Platelets from patients with myocardial infarction can activate T cells

by Elena E. Solomou, Katerina Katsanaki, Elena Kalyvioti, Vasilios Gizas, Angelos Perperis, Dora Babali, Evgenia Verigou, Haralambos Gogos, George Hahalis, Periklis Davlouros, and Dimitrios Alexopoulos

Haematologica 2020 [Epub ahead of print]

Citation: Elena E. Solomou, Katerina Katsanaki, Elena Kalyvioti, Vasilios Gizas, Angelos Perperis, Dora Babali, Evgenia Verigou, Haralambos Gogos, George Hahalis, Periklis Davlouros, and Dimitrios Alexopoulos. Platelets from patients with myocardial infarction can activate T cells. Haematologica. 2020; 105:xxx
doi:10.3324/haematol.2019.243402

Publisher's Disclaimer.
E-publishing ahead of print is increasingly important for the rapid dissemination of science. Haematologica is, therefore, E-publishing PDF files of an early version of manuscripts that have completed a regular peer review and have been accepted for publication. E-publishing of this PDF file has been approved by the authors. After having E-published Ahead of Print, manuscripts will then undergo technical and English editing, typesetting, proof correction and be presented for the authors' final approval; the final version of the manuscript will then appear in print on a regular issue of the journal. All legal disclaimers that apply to the journal also pertain to this production process.
Title: Platelets from patients with myocardial infarction can activate T cells.

Running title: Platelets can activate T cells

Author List:
Elena E Solomou (1), Katerina Katsanaki(1), Elena Kalyvioti(1), Vasilios Gizas(1), Angelos Perperis(1), Dora Babali(1), Evgenia Verigou(1), Haralambos Gogos(1), George Hahalis(1), Periklis Davlouros(1), and Dimitrios Alexopoulos(2).

(1). Department of Internal Medicine, University of Patras Medical School, Rion 26500, Greece
(2). B’ Department of Cardiology, University of Athens Medical School, Athens 12462, Greece

Address of Correspondence:
Elena E Solomou, MD
Assistant Professor Internal Medicine-Hematology
University of Patras Medical School
Rion 26500
Greece
Phone: +306970839894 Fax: +302610993950
Email: elenasolomou@hotmail.com, esolomou@med.upatras.gr
Platelets are not just the cells that mediate coagulation. The last years there is compelling evidence that these cells may act as key regulators in immune responses and participate in the pathogenesis of various immune-mediated diseases (1-3). It was shown that platelets can function as antigen presenting cells and activate T cells through MHC-I (4). Patients with myocardial infarction (MI) have activated platelets but whether these platelets interact with the immune system is not completely clear. It is well established that immediate treatment with factors that prevent platelet aggregation is crucial for these patients; the sooner these agents are given the better the outcome (5). T cells have been implicated in the pathogenesis of myocardial infarction (6). Autoreactive T cells may have a role in the destabilization of the atherosclerosis plaque and plaque rupture. Regulatory T cells (CD4+CD25+hi, Foxp3+; Treg) represent an important subset of T cells essential for immune homeostasis that control activation of T cells (7). Up to date, data on Treg show that these cells have a protective role in atherosclerosis; Treg alterations in patients with myocardial infarction with ST elevation (STEMI) are not fully elucidated (8); there is no available data on the possible interaction of activated platelets with the T cells in these patients (9-12).

The micro RNAs (miRNAs) represent small non-coding sequences with the ability to suppress different genes. In mice the deficient expression of the miR155 was associated with enhanced atherosclerosis, decreased plaque stability and decreased Treg (13). However, the role of miR155 is not established in patients with myocardial infarction.

We aimed to investigate whether T cells can be activated by the circulating activated platelets from patients with STEMI and explore how Treg cell population is affected.
After written informed consent was obtained, peripheral blood mononuclear cells were isolated from 33 patients with STEMI (29 men, 4 women, mean age 62.5 years) at the time of hospital admission at diagnosis, before any treatment, as well as 5 days and 30 days later. Ten healthy subjects and 5 patients with unstable angina served as the control and disease-control group, respectively. We also isolated platelet rich plasma or plasma alone from the patients and healthy subjects and used it in mixed cultures with the isolated peripheral blood mononuclear cells. The membrane expression of CD69 was used as a marker for T cells activation. Three additional patients who received aspirin or clopidogrel before admission were also analyzed as an internal control group (See Supplemental Data). We analyzed the percentages of CD4+CD25high+FOXP3+ cells representing the regulatory subset (Tregs) at all three time points as described above (Day 0, day 5, and at one month of follow-up) (14). The miR155 levels were evaluated using real-time PCR in samples from patients and healthy individuals. In STEMI patients the expression of miR155 was evaluated at admission and when the patients were discharged (Day 0 and day 5, respectively). This study was approved by the Ethics Committee of the Patras University Hospital.

