ELSEVIER

The Journal of Heart and Lung Transplantation

http://www.jhltonline.org

ORIGINAL PRE-CLINICAL SCIENCE

Plasma *CD5L* and non-invasive diagnosis of acute heart rejection

Estefanía Tarazón, PhD,[a,b] Nerea Corbacho-Alonso, PhD,[c] María G. Barderas, PhD,[c] Carolina Gil-Cayuela, PhD,[a,b] María García-Manzanares, PhD,[d] Sandra Feijóo-Bandín, PhD,[b,e] Francisca Lago, PhD,[b,e] José Ramón González-Juanatey, MD,[b,e] Luis Martínez-Dolz, MD,[a,b,f] Manuel Portolés, PhD,[a,b,1] and Esther Roselló-Lletí, PhD[a,b,1]

From the [a]Myocardial Dysfunction and Cardiac Transplantation Unit, Health Research Institute Hospital La Fe, Valencia, Spain; [b]CIBERCV, Madrid, Spain; [c]Department of Vascular Physiopathology, Hospital Nacional de Parapléjicos, SESCAM, Toledo, Spain; [d]Medicine and Animal Surgery, CEU Cardenal Herrera University, Alfara del Patriarca, Valencia, Spain; [e]Cellular and Molecular Cardiology Research Unit, Department of Cardiology and Institute of Biomedical Research, University Clinical Hospital, Santiago de Compostela, Spain; and the [f]Heart Failure and Transplantation Unit, Cardiology Department, University and Polytechnic La Fe Hospital, Valencia, Spain.

BACKGROUND: Acute rejection is one of the most important direct contributors to mortality after heart transplantation. Advances in the development of novel non-invasive approaches for the early identification of allograft rejection are necessary. We conducted a non-targeted proteome characterization focused on identifying multiple plasmatic protein differences to evaluate their diagnostic accuracy for rejection episodes.

METHODS: We included consecutive plasma samples from transplant recipients undergoing routine endomyocardial biopsies. A liquid chromatography−tandem mass spectrometry analysis using isobaric tags (tandem mass tag 10-plex) was performed and concentrations of *CD5L* were validated using a specific sandwich enzyme-linked immunosorbent assay.

RESULTS: A total of 17 altered proteins were identified as potential markers for detecting heart transplant rejection, most involved in inflammation and immunity. *CD5L*, an apoptosis inhibitor expressed by macrophages, showed the best results in the proteomic analysis ($n = 30$). We confirm this finding in a larger patient cohort ($n = 218$), obtaining a great diagnostic capacity for clinically relevant rejection (≥Grade 2R: area under the curve = 0.892, $p < 0.0001$) and preserving the accuracy at mild rejection (Grade 1R: area under the curve = 0.774, $p < 0.0001$). *CD5L* was a strong independent predictor, with an odds ratio of 14.74 ($p < 0.0001$), for the presence of rejection.

CONCLUSIONS: Episodes of acute cardiac allograft rejection are related to significant changes in a key inhibitor of apoptosis in macrophages, *CD5L*. Because of its precision to detect acute cellular rejection, even at mild grade, we propose *CD5L* as a potential candidate to be included in the studies of molecule combination panel assays. This finding could contribute to improving the diagnostic and preventive methods for the surveillance of cardiac transplanted patients.

J Heart Lung Transplant 2020;39:257−266

[1]These authors have contributed equally to this work.

Reprint requests: Estefanía Tarazón, PhD, Myocardial Dysfunction and Cardiac Transplantation Unit, Health Research Institute Hospital La Fe, Avd. Fernando Abril Martorell. 106, 46026 Valencia, Spain. Telephone: +34 96-124-66-44. Fax: +34 96-124-66-00.

