Implication of Inflammation on Coxsackie-Adenovirus Receptor Expression on Cardiomyocytes and the Role of Platelets in Patients with Dilated Cardiomyopathy

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Abstract

Background

Coxsackie-adenovirus receptor (CAR) is involved in the pathogenesis of inflammatory dilated cardiomyopathy (DCM). We aimed to examine the relationship of CAR expression on platelets and cardiomyocytes with virus persistence, local and systemic inflammation and platelet activity in patients with DCM.

Methods

Endomyocardial biopsy (EMB) samples of 38 patients (mean age 39.5±11.3 years, 20 male) with DCM were analyzed for CAR expression, local inflammation grade by immunohistochemistry **and** virus persistence by real-time PCR. Platelet morphology was analyzed in all patients and 30 healthy subjects (HS) using scanning electron microscopy, platelet activity by light transmission aggregation, and CAR persistence by immunofluorescence. Platelets of 20 patients were analyzed for cytomegalovirus and herpes simplex virus 1-2 by immunofluorescence. Serum levels of tumor necrosis factor alpha (TNF α) and Interleukin-6 were assessed using ELISA in all studied subjects.

Results:

CAR expression in EMB samples was related to the heart failure functional class and the level of IL-6. Platelets from DCM patients showed enhanced spontaneous and ADP induced aggregation. Platelets' CAR expression was >4 fold higher in DCM than HS and was observed predominantly at sites of intercellular communications in microaggregates and leukocyte-platelet aggregates. CAR-positive patients showed significantly higher TNF-α and IL-6 serum levels in CAR-negative patients. Platelets of 6 (30%) DCM patients revealed the mature cytomegalovirus and herpes simplex viruses particles.

Conclusion

Tight junction protein CAR may serve as a docking pin creating a new type of contact structure that could be responsible for signaling between neighboring cells in pathological conditions.

Introduction

The coxsackievirus and adenovirus receptor (CAR) is a tight junction protein that structurally belongs to immunoglobulin superfamily and was first described as a cellular protein attachment of coxsackie B viruses and adenoviruses [1, 2]. CAR is widely expressed on cells of various organs and tissues but its physiological functions have not been clearly identified. In normal myocardium, CAR is expressed in complex with other tight and gap junction proteins like ZO-1, β-catenin and connexin-45 found in the intercalated disc; the structure that connect two cardiomyocytes [3, 4]. In patients with inflammatory dilated cardiomyopathy (DCM), increased expression and redistribution of CAR to lateral cardiomyocytes membrane was reported [5, 6]. CAR expression in heart tissue of animals suffering from autoimmune

myocarditis is regulated by cytokines. Tumor necrosis factor alpha (TNFα) upregulates CAR expression while transforming growing factor-beta 1 (TGFβ1) negates the effect of TNF-α and thus inhibits coxsackievirus B3 (CVB3) infection in cardiomyocytes [7]. Animal studies also showed that increased expression of CAR – in the absence of viral infections – may trigger pro-inflammatory cascade activation with increased cytokine response (interferon-γ, interleukin (IL)-12, IL-1β, TNFα and IL-6) and migration into the myocardium of natural killer and macrophages, and lead to the myocardial changes observed in DCM [2].

Expression of CAR is an important determinant of viral tropism for myocardium [8]. Viruses are important causative factors in DCM, which is considered to be a late sequel of acute viral myocarditis. This is due to persistent virus infection or activation of autoimmune processes and damage within the myocardium. Among all viruses, parvovirus B19, Cytomegaloviruses (CMV), Epstein-Barr virus (EBV), Herpes Simplex Virus 1–2 (HSV) and Human Herpes Virus (HHC) 6 are commonly found in endomyocardial biopsy (EMB) samples in patients with suspected myocarditis and DCM. However, the pathological role in inflammatory heart disease was only proven in animal experiments, only observed with for adenoviruses and CVB3 in relation to CAR. [2, 9].

CAR is a widely expressed receptor. All types of blood cells including erythrocytes [10], leukocytes [11] and platelets [12] express CAR. Those cells are the ones capable for the immediate response during systemic inflammation [13–15] and can directly take part in host defense mechanisms [16]. Upon virus infection, activated platelets directly interact with viruses with subsequent internalization. This is described for small RNA viruses such as CVB, EMCV, Dengue virus, HIV-1 (human immunodeficiency virus 1) and hepatitis C viruses [17–20]. In mouse models of myocarditis, platelets were shown to promote the infection by spreading the viral infection as well as contribute to limitation of viremia and long lasting immunity [19, 20]. The role of platelets and CAR receptor in myocarditis and DCM in humans is still unclear. The aim of this study was to examine platelet activation and the relationship of CAR expression on cardiomyocytes and platelets with the degree of inflammation in patients with different phases of the inflammatory process in myocardium.

Patients, Materials And Methods

Ethics statement

The present study was approved by the Local Ethical Committee of National Medical Research Center for Cardiology. The study protocol conformed to the principles outlined in the Declaration of Helsinki. All the participants were informed of the nature of the study and provided written consent.

Patients with dilated cardiomyopathy (DCM)

Thirty-eight patients (mean age 39.5 ± 11.3 years, 20 male) with DCM of presumably inflammatory etiology who had strict clinical indications for EMB at the time of inclusion in the study, were recruited. All patients had clinical and instrumental syndrome of DCM (left ventricular ejection fraction 34.7 ± 9.7%, left

ventricular end diastolic diameter 7.3 ± 1.3 sm, left ventricular end systolic diameter 5.5 ± 1.5 sm, symptoms of heart failure II-IV NYHA functional class) and received standard heart failure therapy including angiotensin-converting enzyme inhibitors or angiotensin receptor blockers or sacubitril/valsartan, beta-blockers, diuretics, mineralocorticoid receptor antagonists and according indications – amiodaron, digoxin and warfarin or direct oral anticoagulants (DOACs). The patients did not receive any antiplatelet therapy. The indications to EMB in all patients followed the Consensus of European Society of Cardiology (ESC), American Heart Association (AHA) and American College of Cardiology (ACC) [21]. Baseline characteristics are included in Table 1.

Endomyocardial biopsy and sample processing

EMB was performed at the end of angiographic studies using biopsy forceps 5.5F x 104cm (*Cordis* Corporation, USA) in the cath lab via femoral vein/artery. In each patient 3–4 biopsies samples of 1 mm^3 from the LV apex, the lateral LV wall or apical-septal portion of the RV were obtained. No complication during the EMB was observed. Each EMB sample was divided into three portions. One was embedded in paraffin, followed by preparing slices of ~ 5–7 microns thickness, and routinely processed for hematoxylin and eosin (H&E) staining. These sections were studied to assess the degree and type of inflammation and fibrosis, along with myocyte hypertrophy. Histochemical staining for collagen (Masson's trichrome) was also performed in all cases to evaluate the presence of fibrosis and was quantitatively evaluated using Aperio Scan Scope software (CS Positive Pixel Count mode) (Fig. 1A,B, supplemental Fig. 1). The second portion was cooled to prepare cryosections followed by immunohistochemical (IHC) evaluation for the presence of immune cells in inflammatory infiltrates, and CAR expression. The third portion of each sample underwent PCR analysis for virus persistence. A panel including the following viruses were evaluated: herpes simplex virus types 1–2 (HSV 1–2), cytomegalovirus (CMV), Epstein-Barr virus (EBV), herpes virus type 6 (HHV6) and parvovirus B19 and coxsackie B3 virus.

