL)-17A, IL-21, IL-10) by in vitro stimulated CD4+ T-cells. Moreover, given their both beneficial and harmful roles in several clinical settings, including vascular injury[11], we quantified the circulating fraction of the CD4+CD25highFoxP3+ naturally occurring regulatory T-cells (Treg). On this basis, newly established phenotypic and functional blood CD4+ T-lymphocyte ratios, that overcome the traditional Th1/Th2 paradigm[12], have been calculated and related to the presence of an aortic aneurysm. We also measured the circulating levels of several cytokines (IFN-γ, IL-6, IL-10, IL-17A, IL-23, transforming growth factor beta (TGF-β)) and chemokines (RANTES, CX3CL1) known to influence CD4+ T-cells function and migration[13-15].

METHODS

Patients and Blood Samples

We have enrolled 20 patients undergoing surgery for aortic valve disease (AVD). TAA group (n=10) included patients undergoing aortic valve surgery with TAA surgery and AVD group (n=10) included patients undergoing surgery only for AVD, like stenosis or regurgitation, or both. Both groups underwent surgery by aortotomy. All patients had an indication for aortic valve surgery, according to current guidelines. TAA surgery was performed, if recommended by current guidelines. Exclusion criteria were the presence of genetic disorders, autoimmune and chronic inflammatory diseases, cancer, or hematological diseases. We enrolled patients with normal, ectasic or entirely aneurysmatic ascending aorta. Comorbidities, risk factors, and medical therapies were recorded, with particular attention to statins and/or angiotensin-converting enzyme (ACE) inhibitors, known to exert also immunomodulating effects[16-18]. Mean and peak aortic gradients were measured by Doppler echocardiography and used to define the grade of stenosis. Aortic valve lesions were classified as predominant stenosis (mean transvalvular gradient ≥40 mmHg and grade of regurgitation <3+/4+), predominant regurgitation (grade of regurgitation 4+/4+ and mean gradient <40 mmHg), and stenoregurgitation, in other cases.

Venous blood was collected at the admission in Vacutainer tubes containing tripotassium ethylenediaminetetraacetic acid (K3-EDTA) or sodium-heparin as anticoagulants. Plasma was obtained after blood centrifugation and immediately frozen at -80°C. The protocol was approved by the local ethics committee and informed consent was obtained from each patient.

Data Acquisition and Analysis

Leukocyte count was carried out with a routine analyzer. Flow cytometry was performed with a FACS scan instrument equipped with a CellQuest software (Becton Dickinson). Quantification of lymphocyte surface markers expression and intracellular cytokines content was based on measurement of fraction (percentage) of positive enzymes. Enzyme-linked immunosorbent assays (ELISA) were performed using the ASYS HITECH Microplate Reader (Eugendorf).

Surface Staining

Blood samples were processed as previously described for monocyte analysis[19]. In brief, K3-EDTA samples were treated with a red cell lysing solution to isolate leukocytes, and then stained with the following combinations of phycoerythrin (PE)/cyanine 5 (Cy5) (PCS)-, fluorescein isothiocyanate (FITC)-, and (PE)-conjugated monoclonal antibodies: CD4/CD28/CD25; CD4/CD28/CCR5; CD4/CD28/CXCR3; CD4/CD28/CX3CR1. Isotype controls were performed. The antibodies used were from Becton Dickinson, Pharmingen, Immunotech, R&D Systems, and MBL International Corporation. The acquisition was stopped after 30,000 CD4+ T-lymphocytes were collected for each sample (Figure 1).

