

Regulatory T cells, TGF- β , FoxP3 in acute myocardial infarction

Quantification of CD4⁺/CD25⁺⁺/CD127⁻ regulatory T cells and gene expression study of TGF- β and FoxP3 in individuals with acute myocardial infarction

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Introduction

Cardiovascular diseases are the most common cause of death worldwide. It is estimated that 80% of the premature deaths provoked by cardiovascular diseases could be prevented by changes in lifestyle [1]. Acute myocardial infarction (AMI) occurs when the blood flow that irrigates part of the heart muscle is interrupted [1, 2]. This is usually due to the occlusion of a coronary artery caused by the disruption of the vulnerable atherosclerotic plaque from the arterial wall, thus originating a lack of oxygen and consequently the ischemia of the irrigated tissues [3–6].

Atherosclerosis is a complex disease in which various components of the vascular, metabolic and immune systems are involved. A critical role in the progression and destabilization of atherosclerotic plaques has been attributed to the T helper 1 (Th1) lymphocytes through the production of proinflammatory cytokines [7–9]. The intensification of the inflammation may result in the

rupture of the atherosclerotic plaque with consequent formation of a thrombus, which can culminate in infarction [9, 10].

A loss of control in the immune response appears to be associated with an amplification and excessive inflammatory response. This loss occurs in patients with a decreased activity of regulatory T cells (Treg), as a result from the activity boost of effector T cells with proinflammatory activity [8–10].

In AMI, the cardiac injury activates immune mechanisms to initiate an inflammatory reaction. The loss in the regulation of the inflammation results in deleterious effects regarding the left ventricular function and in the evolution of the patient, and may also be the cause of the loss of organ function [9, 11]. Experimental studies about the levels of circulating Treg cells in patients with AMI found contradictory results. Several studies have observed impairment in the number and activity [12–15]. Furthermore, a recent study found an increase of these cells in circulation [16].

Treg cells (CD4⁺/CD25⁺⁺) constitute a population of thymus-derived lymphocytes which are involved in the maintenance of self-tolerance and suppression of abnormal or excessive immune responses, thus playing an essential role in regulating responses mediated by auto-reactive effector T lymphocytes [17–19]. Treg lymphocytes were initially characterized by the co-expression of the surface markers CD4⁺ and CD25⁺⁺ (alpha chain of the IL-2 receptor). Subsequently other expressed markers were found such as: cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), glucocorticoid-induced TNF receptor (GITR), OX40 (CD134), CD103, L-selectin (CD62 ligand – CD62L), lymphocyte activation gene 3 protein (LAG-3) and low expression of CD127 (the α -chain of the IL-7 receptor) [19–22].

However, the most defining characteristic of this population is the expression of the transcription factor Forkhead box protein P3 (FoxP3) [15, 18, 20], which is crucial for the development and activity of Treg cells: its translation regulates the expression of molecules associated with Treg cells such as CD25, CTLA-4 and GITR [18, 23, 24]. FoxP3 deficiency is involved in the function loss

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Abstract

Introduction: Acute myocardial infarction (AMI) occurs when the blood flow that irrigates part of the heart muscle is interrupted, usually resulting from occlusion of a coronary artery following the rupture of the atherosclerotic plaque. Several studies suggest a crucial role of T lymphocytes in the pathophysiology of atherosclerosis and AMI.

The CD4⁺/CD25⁺⁺/CD127⁻ regulatory T lymphocytes (Treg) constitute a lymphocyte population with immunosuppressive properties, characterized by the expression of the transcription factor Forkhead box protein P3 (FoxP3), crucial for maintaining its functions. These cells can mediate immunosuppression through the production of transforming growth factor beta (TGF- β).

Objectives: The study intended to determine the frequency of CD4⁺/CD25⁺⁺/CD127⁻ Treg cells in peripheral blood and the gene expression of FoxP3 and TGF- β 1 in these cells, in individuals with a diagnosis of AMI, in two moments (t=0 and t=1).