Immunohistochemical staining of EBM samples

IHC was used for evaluation of the presence of immune cells in inflammatory infiltrates and CAR expression. The degree of severity of inflammation was evaluated by quantitative and qualitative analyses of different populations of immune cells in the infiltrate (CD 3, CD4. CD8, CD68), as well as the level of cardiomyocytes damage and the presence of necrotic cells. Immunostaining was performed on frozen sections of 5 μm thick. After drying, the sections were fixed in cold 10% acetone for 10 min, then 10 minutes. The endogenous peroxidase was inactivated with 0.3% H_2O_2, Sections were then incubated for 30 min in 1% bovine serum albumin in order to prevent nonspecific antibodies adsorption. Primary antibody [1:500 anti-CAR (clone H300, Santa Cruz Biotechnology, USA), 1:100 anti-CD3 (clone UCHT-1, MP Biomedicals, Germany), 1:50 anti-CD4 (clone CLB-159, MP Biomedicals, Germany), 1:50 anti-CD8 (1:50, clone T8, MP Biomedicals, Germany), 1:10 anti- CD45RO (clone UCHL-1, Abcam Biochemicals, UK) or 1:100 anti- CD68 (clone KP-1, Abcam Biochemicals, UK) was applied onto the sections and incubated overnight at 4°C or 1 h at RT. Vectastain ABC-reagent was applied on the sections (MP Biomedicals, Germany) and receptors were stained with diaminobenzidine (DAB, Dako) and counterstained with

Mayer's hematoxylin (Sigma–Aldrich, USA). The slides were coded and examined under light microscopy by a cardiac pathologist in a blinded manner. IHC stained sections were photographed and quantified using Aperio Scan Scope CS (Aperio Technologies Inc, USA). The intensity of CAR staining (rabbit H-300 polyclonal anti-CAR antibody at dilution 1:500, Santa Cruz Biotechnology Inc., Santa Cruz, CA) in EMB samples was evaluated using Aperio Scan Scope software (CS Membrane mode with three-grade scale) (Fig. 1C, D supplemental Fig. 1).

Assessment of virus persistence in EMB

All EMB samples were analyzed for HSV 1–2, CMV, EBV, HHV6 and parvovirus B19 by quantitative and for Coxsackie virus by qualitative real-time PCR. PCR was carried out using "AmpliSens EBV/CMV/HHV-6 screen-FL", "AmpliSens HSV1-2", "AmpliSens Parvovirus B19-FL" and "AmpliSens® Enterovirus-FRT PCR kit (InterLabService Ltd, Russia) by «RotorGene 3000". DNA extraction was performed by "DNA-Sorb B" (InterLabService Ltd, Russia).

Healthy subjects:

Blood samples were collected from thirty apparently healthy subjects (HS) as control for the platelet studies and serum inflammatory markers. All HS (20 male, mean age 38 ± 7 years, 8 smokers) had no signs of cardiovascular pathology according to ECG, transthoracic echocardiography and stress-test and had no pathological deviations according to standard laboratory evaluation.

Platelets studies:

Platelet aggregation, morphology, expression of CAR were analyzed in all 38 DCM patients prior to EMB and in the 30 apparently HS. Blood was collected from the antecubital vein using a 21-G needle into citrated tubes (3.2% sodium citrate in the ratio 1:10) and gently mixed. Platelet rich plasma (PRP) was prepared by centrifugation at 170 g for 17 min at room temperature (RT) and platelet count was adjusted to 200–350 x 10^9 platelets / L.

Platelet aggregation

Platelet aggregation was assessed in PRP using the Biola 230LA aggregometer (Biola, Russia). This analyzer measures simultaneously platelet aggregation and mean aggregate size. The former is based on a conventional turbidometric method measuring changes in light transmission (LT) and expressed in percentages (%) [22]. The latter is based on the analysis of LT produced by the changes in the number of platelets in the optical channel [23]. The relative value of these fluctuations is proportional to the mean aggregate size (expressed in relative units, r.u.). The high sensitivity of this method makes it ideal to study spontaneous platelet aggregation and aggregation induced by low concentrations of agonists, which cannot be evaluated by the standard LT method. Spontaneous and 0.1 μM ADP-induced ability to form small aggregates (3-100 cells) was estimated using this technique.

Platelet morphology assessment using scanning electron microscopy

Scanning electron microscopy was used to evaluate platelet morphological changes and signs of activation including leukocyte-platelet aggregates (LTA) and erythrocyte-platelet aggregates (ETA). The samples were obtained as described elsewhere [24]. Briefly, 20 µl of blood was fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 in ratio 1:30 for 1.5 hours, at RT, then were placed on polycarbonate membranes (Isopore Membrane Filters, Millipore) with 0.40 µm diameter holes, dehydrated in a dilution series of ethanol, and finally stored in 100% ethanol. The samples were dried (HCP-1 critical-point drying apparatus; Hitachi, Japan), coated with carbon-gold and examined with a scanning electron microscope (PHILLIPS PSEM 550x; Netherlands) with magnification x5000.

CAR expression on platelets using immunofluorescent staining

500 µl PRP was fixed in 1 ml 4% paraformaldehyde for 10 min at RT and the platelet pellet was rinsed three times in PBS. Platelets were next incubated with 100 µl rabbit H-300 polyclonal anti-CAR antibody at 1:50 dilution (Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 1.5 hour, 37°C, washed three times in PBS, then incubated with 100 µl goat anti-rabbit IgG F(ab')2 Texas Red secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) in 1:100 dilution for 30 min, 37°C. Platelets were rinsed three times in PBS before applying to a drop of glycerol – gelatin on a glass slide and analyzed with fluorescent microscopy (Leica DM5000B). For quantitative analysis, ten random fields were selected, and the number of platelets stained with Texas Red was counted relative to the total number of platelets and expressed as a percentage (the total number of platelets counted was no less than 1000 in any experiment).

Immunofluorescent staining of platelets for viruses

Platelets of 20 pts were analyzed for virus persistence. 1 ml PRP was fixed in 1 ml 4% paraformaldehyde 10 min at RT and the platelet pellet was rinsed three times in PBS. For permeabilization, the pellet was resuspended in 1% Triton X100 solution and incubated for 10 min RT. After that, the suspension was washed three times with PBS, followed be resuspension in 0.4% casein solution on PBS and incubated for 30 minutes at 37° C to reduce the level of antibodies' nonspecific binding to the membrane. The resulting suspension of platelets was divided into 3 samples and incubated for 30 min at 37° C with monoclonal antibodies to late envelope protein gB (dilution 1:50 on PBS Virosial, Portland, Maine) of CMV or mouse monoclonal IgG_1 gB antibody to HSV1-2 (dilution 1:100 on PBS Santa Cruz Biotechnology Inc., Santa Cruz, CA) or with the second antibodies (Sigma Chemical Co, St. Louis, MO) for control. Platelets were rinsed three times in PBS before applying to a drop of glycerol – gelatin on a glass slide and analyzed with fluorescent microscopy (Leica DM5000B). For quantitative analysis, ten random fields were selected, and the number of platelets stained with FITC was counted relative to the total number of platelets and expressed as a percentage (the total number of platelets counted was no less than 1000 in any experiment).