Intracellular Cytokines

A whole blood staining procedure for intracellular cytokines detection was carried out as described[20]. In brief, heparinized samples were diluted 1:1 with RPMI (acronym for Roswell Park Memorial Institute) medium complete medium and incubated at a density of 2x10^6 leukocytes/ml for 5 hours, at 37°C. Phorbol...
myristate acetate (PMA) (Sigma Chemical Co.) (50 ng/ml) and ionomycin (1 mg/ml) were used as in vitro activators, in the presence of brefeldin A (eBioscience Inc.) (3 mg/ml final) as secretion blocking agent. Samples were then recovered, treated with a red cell lysis solution, and the isolated white blood cells (WBC) were stained with a combination of PC5- and FITC-conjugated monoclonal antibodies against the markers CD3 and CD4, respectively. Then, the cells were fixed with formaldehyde (2%) and permeabilized with saponin (0.5%). Cytokine staining was performed with PE-labeled monoclonal antibodies against IL-17A, IL-21, IFN-γ and IL-10. Isotype controls were performed. The acquisition was stopped after 30,000 CD3+ T-lymphocytes were collected for each sample (Figure 2).

**CD4+CD25highFoxP3+ Regulatory T-cells**

Blood samples were processed as published[21], with minor modifications. In brief, K3-EDTA samples were lysed as described before and the isolated WBC stained with PC5- and PE-conjugated monoclonal antibodies against the markers CD4 and CD25, respectively. Then, cells were fixed, permeabilized, and stained with an Alexa Fluor 488-conjugated anti-human forkhead-box-P3 (FoxP3) monoclonal antibody (eBioscience Inc.). Isotype controls were performed. Treg cells fraction was quantified into the 2% of CD4+CD25high double positive events, as reported[22]. The acquisition was stopped after 30,000 CD4+ T-lymphocytes were collected for each sample (Figure 3).

**ELISA**

Plasma levels of IFN-γ, IL-6, IL-10, IL-17A, IL-23, TGF-β, RANTES, and CX3CL1 were quantified by ELISA kits (R&D Systems) according to the manufacturer’s protocol. Minimum detectable concentrations were 15.6 pg/ml (IFN-γ), 0.156 pg/ml (IL-6), 0.78 pg/ml (IL-10), 31.25 pg/ml (IL-17A), 39.0 pg/ml (IL-23), 0.031 ng/ml (TGF-β), 0.031 ng/ml (RANTES), and 0.156 ng/ml (CX3CL1).
The mean frequency (± SEM) of CD4+ T-lymphocytes was 46.39±1.99; their absolute number (number of cells/ml) was 953.34±61.85, accordingly with published CD4+ T-cell reference values during aging [23]. The mean percentage (± SEM) of CD4+ T-cells belonging to the pro-inflammatory/cytotoxic subset CD4+CD28- was 5.56±1.33; this value, if referred to the age’s range of our patients, is also in accord with literature data [24]. Cumulative analysis of surface markers expression on total CD4+ and CD4+CD28- T-cell subsets are reported in Table 2.

The measurement of plasma cytokines/chemokines is reported in Table 3.

No relationship has been found between the plasma levels of cytokines/chemokines and CD4+ T-cells phenotypes, had an isolated ascending aorta replacement and 2 underwent combined aortic root surgery. Clinical and surgical characteristics of patients are reported in Table 1.

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intracellular cytokines levels, and presence of TAA, respectively. Cumulative quantification of intracellular cytokines is reported in Table 4; also, these data are in accordance with published T-cell cytokines levels in normal aging[25].

**Relationships among CD4+ T-cells Phenotypic Subsets**

The accumulation of CD28- T-cells in aging is driven by a repeated cellular activation, leading also to a modulation of CD4+ T-cells chemokine receptors expression[24,26]. In our patients, a tightly positive correlation between the circulating fraction of CD4+CD28- T-cells and the frequency of CD4+ T-lymphocytes carrying the fractalkine receptor CX3CR1 (CD4+/CX3CR1*) (P<0.0001, R=0.934) has been observed.

**Relationships among CD4+ T-cells Phenotype/Function and TAA**

The sum of blood CD4+CD28±CD4+/CX3CR1+ T-cells fractions was significantly lower in the TAA group than in the AVD group (Figure 4).

For each patient, the sum of CD4+CD28±CD4+/CX3CR1+ T-cell fractions correlated positively with the corresponding values of the above-mentioned cytokine ratio (Figure 5).