Material and Methods: The study focused on 12 patients with AMI and 10 other individuals with no history of cardiovascular disease. The quantification of the CD4⁺/CD25⁺⁺/CD127⁻ Treg cells was made by flow cytometry and the quantification of the TGF- β 1 and FoxP3 mRNA was performed using a real time PCR technique.

Results: There was an increase in the FoxP3 gene expression of Treg cells in patients with AMI at t=0, which was not confirmed in comparison to the frequency of those cells in circulation, since there was a decrease instead. Later, at t=1, a recovery in its number was observed.

Conclusion: There is a commitment in the frequency of Treg cells in AMI which, however, was not reflected in their activity.

Keywords: Regulatory T cells, TGF- β , FoxP3, acute myocardial infarction (AMI).

of Treg cells and severe autoimmune diseases [25].

The mechanism by which Treg cells mediate immune suppression is not fully understood. Several studies have shown that Treg cells can regulate the immune response through the release of immunosuppressive cytokines as IL-10 and transforming growth factor beta (TGF- β). TGF- β is a pleiotropic member of a large family of multifunctional cytokines with a wide range of biological effects, which plays an essential role in T cell tolerance [18, 26, 27]. Studies have demonstrated that a TGF- β deficiency in mice results in a lethal autoimmune phenotype with consequent progressive infiltration of leukocytes in multiple organs [28, 29].

In this context, this work intends to study the frequency of CD4⁺/CD25⁺/CD127⁻ regulatory T cells and its expression of TGF- β 1 and FoxP3 mRNA in individuals with AMI.

Material and Methods

Population

The quantification of Treg cells and of the TGF- β 1 and Foxp3 mRNA expression was performed on a group of 12 patients with diagnosed AMI in 2 times: at the time of the AMI (t=0) and approximately 1 month after (t=1) with a mean age of 53 \pm 13 years. (Table 1)

The samples were obtained at the Cardiology Service of the University Hospitals of Coimbra. The control group consisted of 10 healthy individuals without history of cardiovascular disease with a mean age of 53 \pm 5 years. Peripheral blood samples were collected by venipuncture into tubes with anticoagulant (tripotassium ethylene diaminetetraacetic acid – K₃EDTA).

Quantification of CD4⁺/CD25⁺/CD127⁻ regulatory T cells

Flow Cytometry

The quantification of regulatory T cells was performed by flow cytometry. Monoclonal antibodies were used, using a surface marking protocol: anti-CD4 conjugated with Piperidine Chlorophyll Protein associated with Cyanine 5.5 (PerCP Cy5.5) (BD Biosciences, clone R34.34, San Jose, California, USA), anti-CD25 conjugated with Fluorescein Isothiocyanate (FITC) (BD Pharmingen, clone M-A251, San Diego, USA) and anti-CD127 conjugated with Phycoerythrin (PE) (Beckman Coulter, clone SK3, Brea, California, USA).

After that step, 10 μ L of anti-CD4, anti-CD25 and anti-CD127 were added to the samples and incubated for 10 minutes at room temperature in the dark. By the addition of 2 ml of lysing solution (FACS™ Lysing Solution, BD Biosciences, San Jose, California, USA) diluted 1:10 in distilled water, the lysis of the erythrocytes was carried out

Table 1: Clinical characteristics and biochemical parameters of patients with AMI, enrolled in the study. Results are presented as mean \pm standard deviation.

N		12
Age		
Type of infarction ¹	Anterior	8
	Inferior	3
Number of injured vessels	< 2	8
	\geq 2	4
Troponin I admission (ng/ml)	\geq 0,5	6 (65,58 \pm 89,28)
	< 0,5	6 (0,14 \pm 0,14)
Maximum Troponin I	< 100	8 (40,40 \pm 30,2)
	\geq 100	4 (155,28 \pm 56,97)
PCR ² admission (mg/L)		0,30 \pm 0,2
Maximum PCR		4,51 \pm 5,29
Glucose (admission)		158,17 \pm 63,92
Number of internment days		6,92 \pm 2,68
EF LV ³ %		49,3 \pm 15,8

¹ Type of Infarction (Antero-lateral infarction observed in only one case);

² PCR –Protein C Reactive;

³ EF-LV – Ejection fraction of the left ventricle.

for 10 minutes at room temperature also in the dark. The samples were then centrifuged for 5 minutes at 200G.