Determination of cytokine levels in patients' serum

Assessment of systemic inflammation was done via measuring TNFα, TGF-β1, IL6 concentration in serum of healthy subjects and DCM patients was performed by standard indirect enzyme immunoassay

(ELISA) on the Tablet photometer Anthos-2020 (Anthos Labtec, Austria) using sets of “Human TNF-alpha Platinum ELISA”, “Human TGF-β1 Platinum ELISA”, “Human IL-6 Platinum ELISA” (“eDioscience - Bender Medsystems”, USA, Austria). According to the diagnostic kits’ reference ranges for healthy volunteers, TNFα is not detected, IL-6 is less than 12.7 pg/ml (mean 5.8 pg/ml) and for TGF-β1, 5222–13731 (with a mean of 6723 ± 1978) pg/ml were defined.

Statistical analysis

Statistics data are expressed as mean and SEM or median and quartiles and tested for significance using Student’s *t* test or one-way ANOVA as well as Mann-Whitney-test for comparison of continuous variables. To compare the diagnostic value of the different indicators, we used «ROC-analysis» (Receiver Operating Characteristic). A two-tailed p value of < 0.05 was considered statistically significant. The selected variables were compared using Spearman’s correlation coefficient; $P < 0.05$ was considered statistically significant and $P < 0.001$ was considered highly significant. The results were statistically evaluated using SPSS version 20 (IBM Corporation, USA).

Results

Myocardial inflammation in patients with DCM with persistence of parvovirus B19

According to current quantitative immunohistological criteria, the persistence of more than 14 inflammatory cells (predominantly CD3 + and CD8 + T- cells and less than 4 CD68 + macrophages) per 1 mm^2 together with persistent necrotic cardiomyocytes is sufficient for histological diagnosis of active inflammation in myocardium [25]. Focal or diffuse inflammatory cell infiltration with CD3+, CD4+, CD8+, CD68+, CD45RO surrounded by necrotizing cardiomyocytes was detected in 8 (21%) EMB samples, confirming the diagnosis of active myocarditis in these patients (Fig. 1A). In 11 (29%) cases, extensive focal areas of fibrosis with residual inflammatory cells were detected, indicating resolved myocardial inflammation (Fig. 1B). In 19 (50%) cases, the signs of active inflammation were not detected thus the morphological changes were determined as idiopathic DCM with minimal morphological changes (Fig. 1C).

PCR analysis demonstrated that HSV1-2, EBV, CMV and Coxsackie-virus B3 were absent in EMB samples. In 15 (39.5%) EMB samples 87–250 000 copies of parvovirus B19 per 1 ml lysate were detected. The total number of infiltrating inflammatory/immune cells in virus-positive and virus-negative EMB samples was comparable ($p = 0.49$) while the number of CD68 + cells in virus-positive sample was higher (23,5[8,5;57,5] vs 4,5[1;15] per 1 mm^2 in virus negative samples ($p = 0.04$).

Cytokines levels in DCM patients

TNFα was detected in serum in only 2 out of the 30 control subjects (6.7%) HS. In DCM group it was positive in 23 (60.5%) patients, with an average concentration of 59.12[5.32; 124.96] pg/ml. IL6 and

TGFβ1 were not detected in serum of HS. In 10 (26.3%) DCM patients, the level of IL6 exceeded normal ranges and was 20.94[14.58 ;25.71] pg/ml. TGFβ1 was positive in 6 (15.8%) DCM pts, with an average concentration of 24168 ± 11395 pg/ml (Fig. 2A). Comparison with clinical and instrumental characteristics didn't demonstrate any statistically significant correlation. The increased level of TNFα and IL6 didn't correlate with the severity of inflammation according to EMB and the functional class of heart failure. The increased level of TGFβ1 was not related to the percentage of fibrosis in EMB samples.

CAR expression in cardiomyocytes

CAR expression is indicated by brown staining (Fig. 3). Samples stained for CAR were divided into three subgroups according to the intensity of CAR expression. In samples with maximal (third-degree) expression (n = 10, 26.3%), CAR was expressed in the cytoplasm and sarcolemma of cardiomyocytes as well as on the intercalated discs and the whole cardiomyocytes membrane, observed in the longitudinal (Fig. 3A) and cross sections (Fig. 3B). CAR expression was detected in the interstitial cells and the media of intramural vessels. Samples with second-degree CAR expression (n = 22, 57.9%) were characterized by weak staining of cell membranes, homogeneous cytoplasmic staining of cells and a clear staining of intercalated discs (Fig. 3C). In samples with low (first-degree) CAR expression (Fig. 3D), myocytes showed weak staining of intercalated disc and in lateral membranes and cytoplasm (n = 6, 15.8%). There was no correlation between intensity of CAR expression and virus persistence as well as the degree of inflammation (evidenced by inflammatory cells). A weak negative correlation was found between the CAR expression on the membranes of cardiomyocytes and interstitial cells and the percentage of interstitial myocardial fibrosis (r = -0.42). Moreover, a positive correlation was found between the intensity of CAR expression and the New-York Heart Association functional class of heart failure (r = 0.66). Patients with the most severe illness had the most prominent expression of this receptor with CAR redistribution extending from intercalated disc to whole plasma membrane and interstitium. The level of CAR-cardiomyocyte expression was compared to the level of peripheral markers of inflammation – TNF-α, IL-6 and TGF-β1. The intensity of CAR expression on the membranes of cardiomyocytes was found to be significantly higher in patients with IL-6 levels exceeding the reference values (2.5 ± 0.55 versus 2.0 ± 0.43, p = 0.036). There was no correlation of CAR expression with TNF-α and TGF-β1 level.

Platelet aggregation in DCM patients

In all HS, platelet aggregation was within normal ranges. In DCM, 28 (73.7%) patients showed spontaneous aggregation 1.65 [1.3;1.8] r.u. In 13 patients, spontaneous aggregation increased to 37% ±17.3% which was similar to ADP induced aggregation after 9–14 min. This can be explained by platelet release reaction which was not observed in HS. Increased level of platelet aggregation was observed in response to low doses of ADP (0.1 μM and 1.0μM) in 26 (68.4%) and 24 (63%) patients (Table 2). Aggregation levels were 2.6 [1.6;3.4] r.u. (normal range 1.0–2.0 r.u.) and 40 [19;51]% (normal range < 25%) respectively (Fig. 4A). This indicates the changes in platelets' functional characteristics and increase in their activity enabling them to form small aggregates (from 3 to 100 cells). Only in four (10.5%) patients

the increased level of platelet aggregation in response to high dose ADP (5.0 μmol) was observed. This reflected the platelet ability to form large aggregates.

Scanning electron microscopy revealed increased number of platelet microaggregates (Fig. 4B) and leukocyte-platelet (LPA) and erythrocyte-platelet aggregates (EPA) (Fig. 4C) in DCM patients. Their persistence and high level of platelet aggregation possibly reflect microvascular changes due to inflammatory process in myocardium.

Virus persistence inside platelets of DCM patients

20 DCM patients and all HS were analyzed for mature CMV and HSV1-2 particles inside platelets. Viruses were not seen inside the platelets of healthy subjects. Internalized HSV1-2 virus and CMV virus were detected in platelets of 2 and 4 patients respectively. Viral particles were detected in both singular platelets, and in platelet aggregates, including leukocyte-platelet aggregates (Fig. 5). CMV and HSV 1–2 were not detected in EMB samples indicateing the absence of a relation between virus persistence in the myocardium and in peripheral blood cells.