The mean value (± SEM) of blood Treg, expressed as a percentage of total CD4+ T-cells, was 1.19±0.09; no statistically significant differences have been observed between the 2 groups of patients. The frequency ratio of CD4+CD28±CD4+/ CX3CR1+ T-cells vs. circulating Treg was significantly lower in the TAA group than in the AVD group (Figure 6).
Clinical and experimental studies carried out in abdominal aortic aneurysms by histological and immunohistochemical procedures proved that a predominant Th2-mediated immune response, mainly driven by IL-4, IL-5, or IL-10 cytokines, induces severe aneurysm formations[27]. On the other hand, a prevalent Th1-mediated immune response, sustained by the infiltration of mononuclear cells releasing IFN-γ and IL-12, has been demonstrated to be responsible for transmural inflammation and external vessel wall dilatation in ascending TAA[28]. Nevertheless, independently of their localization, both types of dilating aortic lesions are characterized by common histopathological findings, also including evident modifications of ECM turnover with overexpressed collagen deposition and fibrosis[2-4]. As known, the ECM remodeling, with fibroblasts activation and development of fibrosis, occurring in pathological conditions, such as systemic sclerosis, atherosclerosis, parasitic infections, and after the use of silicone mammary implants, is suppressed by a locally polarized Th1 IFN-γ-driven immune response[5,6].

In spite of the histological demonstration of a prevalent Th1-mediated immune response into the wall of dilated and/or aneurysmatic ascending thoracic aortas, so far little is known about the systemic immunological status of these patients. Therefore, in our study, we evaluated several phenotypic and functional CD4+ T-cell features.

CD4+ T-cells Phenotype/Function, Aortic Valve Pathology, and Clinical Features

No correlation has been found between all the above-mentioned phenotypic and functional CD4+ T-cell features and the patients’ ages. Student's t-test and linear regression statistical analysis did not show significant associations between immunological parameters and patients' clinical characteristics, such as the prevalent type of valve pathology, aortic valve mean gradient values, associated risk factors, and current medical therapies. The mean functional ratio of IFN-γ vs IL-17A+IL-21 cytokine-producing CD4+ T-cells was significantly higher (P=0.0228) in female (2.144±0.147) than in male patients (1.398±0.07). On the other hand, the Chi-squares statistical analysis did not evidence a significant association between sex differences and the presence of TAA.

DISCUSSION

Clinical and experimental studies carried out in abdominal aortic aneurysms by histological and immunohistochemical procedures proved that a predominant Th2-mediated immune response, mainly driven by IL-4, IL-5, or IL-10 cytokines, induces severe aneurysm formations[27]. On the other hand, a prevalent Th1-mediated immune response, sustained by the infiltration of mononuclear cells releasing IFN-γ and IL-12, has been demonstrated to be responsible for transmural inflammation and external vessel wall dilatation in ascending TAA[28]. Nevertheless, independently of their localization, both types of dilating aortic lesions are characterized by common histopathological findings, also including evident modifications of ECM turnover with overexpressed collagen deposition and fibrosis[2-4]. As known, the ECM remodeling, with fibroblasts activation and development of fibrosis, occurring in pathological conditions, such as systemic sclerosis, atherosclerosis, parasitic infections, and after the use of silicone mammary implants, is suppressed by a locally polarized Th1 IFN-γ-driven immune response[5,6].

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**Table 1.** Patients’ clinical characteristics (n=20).

| Variables                      | Values (mean ± SEM or number (%)) |
|--------------------------------|-----------------------------------|
| Age (years)                    | 67.9±1.33                        |
| Sex (male)                     | 12 (60%)                          |
| Smoker                         | 11 (55%)                          |
| Obesity                        | 7 (35%)                           |
| Diabetes                       | 2 (10%)                           |
| Hypertension                   | 13 (65%)                          |
| Statin therapy                 | 12 (60%)                          |
| Angiotensin-converting enzyme inhibitors therapy | 6 (30%) |
| Predominant aortic valve pathology |                                    |
| Bicuspid                       | 6 (30%)                           |
| Regurgitation                   | 8 (40%)                           |
| Stenosis                       | 10 (50%)                          |
| Steno-regurgitation             | 2 (10%)                           |
| Mean gradient* (mmHg)          | 51.50±2.70                        |
| Type of surgery                |                                   |
| Isolated AVR                   | 10 (50%)                          |
| AVR + TAS                      | 10 (50%)                          |