After decantation, the cells were washed in 2 ml phosphate buffered saline (PBS) (Gibco – Invitrogen, Carlsbad, California, USA) diluted 1:10 in distilled water and the centrifugation and decanting was repeated. The cells were resuspended in 250 μ l of PBS and then proceeded to acquisition in the FACS Calibur flow cytometer (Becton Dickinson, San Jose, California, USA) using Cell Quest Software (Becton Dickinson, San Jose, California, USA). In a first step, 20000 total events were acquired from peripheral blood nucleated cells. The second step acquisition was made via an electronic “gate” in CD4 positive cells. Data analysis was carried out in Infinicity Software 1.4 (Cytognos, Salamanca, Spain).

Quantification of TGF- β and FoxP3 mRNA in Treg cells by Real-Time PCR

Sorting and RNA extraction

The CD25^{high}CD127^{low}CD4⁺ Treg cell population was separated using the FACS Aria Sorter cell sorter (BD, San Jose, CA, USA). For the RNA extraction from the Treg cells, RNAeasy Micro Kit (Qiagen, Hilden, Germany) was used.

cDNA synthesis (Real-Time PCR)

The quantification and integrity of the RNA was analyzed using the 6000 Nano Chip® kit in the Agilent 2100 analyzer (Agilent, Walbronn, Germany). The cDNA synthesis from the extracted RNA was performed with the SuperScript® VILOTM RT-PCR kit (Invitrogen, California, USA) according to the manufacturer’s instructions.

Relative quantification of gene expression

The relative quantification of the gene expression in the samples under study was performed by Real-Time PCR using the LightCycler 480 II thermal cycler (Roche, Basel, Switzerland).

The normalization of the relative gene expression quantification was performed with the Selection Gene Human kit (Primer Design, Southampton, UK) and with the geNorm software (Ghent University Hospital, Center for Medical Genetics, Ghent, Belgium) in order to select the suitable housekeeping genes for this study (ATP5B and B2M).

The real time PCR reactions were carried out using QuantiTect Primer Assays (Qiagen, Hilden, Germany) with optimized primers for FoxP3 (QT00048286) and TGF- β 1 (QT00000728) together with the QuantiTect SYBR Green PCR kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

The amplification reactions of cDNA samples by real-time PCR were performed in the Lightcycler 480, with an initial step of 10 minutes at 95°C, to activate the DNA polymerase, followed by 40 cycles of amplification with 15 seconds at 95°C for denaturation and 30 seconds at 60°C, plus 30 seconds at 72°C for the primer annealing and extension of the complementary chain.

The results of the quantitative real-time PCR were analyzed with the LightCycler 480 software (Roche, Basel, Switzerland) and the quantification was performed in the qBasePlus software (Biogazelle, Zulte, Belgium)

Statistical Treatment

Statistical analysis was performed using the Statistical Package for Social Sciences software (SPSS) 15.0 for Windows (SPSS Inc. Chicago, USA).

When comparing the results between the different groups, the test used was Student's t test. The results are presented as mean \pm standard deviation. The differences between groups were considered statistically significant when p-value <0.05.

Results

Absolute values of circulating leukocytes

In the analysis of the absolute values of leukocytes, it was found that these were higher in individuals with AMI at the first time (t=0) compared with the control group and with the second study time (t=1). There was also a decrease in lymphocytes and an increase of neutrophils in the AMI group at t=0, when compared to the control group and the AMI group at t=1. For the t=1 absolute values were similar to the control group values. (Table 2)

Frequency of regulatory T cells (CD4+/CD25+/CD127-)

Regarding to the CD4+ lymphocytes population there was a reduction in their rate in t=0 in the with AMI group, comparing to the control group and to t=1. It was also observed that the percentage of CD4+/CD25+/CD127- regulatory T cells within the CD4+ cell population was similar in all three groups. (Table 3)