E-mail address: tarazon_est@gva.es

https://doi.org/10.1016/j.healun.2019.11.004

Heart transplantation is still the only possible life-saving treatment for end-stage heart failure.[1] Nowadays, the conclusive diagnosis of solid organ allograft status depends on the histological assessment of the allograft biopsy.[2,3] Because of the invasive nature and technical limitations of endomyocardial biopsies (EMBs),[4,5] many studies in recent years have focused on the search for circulating biomarkers as non-invasive alternatives.[6–12] Numerous researchers have made an effort to detect molecules such as microRNAs[6–9] and gene-[3,10] or protein-based blood biomarkers[11,12] of cardiac allograft rejection. Their success has been limited or still incomplete and therefore, non-invasive monitoring of these patients remains a challenge. Improvements toward clinical implementation of new markers are laborious and costly, and a prospective, uniform, and standardized validation in large patient cohorts is necessary. In fact, very few biomarkers for acute rejection have obtained Federal Drug Administration approval to be used in transplantation.[10,13] Gene expression profiling (Allomap) is the first and only non-invasive analytic test included in International Society for Heart and Lung Transplantation guidelines to identify the risk of acute cellular rejection in heart transplant recipients. It still presents some limitations. It lacks a good positive predictive value and it can only be used to rule out the presence of acute cellular rejection of Grade 2R or greater in appropriate low-risk patients, between 6 months and 5 years after heart transplantation.[3] Therefore, a novel set of biomarkers is greatly required to complement these assays to detect early stages of organ injury and improve routine clinical practice because current procedures detect pathological alterations at advanced and often irreversible stages of allograft damage.

Advances in proteomics technologies provide immense opportunities for biomarker-related clinical purposes.[14,15] Because of the heterogeneity of the patients and the variability in the treatment and clinical characteristics, a panel of proteins rather than a single biomarker may be essential to reach the high sensitivity, specificity, positive predictive value, and negative predictive value required for detecting cardiac rejection. Currently, this is possible because biomarker analysis could integrate information from multiple platforms and studies to incorporate new promising molecules to the studies in progress. In this sense, in a previous study, we proposed SERCA2a as a potential marker for the diagnosis of cardiac rejection with the aim of allowing the possibility of contributing to obtaining improved panels with this candidate.[11]

In this study, we have conducted a non-targeted proteome characterization focused on identifying multiple protein differences as candidate biomarkers of acute rejection in cardiac transplantation. We found relevant changes in circulating proteins involved in inflammatory and immune response, and we focused specially on the apoptosis inhibitor expressed by macrophages, *CD5L*, as a potential non-invasive marker of cardiac rejection.

Methods

Sample collection

This study included a total of 218 consecutive plasma samples from heart transplant patients (>18 years) who were referred for EMB as a scheduled routine screening from a single center. The samples and associated clinical data were collected from follow-up visits of cardiac transplantation recipients from the University and Polytechnic Hospital La Fe (February 2015 to June 2018). At the time of EMB, blood samples were collected for laboratory analysis. The plasma was separated by centrifugation at 1,500*g* for 10 minutes at 4°C, aliquoted, and immediately stored at −80°C. First, a preliminary proteomic-based study included 30 samples from heart transplant patients. The participants were divided into 3 groups: patients transplanted without allograft rejection (Grade 0R, $n = 10$) and 2 subsets of patients with biopsy-proven allograft rejection (Grade 1R, $n = 10$; Grade ≥2R, $n = 10$). To compare groups with each other, the same sample size was established for all groups, so that patients were consecutively included according to their grade until the number of patients per group was equal. Next, we used an additional validation cohort of 218 consecutive samples (Grade 0R, $n = 98$; Grade 1R, $n = 82$; Grade ≥2R, $n = 38$).

Patients were maintained on a standard immunosuppression regimen, and rejection episodes were assessed according to the International Society for Heart and Lung Transplantation consensus report.[16] For each sample, we recorded age, sex, body mass index, primary heart disease, interval between transplantation and study enrollment, biochemical markers, echocardiographic parameters, and other clinical characteristics at the time of each biopsy (Table 1). Experimenters were blind to group assignment and outcome assessment for all the experiments.

The study was approved by the Ethics Committee (Biomedical Investigation Ethics Committee of University and Polytechnic Hospital La Fe of Valencia, Spain) and was conducted in accordance with the principles outlined in the Declaration of Helsinki.[17] Before sample collection, an informed consent was obtained from each patient.

Proteomic study

This information is available in the Supplementary Material online at www.jhltonline.org.