*Calculated in aortic stenosis and steno-regurgitation. AVR=aortic valve replacement; SEM=standard error of the mean; TAS=thoracic aortic surgery for aneurysm.

**Table 2.** Surface marker expression on blood total CD4+ and CD4+CD28- T-cell subsets (percentage of positivity, mean ±SEM) (20 patients).

|                     | CD25     | CCR5     | CXCR3     | CX3CR1    |
|---------------------|----------|----------|-----------|-----------|
| Total CD4+          | 42.21±1.64| 14.51±0.82| 44.65±2.54| 8.98±1.54 |
| CD4+CD28-           | 3.98±2.64| 28.65±2.42| 71.75±3.70| 91.77±1.45|

SEM=standard error of the mean.

**Table 3.** Blood levels of cytokines and chemokines (mean ± SEM) (20 patients).

| Variables          | Mean ± SEM |
|--------------------|------------|
| IFN-γ (pg/ml)      | 10.36±3.25 |
| IL-6 (pg/ml)       | 2.65±0.50  |
| IL-17A (pg/ml)     | 13.51±2.37 |
| IL-23 (pg/ml)      | 20.76±3.90 |
| IL-10 (pg/ml)      | 0.55±0.07  |
| RANTES (ng/ml)     | 17.35±1.52 |
| TGF-β (ng/ml)      | 6.59±0.58  |
| CX3CL1 (ng/ml)     | 0.47±0.02  |

IFN-γ=interferon gamma; IL=interleukin; SEM=standard error of the mean; TGF-β=transforming growth factor beta.
We have found out that the cumulative CD4+ T-cell fraction calculated by percentage addition of circulating CD4+CD28− T-cells, a subset of cytotoxic T-lymphocytes producing large amount of IFN-γ[29], plus CD4+ T-cells carrying the fractalkine receptor CX3CR1 (CD4+/CX3CR1+), preferentially expressed on Th1 IFN-γ-producing cells[30], is significantly lower in the TAA group than in the AVD group.

A prevalent pro-fibrotic IL-17A/IL-21-driven polarization of blood CD4+ T-lymphocytes in the TAA group seems to be demonstrated, in our study, by the significantly lower mean ratio observed between the frequency of CD4+ T-lymphocytes producing the anti-fibrotic IFN-γ vs. the total fraction of IL-17A+IL-21 cytokine-producing CD4+ T-cells[6,31,32].

Previous papers demonstrated that human fibrocytes are potent antigen-presenting cells (APC) capable of priming naive functional features of peripheral blood CD4+ T-lymphocytes in patients undergoing a cardiac operation for aortic valve replacement associated, or not, with elective surgery for TAA.

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10-producing CD4+ T-lymphocytes separately suppress collagen among different phenotypic/functional characteristics of that the evaluation of balanced dynamic inter-relationships Th17- than for Th1 IFN-γ-mediated inflammatory responses[34,35], APC cross-talk via CD40-CD40L interactions for generation of CD4+CD28±CD4+/CX3CR1+ T-cell fraction indicates that the values of the IFN-γ/(IL-17A + IL-21) functional ratio and the production, and pro-fibrotic tissue remodeling.

Fig. 6 – Frequency ratio of blood CD4+CD28±CD4+/CX3CR1+ T-cells vs. Treg in AVD (white bar) and TAA (black bar) patients. Statistical comparison between ratios (mean ± SEM) was performed with ANOVA Student’s t-test for unpaired data. Statistically significant differences (P<0.05) were detected.

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