CAR expression on platelets from DCM patients

It was previously published that only 1.75[0.2; 6.4]% platelets in HS are CAR positive [26]. Homogeneous staining of singular platelets (non-aggregates), with even CAR distribution along the entire platelet membrane can be seen (Fig. 6A,B). A very few microaggregates, leukocyte-platelet aggregates and erythrocyte-platelet aggregates were detected in HS, their CAR expression was an extremely rare finding. In DCM patients (Fig. 6C, D) the number of CAR positive platelets was significantly (more than 4 fold) higher in DCM (8.0 [4.0;19.0]%) compared to healthy subjects (1.7%) ($p = 0.0002$) (Fig. 6E). The number of both homogeneously stained single leukocytes, platelets, and those in large aggregates, as well as microaggregates with linear staining at the sites of intercellular contacts, were also increased.

Similar to cardiomyocytes, platelets expressed CAR along either the entire plasma membrane or at intercellular communication sites (areas between cells in the aggregates) and strongly remained in intercalated disc in myocardium. The comparison between CAR expression on platelets and cardiomyocytes in DCM patients revealed no difference.

Taking into account that in HS group the maximum number of platelets expressing CAR was 6.4%, 7% cutoff level of CAR-positivity was chosen to identify the factors influence its overexpression. In 25 (65.8%) DCM patients, the percentage of platelets expressing CAR exceeded 7% - they were defined as CAR-positive. The remaining 13 (34.2%) patients were defined as CAR-negative. Among CAR-positive patients, TNF-α and IL-6 were significantly higher than in CAR-negative (4.9 [0; 75.1], pg/ml vs. 0 [0, 3.5], $p = 0.0001$ and 3.8[0.2, 13.6] pg/ml vs. 0.2[0; 0.1] pg/ml, $p = 0.024$ respectively, Fig. 2B). ROC-analysis confirmed the importance of IL6 and TNFα for increased CAR expression: $S = 0.746$ and 0.698 respectively.

During the course of study a few patients with acute myocarditis according EMB, with increased number of CAR-positive platelets (47% in first case and 18% in the second) were re-analyzed for CAR receptor for the second time. Those patients have had the course of anti-inflammatory prednisolone therapy during 6 months with significant clinical improvement. On re-examination, we found significantly decreased CAR expression (1% in first case and 2.5% in second case). Only cells with homogenous CAR staining were seen (data not shown).

Discussion

Local inflammatory reaction leads to cardiomyocyte necrosis with subsequent redistribution of gap junctions and dissociation of tight junctions. Among the many tight junction proteins, the greatest attention is given to coxsackie-adenovirus receptor (CAR). CAR participation in the pathogenesis of inflammatory diseases of the myocardium suggests that regulation of its expression depend on the degree of inflammation. CAR overexpression is known to maintain the inflammation in myocarditis [2]. Whether the increased expression of this receptor on cardiomyocytes, is a result of an inflammatory condition of the myocardium, or merely a pathophysiologic finding contributing to the inflammatory process and myocardial remodeling, remains unclear. To address this question, we evaluated the intensity of the expression of CAR on the cardiomyocytes in relation to the degree of cellular infiltration of the myocardium in patients with DCM. Our data showed heterogeneous morphological changes in EMB in patients with similar clinical (symptoms of heart failure) and instrumental data (heart chamber dilatation and reduced left ventricular ejection fraction). Experimental models showed differntial CAR expression in different disease stages with maximum level appearing in the necrotic phase. CAR expression can be found in the cardiomyocytes not only in the inflammatory lesions but also in the intact area [9]. Our study shows similar finding without CAR expression in the necrotic stage. However, similar to Kaur T. et al.[23] who had shown no direct relationship between high CAR expression and severity of myocarditis in the autopsy material of DCM hearts, we failed to identify such relationship in our investigation.

Recognizing the important role of CAR in the pathogenesis of inflammatory DCM led to direct investigation of the factors influencing its expression. It is known that the expression of CAR is enhanced by increasing concentration of TNFα that can be seen in both the animal models of CVB3 induced myocarditis [7] and in cultured oncological cell [27]. At the same time TGF-β1 was shown to block the TNFα influence and return the CAR concentration on membranes to baseline [7]. This observation prompted us to examine serum cytokines. Our study revealed a relationship between myocardial and platelet CAR and high level of serum IL-6. Analysis of the data also indicates that CAR overexpression is typical for patients with severe heart failure. Taking into account the fact that the high concentration of IL-6 has cardiodepressive effect, leads to an increased collagen volume fraction, and consequently, myocardial stiffness [28], we can assume that increased IL-6 level enhances functional role of CAR as an intercellular tight junction protein. It is well known that in DCM membrane of intercalated disc are curved with a concomitant increase in the expression of adhesive contact, which may lead to a loss of flexibility and high rigidity in the sites of intercellular contacts [29]. Besides changes on the tissue level changes of the cardiomyocytes' structure contribute to the reduction of the heart contractility. The main event of the

pathological remodeling of cardiomyocytes is a disruption of intercellular contacts. Pathological conditions lead to a significant decrease in the expression of other type of cell-cell contacts - gap junctions, which normally carry out electromechanical coupling between adjacent cardiomyocytes [30]. We can propose that diffuse reduction in myocardial contraction of the left ventricle in patients with DCM can be determined by replacement of gap junction proteins, connexins, to proteins of tight junctions. It is likely that the observed CAR overexpression may reflect an attempt to restore intercellular contacts in the affected myocardium with progressive dilatation. And perhaps the purpose of tight junctions proteins, CAR for example, is to distinguish the intact cells from necrotic lysis products. A strong expression of the CAR protein can be observed in the intercalated discs and sarcolemma not only in the end stage human dilated cardiomyopathy (DCM), an up-regulation of CAR mRNA was revealed in ischemic cardiomyopathy (ICM), in myocardium of patients with valve-failure associated heart disease and in animal models of myocardial infarction [31]. Thus, our results and results of other studies suggest a role for CAR in myocardium remodeling during pathological conditions regardless of its etiology.

The fact that peripheral proinflammatory cytokine – IL6 – is related to CAR expression on the membrane of platelets, blood cells, which are capable of immediately responding to systemic inflammation reminds us about their complex role in virus infection [12]. Viral infection even that transient is considered to be the etiological factor of myocarditis and can induce platelet activation. Platelet activation can lead to elimination of virus laden platelets but also to the clearance of virus particles through release their contents from α-granules including high amount of CXCL4 which in turn up-regulates coagulation and leukocyte recruitment [32]. Previous studies have confirmed platelet changes in patients with idiopathic DCM [33]. It is likely that these changes contribute to the impairment of microcirculation, and thus to exacerbating of the disease. CAR expression on peripheral blood cells in patients with DCM was first described by Liu Q. et al. [11]. They showed -in 50 patients with DCM-, CAR expression on leukocytes and CVB3 persistence inside them together. This is contradictory to our results which demonstrated that, CAR expression and persistence of Coxsackie virus in myocardium were not related. In our study 39.4% EMB samples were parvovirus B19-posititve. Virus persistence was not related to histological signs of myocarditis. Our data is fully consistent with the data of Marburg registry which showed that herpes virus type 6 and parvovirus B19 are typical for EMB samples in European population, while the coxsackie virus is a rare finding [25].