Circulating *CD5L* validation

CD5L was determined using a specific sandwich enzyme-linked immunosorbent assay (ab213760-Human *CD5L*/CT2 Elisa Kit, Abcam). The *CD5L* test has a limit of detection of <10 pg/ml. The intra- and interassay coefficients of variation were of 4.5% and 5.6%, respectively. No significant cross-reactivity or interference between *CD5L* and analogues was observed. The tests were

Table 1 Patient Characteristics at the Time of Biopsy and Blood Sample Extraction

	Proteomic study		Validation cohort	
Characteristic	Non-rejection ($n = 10$)	Rejection ($n = 20$)	Non-rejection ($n = 98$)	Rejection ($n = 120$)
Age, years	50 ± 13	49 ± 11	50 ± 13	51 ± 12
Male sex (%)	90	95	82	92[a]
White race (%)	97	87	95	91
Indication for cardiac transplantation				
Ischemic cardiomyopathy (%)	20	30	36	41
Idiopathic dilated cardiomyopathy (%)	50	45	48	46
Other (%)	30	25	16	13
Time between Tx and study enrollment (months)	5.6 ± 4.3	3.0 ± 2.7	5.9 ± 4.3	5.7 ± 4.1
Body mass index (kg/m^2)	25 ± 4	24 ± 4	24 ± 4	25 ± 4
Hypertension (%)	10	45	42	43
Diabetes mellitus (%)	30	35	34	45
Dyslipidemia (%)	40	20	49	38
Primary graft failure (%)	30	30	26	29
Infection (%)	30	40	22	34
Ventricular assist device before Tx (%)	60	35	42	37
Echo-Doppler study				
Ejection fraction (%)	64 ± 7	63 ± 8	61 ± 7	60 ± 6
LV end systolic diameter (mm)	28 ± 4	28 ± 4	28 ± 5	28 ± 4
LV end diastolic diameter (mm)	45 ± 4	44 ± 4	44 ± 5	45 ± 4
Hemodynamic parameters				
Mean right atrial pressure (mm Hg)	5.7 ± 2.7	6.9 ± 2.8	6.4 ± 4.3	7.4 ± 4.1
Systolic right ventricular pressure (mm Hg)	36 ± 8	36 ± 8	38 ± 9	38 ± 8
Diastolic right ventricular pressure (mm Hg)	5.8 ± 3.4	7.1 ± 3.2	6.7 ± 4.4	7.2 ± 4.2
Immunosuppressive therapy				
Tacrolimus (%)	100	100	100	100
Mycophenolic acid (%)	100	95	100	98
Steroids (%)	100	95	100	99
Neutrophils (thousands/mm^3)	4.1 ± 2.4	6.3 ± 3.8	5.9 ± 7.3	4.9 ± 3.8
Leukocytes (thousands/mm^3)	6.1 ± 2.6	8.8 ± 4.0[b]	7.8 ± 4.9	7.3 ± 4.0
Lymphocytes (thousands/mm^3)	1.3 ± 0.47	1.8 ± 0.99	1.8 ± 1.3	1.9 ± 2.5
Hemoglobin (mg/dl)	12.0 ± 2.8	12.1 ± 2.1	11.8 ± 1.9	12.0 ± 1.2
Hematocrit (%)	37 ± 9	37 ± 6	36 ± 7	37 ± 6
NT-proBNP (pg/ml)	144 (110–235)	770 (391–1,470)[c]	314 (158–709)	440 (267–1,289)[a]
Troponin T (pg/ml)	11 (6–15)	39 (26–59)[c]	17 (11–39)	25 (13–54)

LV, left ventricular; NT-proBNP, N-terminal fragment of B-type natriuretic peptide; Tx, transplantation.
[a]$p < 0.05$ (non-rejection vs rejection group, validation cohort).
[b]$p < 0.05$ (non-rejection vs rejection group, proteomic study).
[c]$p < 0.01$ (non-rejection vs rejection group, proteomic study).

quantified at 450 nm in a dual-wavelength microplate reader (Sunrise; Tecan) using Magellan version 2.5 software (Tecan).

Statistical analysis

Basal characteristics were expressed as mean ± standard deviation for continuous variables and percentages for discrete variables. Results for each variable were tested for normality using the Kolmogorov-Smirnov method. Continuous variables not following a normal distribution were compared using the Mann-Whitney test, and categorical clinical variables were compared using the chi-square test. Variables with a normal distribution were compared using the Student's *t*-test for continuous variables and the Fisher's exact test for discrete variables.

The relativity, sensitivity, specificity, and predictive value of plasma *CD5L* levels for the presence of transplant rejection were assessed by the construction of receiver operating characteristic (ROC) curves. Logistic regression was performed to evaluate the power of circulating *CD5L* in combination with age, sex, body mass index, hypertension, diabetes mellitus, dyslipidemia, time between transplant and EMB, immunosuppression, infection, primary graft disfunction, and use of ventricular assist device before transplantation for prediction of heart transplant rejection. A *p*-value < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS software (version 20.0; IBM SPSS Inc).