As for the frequency of CD4+/CD25+/CD127- Treg cells in the total number of leukocytes in peripheral blood, there was a statistically significant decrease in its percentage in individuals with AMI at t=0, relatively to the control group. This decrease was also significant in terms of absolute values. For t=1 it was found that the percentage of Treg cells was superior in individuals with AMI in comparison with t=0 as well as in comparison to the control group. However, in this last case, the difference was not statistically significant. This situation was equally verified in terms of absolute values. (Table 3)

Gene expression levels of FoxP3 and TGF- β by Treg cells

In the study of FoxP3 gene expression by regulatory T cells, it was found that at t=0, individuals with AMI showed higher levels relatively to the control group, and this difference was statistically significant (p=0.044). At t=1, the AMI group showed no difference in the FoxP3 expression comparing to the control, verifying, however, a significant decrease relatively to t=0. (Figure 1)

Table 2: Absolute values (number of cells per μ L) of leukocytes, lymphocytes, monocytes and neutrophils. Results are presented as mean \pm standard deviation.

	Absolute values of leukocytes	Absolute values of lymphocytes	Absolute values of monocytes	Absolute values of neutrophils
Controls	6520 \pm 1472	1711 \pm 598	343 \pm 203	4257 \pm 1106
Time 0	12942 \pm 2968*	*1309 \pm 612	391 \pm 187	*10832 \pm 2636
Time 1	7042 \pm 1531	1587 \pm 529	322 \pm 94	4724 \pm 1234

* p-value < 0,05

Table 3: Percentage of TCD4+ lymphocytes and Tregs lymphocytes (CD4+/CD25+/CD127-) and absolute values (number of cells per μ L) in peripheral blood cellularity. Results are presented as mean \pm standard deviation.

	% CD4+ Lymphocytes	% CD4+/CD25+/CD127- Lymphocytes		Absolute values of CD4+/CD25+/CD127 Lymphocytes
		CD4+	Total	
Controls	11,97 \pm 3,90	4,91 \pm 2,17	0,61 \pm 0,14	38,70 \pm 7,91
Time 0	*4,67 \pm 3,37	5,66 \pm 1,97	*0,24 \pm 0,13	*26,96 \pm 11,84
Time 1	10,82 \pm 3,33	6,85 \pm 1,69	0,76 \pm 0,40	44,31 \pm 14,66

* p-value < 0,05

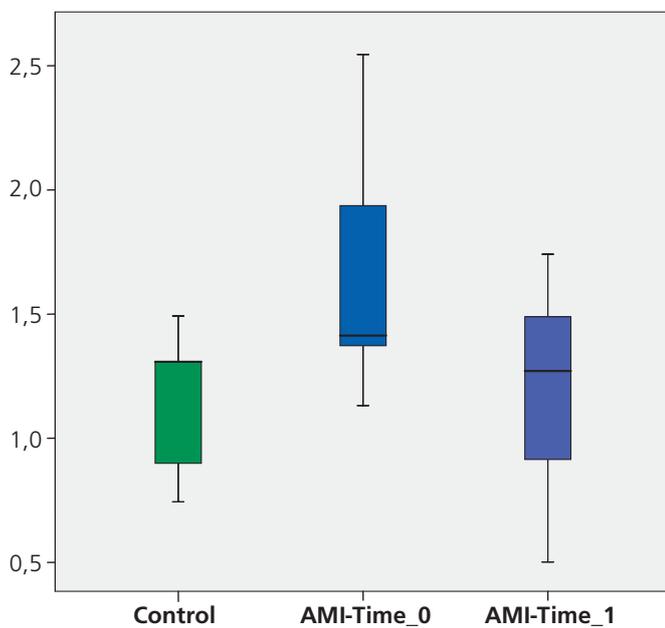


Figure 1 (left): Normalized gene expression levels of FoxP3 by CD4+/CD25+/CD127- by regulatory T cells in the control group and group with AMI, at the first (t=0) and second (t=1) study time.