The notion that viruses can persist in peripheral blood together with platelet ability to internalize virus particles led us to identify -in a pilot study- 6 patients (30%) with mature CMV and HSV 1–2 virus particles inside platelets. Their persistence in platelets was not related to virus persistence in myocardium. It remains unclear whether these viruses are related to pathogenesis of inflammatory heart diseases or is just a bystander maintaining the systemic inflammatory process. Nevertheless, the fact that platelets can internalize viruses during cardiovascular pathology corresponds well with the previous *in vitro* and *in vivo* studies [16–20, 34].

Our data revealed that platelets of DCM patients are activated as evidenced by their increased aggregation. In most patients an increased level of spontaneous and low dose ADP-induced aggregation

were observed. This indicates the changes in the platelets' functional characteristics, and increased activity which enable them to form small aggregates (from 3 to 100 cells). The persistence of microaggregates, leukocyte- and erythrocyte-platelet aggregates (LPA, EPA) was confirmed by scanning electron microscopy. These findings correspond with the observation that in patients, CAR is expressed predominantly at the sites of intercellular communications in the small aggregates. One can suspect that platelet activation upon the inflammatory conditions regardless of its inducer lead to microaggregate, LPA and EPA formation, and CAR is one of the major proteins involved in this process. It seems that CAR is involved in the special 'docking membrane' formation resembling the intercalated disc in myocardium which can enable intercellular communication and signal transduction.

In summary, this study showed some novel key findings. First, CAR expression in myocardium is not related to the severity of inflammation or persistent virus infection but likely involved in the remodeling process during the pathological inflammatory conditions. Moreover, CAR overexpression both in myocardium and platelets is associated with systemic inflammation as evidenced by increased level of IL-6 in peripheral blood. Second, platelets of DCM patients are activated as evidenced by increased aggregation and microagregates; LPA and EPA. CAR is localized predominantly in such aggregates in the sites of intercellular contact forming the 'docking membrane resembling the intercalated disc in myocardium. Third, 30% of assessed DCM patients revealed platelets which carried the internalized CMV and HSV-1 particles. Virus persistence inside platelets doesn't reflect virus persistence in myocardium in DCM patients and may not be the etiological factor of inflammatory heart disease but rather a bystander in the systemic inflammatory process.

We conclude that tight junction protein CAR may be serving as docking pin forming a new type of contact structures which might be responsible for signaling between neighboring cells during inflammation or other pathological conditions. Future investigation should examine further the physiological role of CAR expression in myocardium and its interaction with other types of intercellular connections especially gap junctions.

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Tables

Table 1
Patients' characteristics (DCM)

		n = 38
Mean age, y		39.5 ± 11.0
male – n (%)		20 (52.6)
Disease duration (at the time of endomyocardial biopsy), months		1.25[0.69; 4.85]
NYHA heart functional class		2.4 ± 1.44
ECHO data	Left ventricular end diastolic volume, ml	227 ± 82.6
	Left ventricular end diastolic diameter, mm	72 ± 8.4
	Left ventricular end systolic diameter, mm	52 ± 11
	Left ventricular ejection fraction (%)	31.9 ± 8.2
ECG data	Atrioventricular block II-III grade	4 (10.5)
	Sustained ventricular tachycardia	8 (21.1)
	Left bundle branch block	11 (28.9)
	Atrial flutter/atrial fibrillation– n (%)	7 (18.4)
	• paroxysmal	2
	• permanent	5
Therapy	iACE or ARB or sakubitril/valsartan – n (%)	36 (94.7)
	Beta-blockers – n (%)	32 (84.2)
	Loop diuretics – n (%)	23 (60.5)
	• MRA – n (%)	29 (76,3)
	Amiodaron – n (%)	6 (15.8)
	Digoxin – n (%)	8 (21.1)
	Warfarin/DOAC	10 (26.3)

iACE – angiotensin-converting enzyme inhibitors or; ARB – angiotensin receptor blockers; MRA – mineralocorticoid receptor antagonists, DOAC – direct oral anticoagulants

Table 2
Platelet aggregation in DCM patients

	n	Mean ± σ	M[Q1;Q2]	Normal ranges	Number of pts with platelet aggregation exceeding normal range
Spontaneous aggregation (r.u.)	38	1.6 ± 0.5	1.65[1.3;1.8]	1-1.5	28 (73.7%)
0.1 μM ADP induced (r.u.)	38	2.5 ± 1,1	2.6 [1.6;3.4]	1.0–2.0	26 (68.4%)
1.0 μM ADP induced (%)	38	36 ± 20	40[19;51]	< 25	24(63%)
5.0 induced ADP (%)	38	54 ± 14	53[44;64]%	25.0–68%	4 (10.5%)

Figures

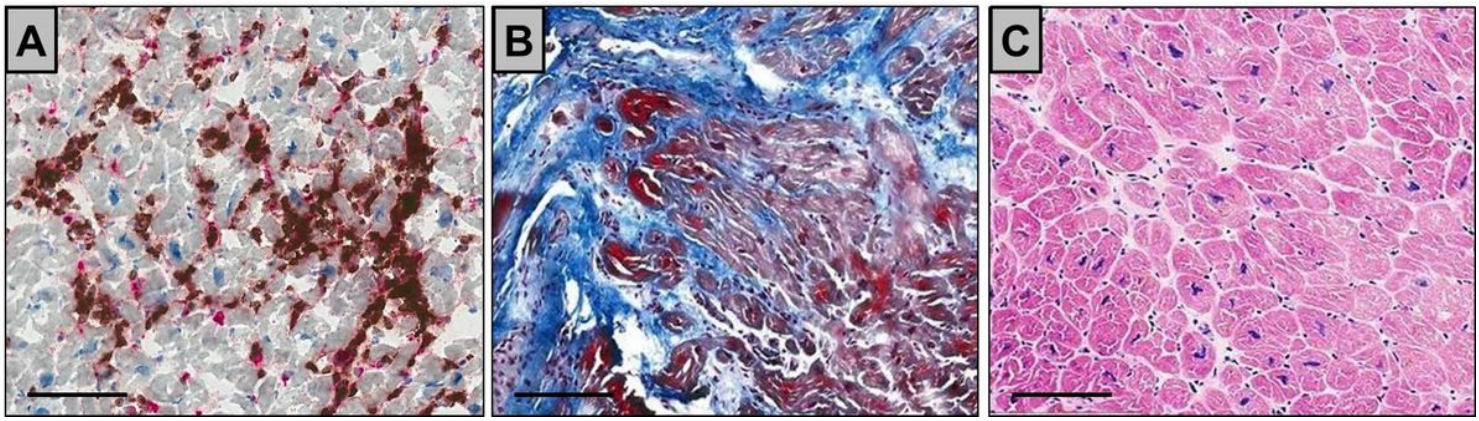

Figure 1

The H&E and immunostaining of EMB samples in DCM patients*. A – EMB sample of patients with active myocarditis showing focal inflammatory cell infiltration (immunohistochemistry double staining for CD3+ (pink) and CD68+ (brown) cells); B – EMB sample of patients with resolved myocarditis (no inflammatory cells infiltration) and huge areas of focal fibrosis. Masson's trichrome staining; C – EMB sample of patients with idiopathic DCM with no signs of active inflammation, H&E. 1 bar= 100μm.*