We first examined the activation of T cells in mixed cultures as described above (See Supplemental data). The expression of CD69 on the surface of T cells was used as a marker for T cell activation. T cells that were incubated with platelets from patients with STEMI showed statistically significant increase of CD69 expression (2.163% vs 0.575%, p =0.013) compared to the T cells that were incubated with platelets from healthy individuals (Figure 1A). We did not record any activation in T cells that were incubated with plasma alone neither from patients with STEMI nor from healthy individuals. These re-
results show that the activation of T cells incubated with platelets is specific only for platelets from patients with STEMI. It is also of note that T cells that were incubated with platelets from patients previously treated with aspirin or clopidogrel did not show any activation (data not shown) and the T cell-CD69 surface expression was comparable to that seen in T cell co-cultured with platelets obtained from healthy individuals. It has been proposed, and our data are in agreement with this, that platelets may also have a role in the activation of the immune system and may represent key players in the immune homeostasis.

Tregs can recognize self from non-self antigens and can down regulate activation of T cells (7). Based on our results that activated platelets can activate T cells, we next examined the percentages of Tregs using flow cytometry to explore whether Tregs are altered in order to control the activation that platelets initiate. Upon presentation, patients with STEMI did not show any statistically significant different numbers in Tregs compared to healthy individuals. However, 5 days later, patients with STEMI displayed a statistically significant increase in Treg numbers compared with the 2 control groups. One month later, Treg numbers returned to the initial presentation levels (Figure 1B). To our knowledge, this is the first time that Tregs were studied serially at different time points following a STEMI. Tregs increase during the first days after the STEMI and possibly represent the T cell subset that is trying to eliminate the activation of the immune system and the inflammatory response.

Recently it was shown that miR155 is upregulated in patients with viral myocarditis (15). Also, differential expression of the Tregs was shown to correlate with the expression of miR155 (13). To explore the mechanism of Treg up-regulation the levels of miR155 were evaluated. Patients with STEMI displayed comparable levels of miR155 at presentation to healthy
individuals. Five days later though, patients with STEMI had a statistically significant decrease of miR155 levels ($p <0.001$) that was inversely correlated with the increased Treg numbers observed at the same time point (Figure 1C). Alterations in different miRNAs expression have been associated with rheumatoid arthritis and psoriasis, but also with solid tumors. Results from mouse models implicate deficient expression of the miR155 with decreased plaque stability and decreased Tregs (13). Up to now, the role of miR155 is not established in patients with myocardial infarction. Herein, we show for the first time that patients with STEMI have decreased miR155 levels that inversely correlate with Treg numbers.

Activated T cells may have a dual role in plaque rupture: T cells secrete IFN-gamma which inhibits the formation of new collagen, essential for the stability of the plaque in coronary arteries. Additionally, such T cells interact with macrophages leading to increased collagen degradation. Decreased formation combined with increased degradation of collagen contributes to plaque rapture (6). We describe that platelets from patients with STEMI can activate T cells ex vivo. This activation of circulating platelets may lead to an increase of the regulatory T cells subset, possibly via the downregulated expression of miR155. The increase in Treg observed in parallel with the decreased miR155 expression, represent perhaps, an effort of the immune system to control those auto-reactive T cells that participate in plaque rapture. We cannot discriminate from the current study whether platelets can only be activated by necrosis alone or whether necrosis directly activates the immune response. Our observations are preliminary and further studies are needed to establish a causal link between activated platelets and T
cells. Moreover, the precise characterization of the mechanisms implicated in activated platelet-T cell interaction might lead to better prevention of myocardial infarction.

**Author Contribution:** EES designed research, performed experiments, analyzed data and wrote the paper. K.K and E.K. analyzed data and performed experiments, V.G., A.P, and D.B. performed experiments, E.V. analyzed data, H.G, G.H., and P.D., analyzed data, D.A. analyzed data and wrote the paper.

*The authors have nothing to disclose*
References


Figure Legend: Platelets from patients with STEMI can activate T cells and increase regulatory T cells

1A. Platelets from patients with STEMI activate T cells ex vivo. T cells incubated with platelets obtained from patients with STEMI displayed a statistically significant increased expression of the surface activation marker CD69 (p=0.013) compared to T cells incubated with platelets from healthy individuals. There was no activation in T cells when incubated with plasma from patients with STEMI. PLT control; activation in T cells when treated with platelets from healthy control subjects, PLT STEMI; activation in T cells when treated with platelets from patients with STEMI. Plasma STEMI; activation of T cells in cultures when treated only with plasma from patients with STEMI.