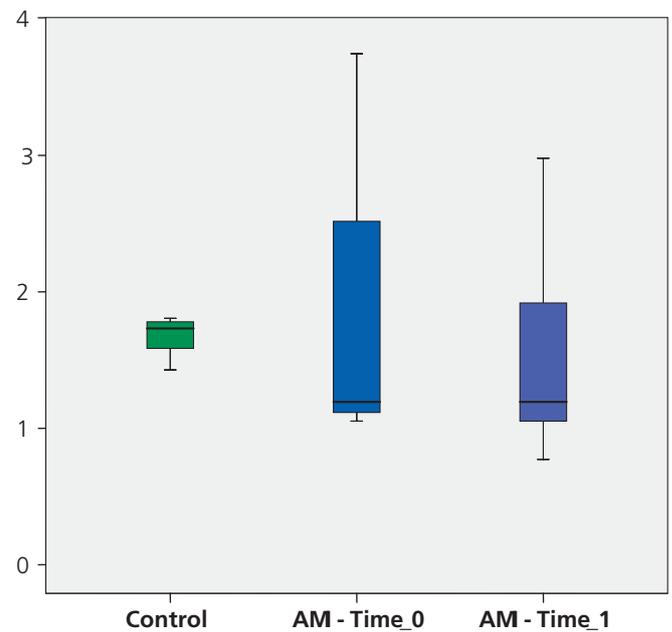


Figure 2 (right): Normalized gene expression levels of TGF-β1 by CD4+/CD25+/CD127- by regulatory T cells in the control group and group with AMI, at the first (t=0) and second (t=1) study time.

Regarding the TGF-β1 gene expression by regulatory T cells, it was found that there were no differences between the levels expressed by the control group and the AMI group at t=0, but there was a small decrease of its expression at t=1. Nevertheless, the differences encountered were not statistically significant. (Figure 2)

Relationship between the number of circulating Treg cells, the levels of FoxP3 and TGF-β1 mRNA expression and the studied clinical parameters.

An increased expression of FoxP3 was found in the group with AMI, which presented troponin values inferior to 0.5 mg/dl (p=0.039) at the admission. (Table 1, Figure 3)

For the remaining studied variables (type of infarction, troponin peak values and number of affected vessels) there were no significant differences in the levels of Treg cells and their gene expression of FoxP3 and TGF-β1 (results not shown).

Discussion

Absolute values of circulating leukocytes

The increase of the absolute values of leukocytes observed in the peripheral blood of individuals with AMI at t=0 compared with the values of the control group is probably linked to the inflammatory environment that is generated due to the ischemia of the heart tissue. In consequence, there is a response by the leukocytes, a finding which is in agreement with other previously pub-

lished studies [9, 11]. Moreover, according to Bodi V *et al* [9] and Munir T *et al* [30], the degree of leukocytosis reflects the extension of the infarcted area. This increase was mainly due to the elevated number of neutrophils, which are in fact early recruited after cardiac injury involving interaction with adhesion molecules (selectins) and activated vascular endothelial cells as described by Frangiogiannis N *et al* [11] and Bodi V *et al* [9]. There was also a decrease in the number of lymphocytes at this stage, which is in agreement to what was reported by Bodi V *et al* [9], confirming that it was found a higher prevalence of cardiovascular events in people who have lymphocytopenia.

At t=1 there is a restoration of the leukocyte values to normal levels, which may indicate the resolution of the inflammation [11, 30]. Several authors refer to the importance of these levels, which can be predictive indicators of the consequences of an AMI: a leukocyte count greater than 10,000 cells/μL has been associated with higher mortality rate [9, 31, 32]. At this stage it has also been observed that there is a decrease in the number of neutrophils and an increase of lymphocytes, approaching the values of the control group, which is indicative of good prognosis according to Nunez J *et al* [33] and Bodi V *et al* [9] that refer that an increased neutrophil/lymphocyte ratio is associated with higher risk of dying during the follow-up.