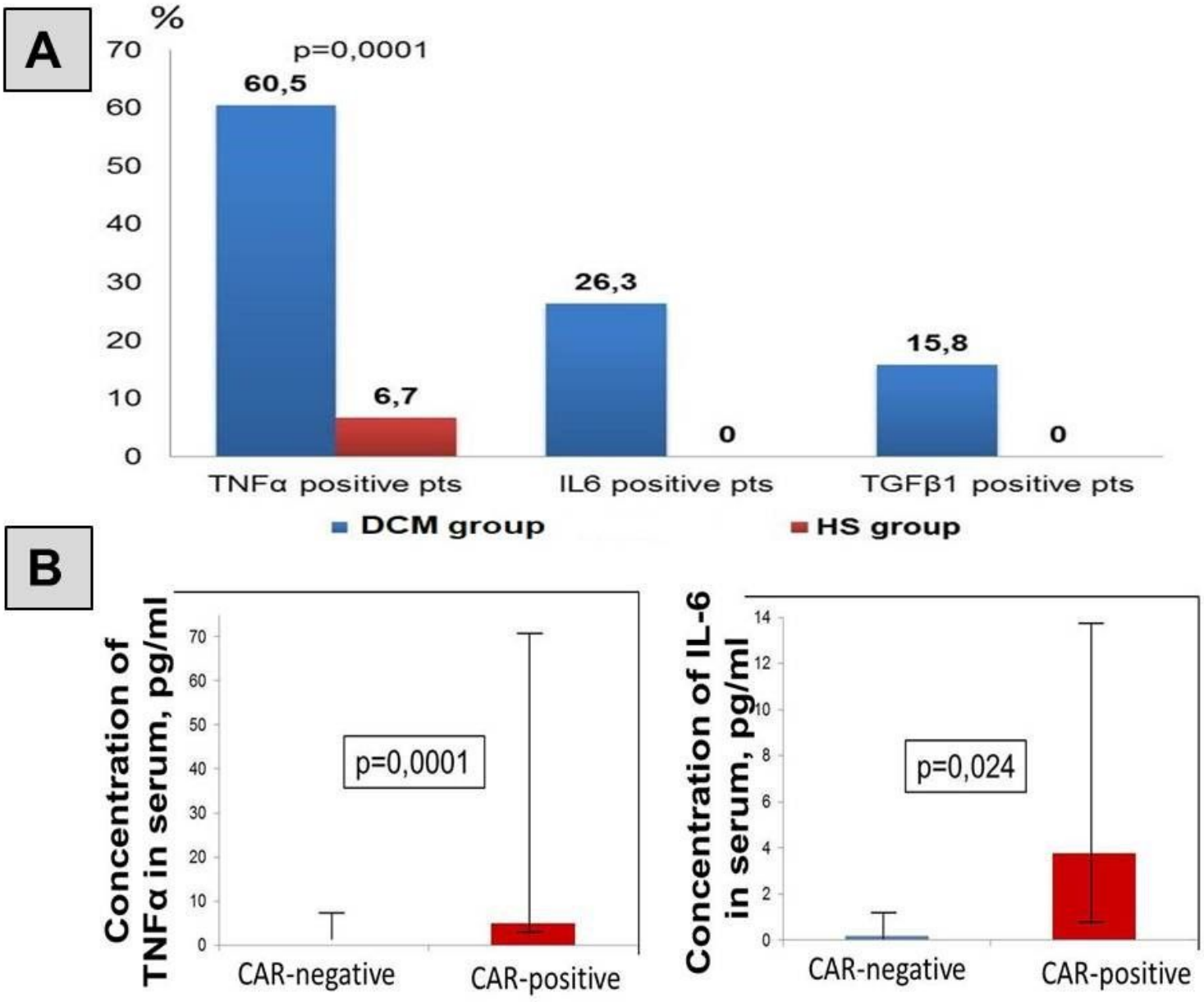

Figure 2

Serum cytokines and its relationship with CAR expression in DCM and HS groups. *A- percentage of patients with increased Levels of TNF-α, IL-6 and TGF. B - the relation of platelets' CAR expression to serum cytokines levels. TNF-α and IL-6 were significantly higher in Among CAR-positive compared to that in CAR-negative patients.*