Results

As shown in Table 1, both populations of study, primary analysis and validation cohort, presented similar clinical characteristics. All groups of patients were comparable with regard to

variables such as age, sex, body mass index, diabetes mellitus, dyslipidemia, echo-Doppler and hemodynamic parameters, presence of primary graft failure, use of ventricular assist device, and immunosuppressive therapy. However, in the validation cohort, we found a higher percentage of male patients and an increase in N-terminal pro−B-type natriuretic peptide levels in the group with rejection. In addition, we found a higher percentage of patients with infection in the group with rejection, but it did not reach statistical significance.

A total of 679 proteins were identified by mass spectrometry in plasma samples. We focused on the identification of up- and downregulation of protein intensities where the fold change was ≥1.5 ($p < 0.05$). A total of 17 proteins were identified that could distinguish at least 1 group from the rest of the dataset with a fold change of 1.5 for up- and downregulation. Of these 17 proteins, 11 play a role in inflammation and immunity (Figure 1A and Table 2) and 6 are involved in different processes, such as oxygen or lipid transport (Figure 1B and Table 2).

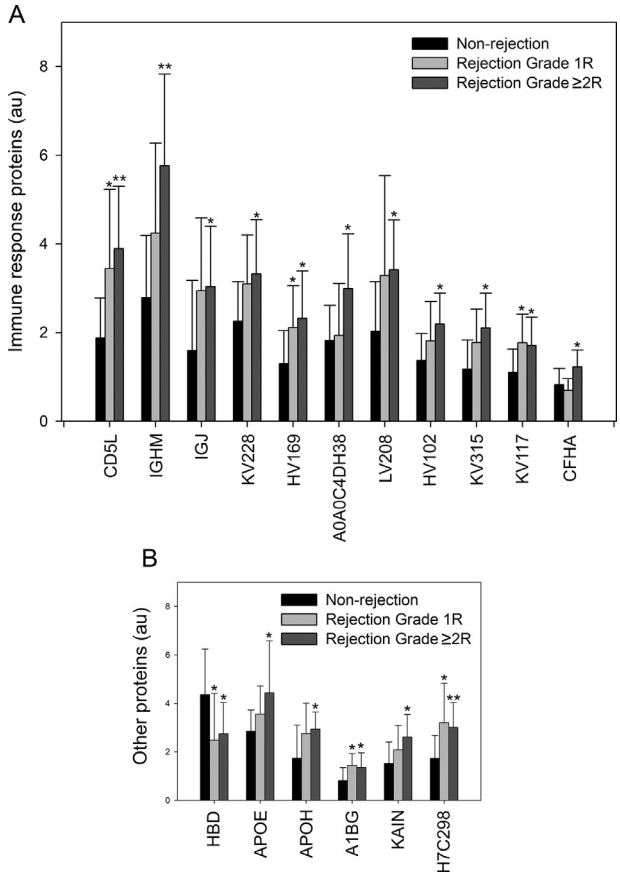

Figure 1 Plasmatic proteins differentially expressed between normal and rejecting heart allografts. The graph shows the values obtained by LC-MS/MS analysis using isobaric tags (TMT 10-plex). Arbitrary units (au) represent the protein relative expression levels for (A) molecules involved in inflammation and immune response and for (B) molecules involved in other processes such as transport of oxygen and lipids in each group of patients (non-rejection, rejection Grade 1R, and Grade ≥2R). Bars indicate protein expression levels ± SD. *$p < 0.05$ and **$p < 0.01$ versus the non-rejection group. au, arbitrary unit; LC-MS/MS, liquid chromatography−tandem mass spectrometry; TMT, tandem mass tag.

A heat map and a hierarchical clustering were performed using the MeV (v. 4.9.0) program to compare the altered proteins in samples from patients with rejection with the corresponding proteins in non-rejection samples. Notably, this analysis identified 2 divergent protein expression profiles, showing a clear demarcation between the rejection Grade ≥2R and the non-rejection group, being more significant in the heat map corresponding to the proteins involved in inflammatory and immune processes (Figure 2).