Frequency of regulatory T cells (CD4+/CD25+/CD127-)

For the quantification of CD4+/CD25+/CD127- regulatory T cells, it was verified that at t=0, individ-

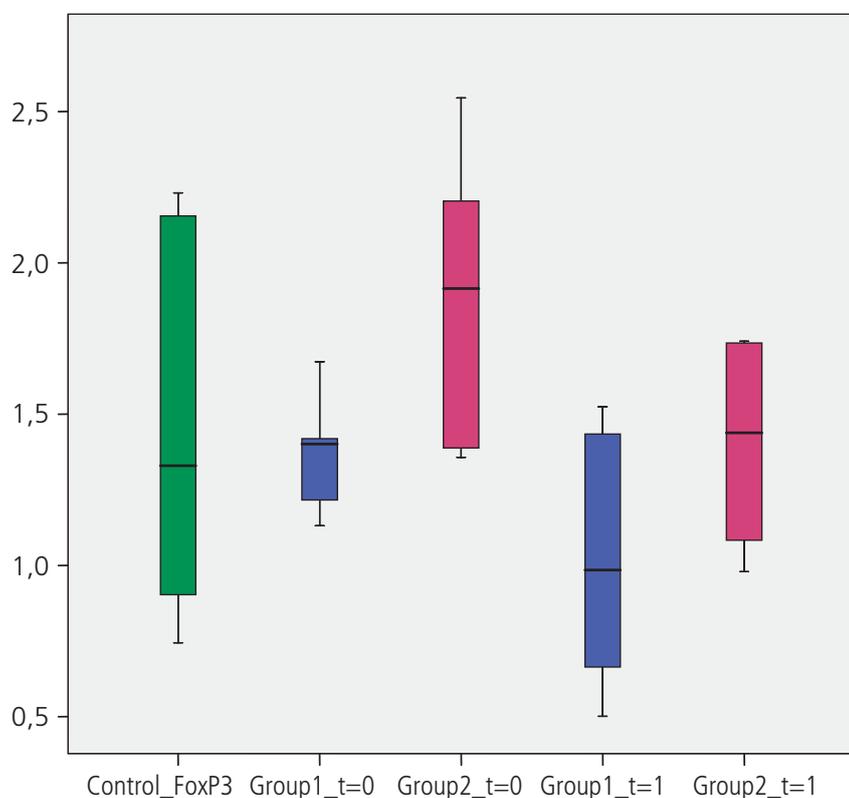


Figure 3: Normalized gene expression levels of FoxP3 by CD4⁺/CD25⁺/CD127⁻ by regulatory T cells on 2 groups with AMI, respectively in the 2 study moments (t=0 and t=1). Group 1 – Troponin in admission less than 0,5 ng/ml; Group 2 – Troponin in admission greater than or equal to 0,5 ng/ml.

uals with AMI presented a decrease in the percentage of these cells in comparison to the control group, which was also followed in terms of absolute value. There is in fact a decrease in the number of circulating Treg cells, however its proportion in the population of CD4⁺ T lymphocytes is stable. Possible explanations for this could include the general decrease of lymphocytes, as the population of CD4⁺ T lymphocytes, or because there is a loss of its activity. Bodi V *et al* [9] and Hansson L *et al* [6] defend that underlying the AMI is a loss in the control of immune response, with reduced and impaired function of regulatory T cells.

Experimental evidence found contradictory results regarding the behavior of regulatory T cells in AMI. Several authors (Mor *et al* [12], Han SF *et al* [13], Sardella G *et al* [14], X and Cheng *et al* [15]) observed a decrease in the frequency of Treg cells in circulation, which is concordant with the findings of this study. Also, according to Han SF *et al* [13] and George J [10], this reduction is consistent with a Th1 cell expansion and increased inflammatory activity, assigning to this imbalance a key role in destabilization and rupture of the atherosclerotic plaque. Yet, Ammirati E *et al* [16] presented different results, since they observed increased levels of Treg cells during the acute phase of AMI, further stating that this increase was not the result of a myocardial response to injury. In a recently published study of Caligiuri G *et al* [34], some hypotheses were raised to explain these various findings, indicating that a decrease of Treg cells in circulation may be due to the fact that they are recruited to the inflammation site.