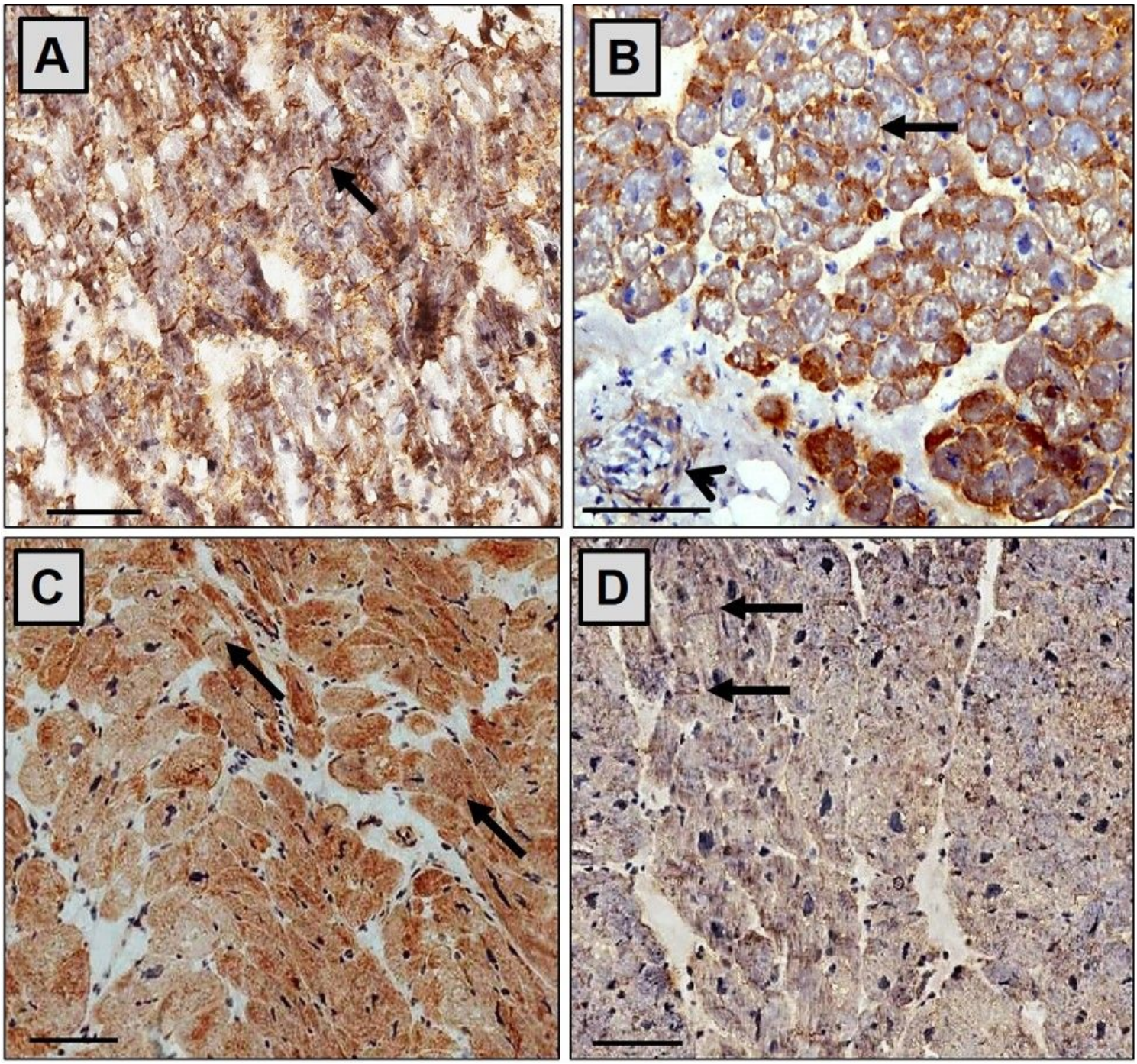

Figure 3

CAR receptor in EMB samples of DCM patients *(A-D). A, B – the EMB samples with maximal (third-degree) CAR expression. A - Longitudinal section. Intense staining of intercalated disks, moderate staining of lateral membranes and interstitial space (arrows indicate the intercalated disks). B - Cross-section showing intensive staining of cardiomyocytes membranes (arrows), the interstitial space and media of intramural vessel (arrowhead). C – Medium (second-grade) CAR-expression. There is weak staining of cell membranes, a moderate staining of the cytoplasm, interstitium and intercalated disc (arrow). D – Low CAR expression (first-degree) with barely noticeable staining of cell membranes. Immunohistochemistry, 1 bar = 100 μm. The cell nuclei were counterstained with hematoxylin.*

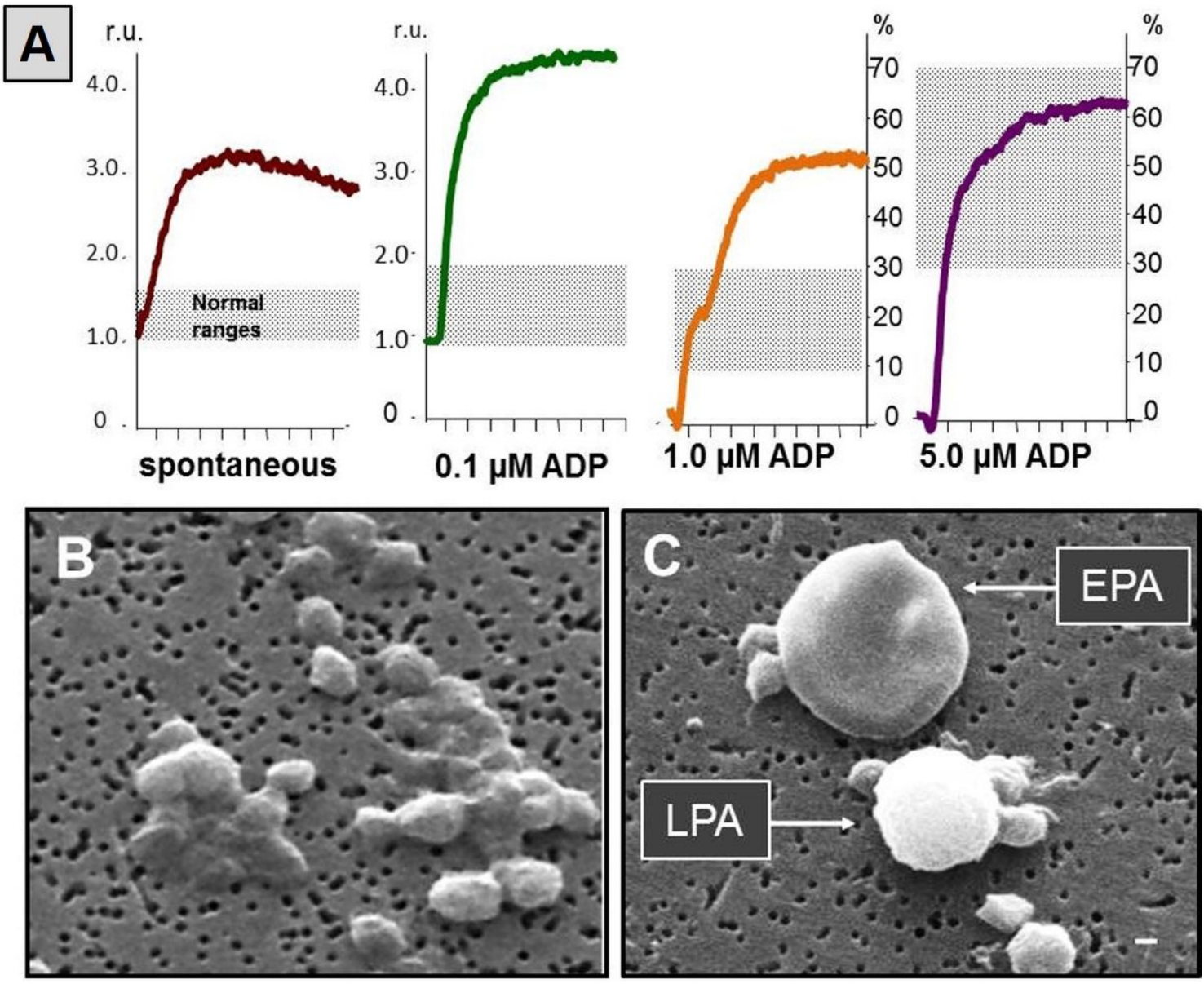

Figure 4

A. *Platelet morphology and aggregation in DCM patients.* A) platelet aggregation curves showing *spontaneous, 0.1, 1.0, 5.0 ADP-induced platelet aggregation. Platelet aggregation was measured simultaneously using traditional turbidometric method and aggregate size formation. Small aggregates are seen with spontaneous and with 0.1 mM ADP-induced aggregation (measured in r.u.), large aggregates were seen and the percentage of light transmission increases with high dose (5 mM ADP) (**B, C) B-** Scanning electron microscopy showing platelets morphology in whole blood sample from DCM patients. B – Microaggregates (magnification x5000). C - Erythrocyte-platelet (EPA) and leukocyte-platelet (LPA) aggregates (magnification x5000). 1 bar = 1 µm.*