1B. Patients with STEMI display a statistically significant increase in Treg numbers. Patients with STEMI at admission (Day 0) show comparable levels of Tregs with the healthy control group and with patients with unstable angina. Five days after admission patients with STEMI displayed statistically significant increase in Tregs compared to controls and one month later, Treg numbers in STEMI patients return to the admission levels. Treg at presentation vs. 5 days after admission; 2.875% vs 4.521, p=0.0002. Treg 5 days after admission vs. 1 month follow-up after admission; 4.521% vs. 2.745%, p=0.0004. Control: healthy control subjects; Myocardial infarction day 0: STEMI patients at admission; Myocardial infarction day 5: STEMI patients 5 days after admission; Myocardial infarction one month fu: STEMI patients one month after initial admission at follow up; Unstable angina: Control group with unstable angina.

1C. Decreased levels of miR155 in patients with STEMI
Patients with STEMI at admission (MI Day 0) show comparable mRNA levels of miR155 with the healthy control group (p=0.79). Five days after admission patients with STEMI displayed statistically significant decrease in miR155 mRNA levels compared to controls (p=<0.001) and this decrease was associated with the increase in Tregs observed in the same patients at the same time point. Control miR155: Healthy control group; MI Day 0 miR155: patients with STEMI at admission; MI Day 5-Exit miR155: patients with STEMI 5 days after admission.
Figure 1C

- Control miR155
- MI Day 0 miR155
- MI Day 5-Exit miR155

- p < 0.001
- p = 0.79
- p = 0.038

mRNA quantity
Materials and Methods

1. **Patients and controls:** After written informed consent was obtained, we examined 33 patients (29 men, 4 women, mean age 62.5 years) with myocardial infarction with ST elevation (STEMI) and 10 aged-matched healthy volunteers. None of the patients with STEMI was receiving low dose aspirin before the event. Thirty one out of the 33 patients examined underwent angioplasty after the event. Only two patients of all patients included in the study did not go into angioplasty since coronary artery bypass graft surgery was suggested. All patients after angioplasty received dual anti-platelet therapy with aspirin along with clopidogrel or ticaglerol. The subjects in the healthy control group were not taking any medications and had similar characteristics with that of the patients examined. Another 5 patients with unstable angina served as the disease-control group. None of the patients was on any immunosuppressive treatment and none was diagnosed with an autoimmune disease. Three additional patients with STEMI received aspirin or clopidogrel before admission, and these patients were also analyzed as an internal control group. This study was submitted and approved by the Ethics Committee of the Patras University Hospital.

2. **Time points:** We analyzed the patients at the following time points; Day 0 was the day that the patient presented at the hospital and was just diagnosed with STEMI, before receiving any treatment. Day 5, was 5 days after the admission and after the diagnosis. Whenever patients didn’t have any complications, on the fifth day after admission they were discharged. One month later the patients were also re-examined and blood samples were collected for analysis. In all time points described
above, heparinized peripheral blood was obtained and was used immediately after
venipuncture, for the isolation of peripheral blood mononuclear cells (PBMCs) by
density gradient centrifugation with lymphocyte separation medium (Organon,
Durham, NC) as previously described (14). The same sample was used for the iso-
lation of platelet rich plasma and plasma alone and these were used immediately in
short term cell cultures as described below.

3. **Short term cultures.** In short term cultures we incubated isolated T cells from
healthy volunteers with: a. Platelet-rich plasma from patients with STEMI (obtained
as soon as their admission before receiving any treatment) or from healthy volun-
teers, b. Plasma alone from patients with STEMI or healthy volunteers, and c. With
RPMI only and culture medium. In all these different culture settings we examined
the percentages of activated CD4 T cells using the CD69 monoclonal antibody as an
activation marker, by flow cytometry. Also, platelet rich plasma was used in three
different cultures from patients with STEMI who received aspirin or clopidogrel im-
mediately before their admission to examine possible differences between those pa-
tients on anti-platelet treatment or not.

4. **Tregs.** Based on our previous experience (14), we analyzed the percentages of
Tregs in patients with STEMI using flow cytometry, at all three time points as de-
scribed above (Day 0, day 5, and at one month of follow-up after the initial admis-
sion). We analyzed the percentages of CD4+CD25high+FOXP3+ cells representing
the Tregs. All experiments were performed using patients’ samples and healthy
subjects samples in parallel to eliminate any potential discrepancies between exper-
iments.
5. **Expression of miR155.** The miR155 levels were evaluated using real-time PCR in samples from patients and healthy individuals. In STEMI patients the expression of miR155 was evaluated at admission and when the patients were discharged (Day 0 and day 5, respectively). The correlation of the expression of miR155 and Tregs was analyzed as described in the manuscript.