Nevertheless, its increase in peripheral blood can be linked to insufficient mobilization for that same location.

In the second study moment (t=1), there was an increase in the percentage of Treg cells in circulation as well as in terms of absolute values, which can be explained by an increase of lymphocytes, including the proportion of CD4⁺ cells, close to the normal range, suggesting a favorable recovery, since it is mentioned in earlier studies that a low CD4⁺ lymphocyte count during follow-up is associated with a larger loss of cardiomyocytes and repeated AMIs [9, 31, 32]. This recovery in the number of Treg cells may also be indicative that the inflammatory response is controlled, since according Bodi V *et al* [9] an excessive inflammatory response has been associated with patients who show a decrease of regulatory T cells. Sardella G *et al* [14] showed experimentally that after coronary intervention there is a restoration of the Treg cell levels, which promotes the cardiac recovery for its anti-inflammatory activities. Ammirati E *et al* [16] also found increased levels of Treg cells 55 days after AMI, justifying this increase as a way of compensation due to the myocardial damage and in order to reestablish a steady state between regulatory T cells and effector T cells.

Gene expression levels of FoxP3 and TGF-β by Treg cells

At the first moment of the study, there was an increase in gene expression levels of FoxP3 by regulatory T cells, thus it can be concluded that although there was some reduction of these cells, the results are indicative that its normal function is not compromised, and there may even be a greater activity in a compensatory way. Mor A *et al* [12] and Han SF *et al* [13] reported contradictory results, showing a decreased expression of FoxP3 by Treg cells, which could be the origin of the loss of activity by these cells in AMI. At t=1, the gene expression of FoxP3 by Treg cells decreased relatively to t=0, approaching the levels expressed by the control group, which may indicate that the activity of these cells is stabilized, revealing a controlled immune response.

The levels of TGF-β1 expressed by regulatory T cells at t=1 showed no changes comparing to the control group. Several authors emphasize the importance of TGF-β in the stabilization of the arteriosclerotic plaque for their immunosuppressive effects [10, 11, 35]. George J [10] further states that there is a disturbance in the function of Treg cells in acute coronary diseases, namely by the decrease of the secreted products such as TGF-β1, which was not disclosed in our findings. Regarding t=1, there were also no changes found in gene expression of TGF-β1 by Treg cells. Papers published by other researchers emphasize the central role of TGF-β in the resolution of inflammation in the infarcted myocardium, including

the suppression of cytokines and chemokines expressed by leukocytes and endothelial cells [11, 35, 36]. Tashiro H *et al* [37] reported that patients with higher levels of TGF- β in plasma present a better outcome.

Relationship between the number of circulating Treg cells, levels of FoxP3 and TGF- β 1 gene expression and studied clinical parameters

In the analysis of the association with the studied clinical parameters, the group in which the troponin values on admission were less than 0.5 mg/dl showed a higher expression of FoxP3 by Treg cells, demonstrating a greater activity. Troponin I is released in response to myocardial necrosis, and its release is proportional to the length of the infarcted area as Somani D *et al* [38] state in their studies. This finding leads us to the idea that an increased activity of regulatory T cells may be beneficial when monitoring the injury of cardiomyocytes.

In fact, on average, this group reached lower maximum levels of Troponin I (despite not being statistically significant), a situation associated with a better prognosis [38]. Some authors refer that there is an auto-immune response by T cells against the released Troponin I [39, 40]. Frenkel *et al* describe the importance of the tolerance induction to the antigens of troponin, mainly through the secretion of anti-inflammatory cytokines (TGF- β 1 and IL-10) by regulatory T cells, which would be beneficial to decrease myocardial damage [39].

Conclusion

The results revealed an increase in FoxP3 gene expression of Treg cells in patients with AMI at $t=0$, a finding not followed by the frequency of these cells in circulation, which suffered a decrease.

It can be concluded that there is a commitment in the frequency of Treg cells in AMI, which, however, was not followed in terms of their activity.

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