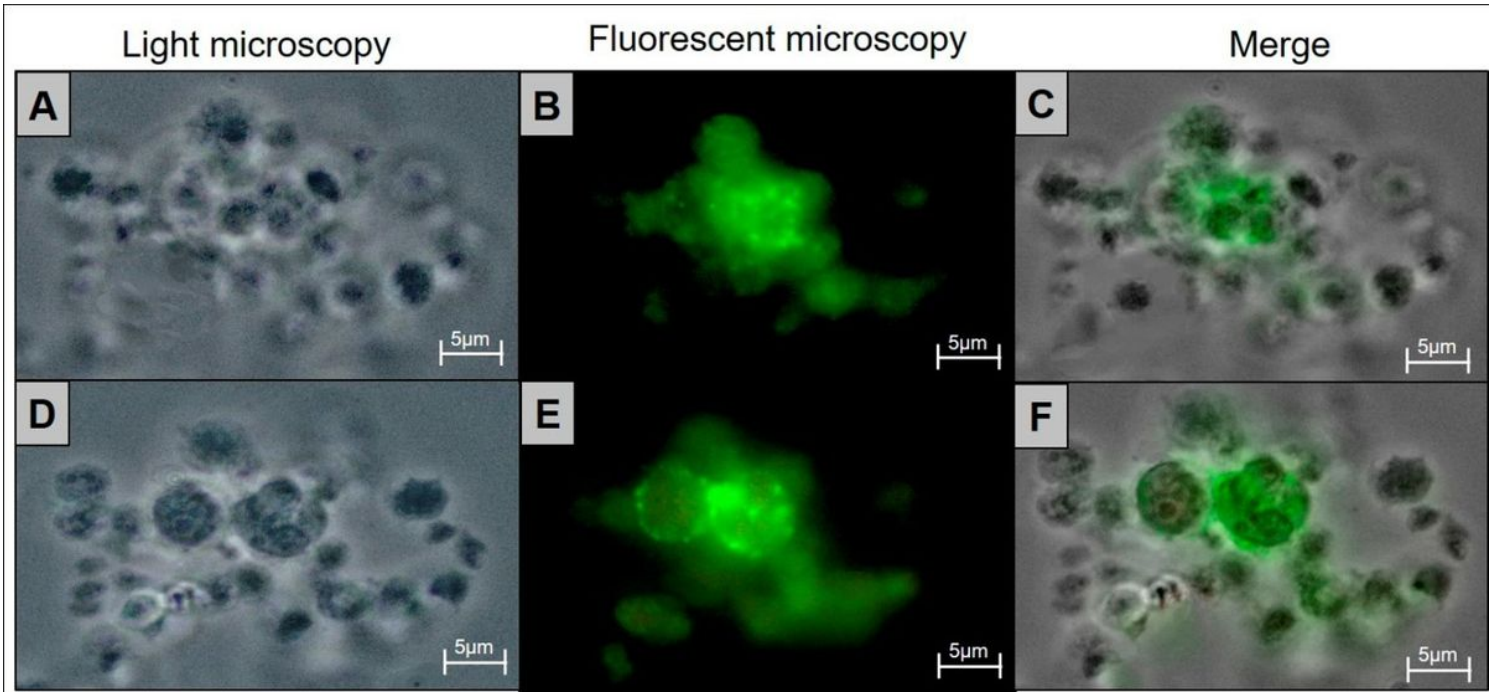

Figure 5

Visualization of HSV 1-2 inside platelets of DCM pts using fluorescence microscopy. *Light (A, D) and fluorescent microscopy (B, E) of the same leukocyte-platelet aggregate A. The surface of leukocytes is covered by platelets. B. Same focal length. A large number of incorporated viral particles are visualized in platelets. C – merged images using light (A) and fluorescence microscopy (B). D – the same aggregate. The surface of leukocytes is in focus. E – viral particles in leukocytes are concentrated in the immediate vicinity of the surface membrane. F – merge of images obtained using light (D) and fluorescence microscopy (E). Magnification x 1000. 1bar =5 μm.*

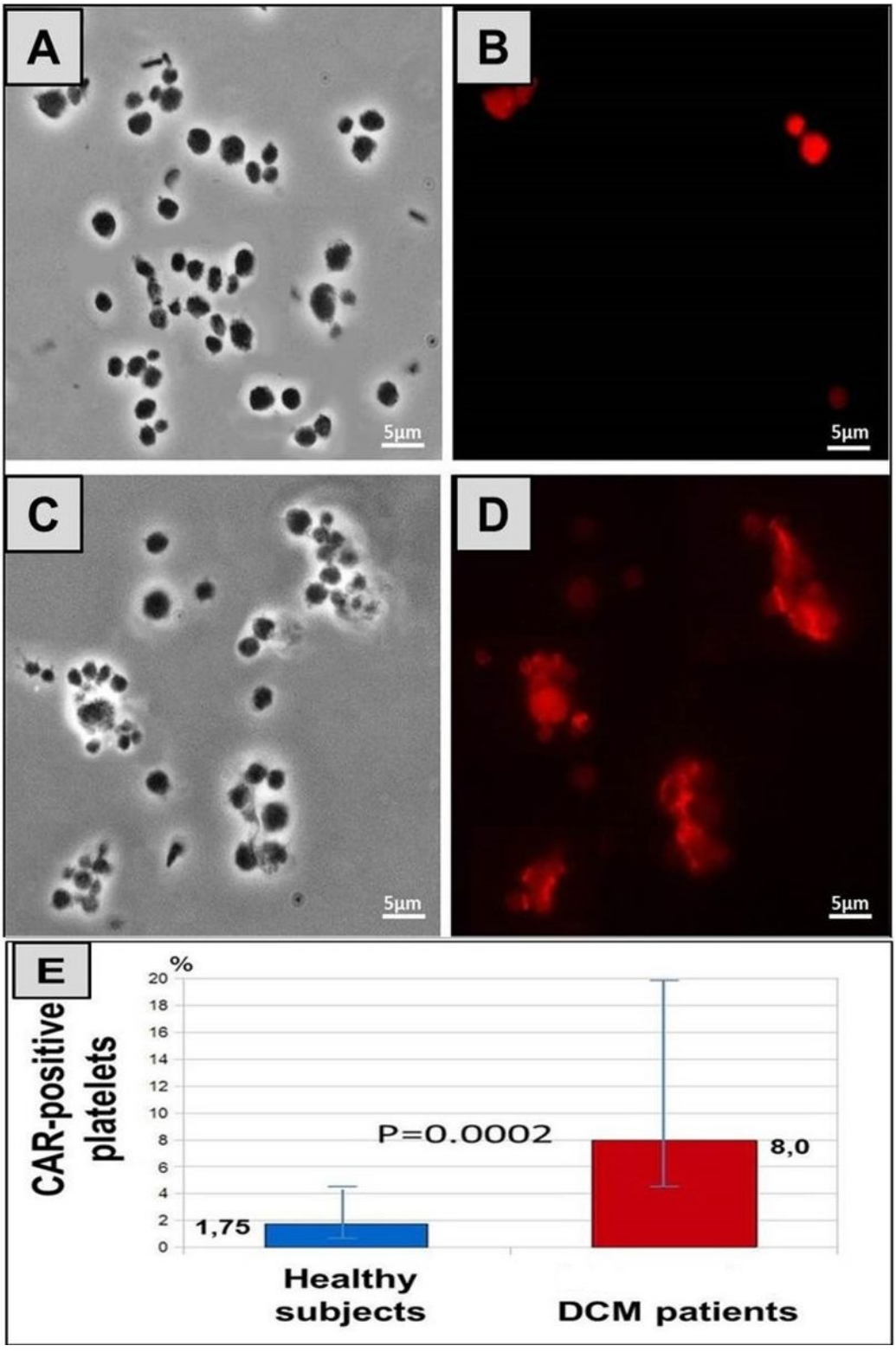

Figure 6

CAR expression on platelets. A-B) platelets from healthy subjects. C,D) DCM patients A, C – light microscopy. B, D – fluorescent microscopy. E – graphic illustration of comparison between CAR expression on platelets. Data presented as Me [Q_1; Q_3].

Supplementary Files

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- Figure1supp.jpg