ROC curves were performed to analyze the capability of the proteins altered for detecting heart transplant rejection. These data are summarized in Table 3. As we show, the molecules with the greatest diagnostic capacity for rejection are those related to the inflammation and immune response. Specifically, the best ROC curves to detect heart transplant rejection (all grades) correspond to *CD5L* (area under the curve [AUC] = 0.850 ± 0.07, $p < 0.01$) and *IGHM* (AUC = 0.830 ± 0.075, $p < 0.01$), two closely related molecules (r = 0.938, $p < 0.0001$). However, our results show that *CD5L* is the only molecule that presents an AUC greater than 0.90 to detect patients with a moderate or severe degree of rejection (Grade ≥2R) and that at the same time presents significant differences in its levels when we compare the group of patients without rejection with the group of patients with mild rejection (Grade 1R), maintaining a good detection capability (AUC = 0.790 ± 0.120, $p < 0.05$) (Figure 3). Multivariate ROC curves combining the most significant molecules involved in inflammation and immune response did not improve the results obtained from individual curves for *CD5L* (data not shown).

Taking into account the excellent diagnostic value observed in *CD5L* levels, we decided to validate these results in a larger patient cohort. *CD5L* plasma levels were higher in patients with heart transplant rejection (median 286 [164−375] pg/ml vs 467 [365−604] pg/ml, $p < 0.0001$). When we compared patients without allograft rejection and patients with allograft rejection of Grade 1R and ≥2R independently, we found significant differences (286 [164−375] pg/ml vs 441 [319−605] pg/ml and 551 [451−635] pg/ml, respectively, $p < 0.0001$ for both comparisons) (Figure 4A). In addition, we confirmed that circulating *CD5L* found in the primary analysis highly discriminated patients with rejection from those without (Figure 4B−D). ROC curves confirm the capability of *CD5L* for detecting heart transplant rejection (all grades combined), obtaining a significant AUC (0.802 ± 0.030, 95% confidence interval [CI], 0.744−0.861; $p < 0.0001$). When we divided the heart transplant rejection group into different grades, we observed that *CD5L* detection capability improves in higher grades, for Grade ≥2R (0.892 ± 0.031, 95% CI, 0.832−0.952; $p < 0.0001$), but preserves a good discrimination capability for Grade 1R (0.774 ± 0.035, 95% CI, 0.705−0.844; $p < 0.0001$). The sensitivity, specificity, positive predictive value, and negative predictive value for the diagnosis of rejection are summarized in Table 4.

A logistic regression was also applied to determine whether circulating *CD5L* was an independent predictor of heart transplant rejection. Age, sex, body mass index, hypertension,

Table 2 Proteins Differentially Regulated in Cardiac Rejection

Entry name	Protein name	Fold change, *p*-value		
		Non-rejection vs rejection ($n = 10$)	Non-rejection vs Grade 1R ($n = 10$)	Non-rejection vs Grade ≥2R ($n = 10$)
Immune response				
*CD5L*_HUMAN	Apoptosis inhibitor expressed by macrophages	1.95[a]	1.84[b]	2.07[a]
*IGHM*_HUMAN	Immunoglobulin heavy constant mu	1.80[a]	1.52	2.07[a]
IGJ_HUMAN	Immunoglobulin J chain	1.88[b]	1.85	1.90[b]
KV228_HUMAN	Immunoglobulin kappa variable 2-28	1.43[b]	1.38	1.48[b]
HV169_HUMAN	Immunoglobulin heavy variable 1-69	1.71[b]	1.63[b]	1.79[b]
A0A0C4DH38_HUMAN	Immunoglobulin heavy variable 5-51	1.35	1.06	1.64[b]
LV208_HUMAN	Immunoglobulin lambda variable 2-8	1.65[b]	1.62	1.68[b]
HV102_HUMAN	Immunoglobulin heavy variable 1-2	1.46[b]	1.32	1.60[b]
KV315_HUMAN	Immunoglobulin kappa variable 3-15	1.65[b]	1.51	1.79[b]
KV117_HUMAN	Immunoglobulin kappa variable 1-17	1.57[a]	1.60[b]	1.55[b]
CFAH_HUMAN	Complement factor H	1.17	−1.18	1.48[b]
Others				
HBD_HUMAN	Hemoglobin subunit delta	−1.58[b]	−1.66[b]	−1.50[b]
APOE_HUMAN	Apolipoprotein E	1.40	1.25	1.55[b]
APOH_HUMAN	Beta-2-glycoprotein 1	1.64[b]	1.59	1.69[b]
A1BG_HUMAN	Alpha-1B-glycoprotein	1.72[b]	1.78[b]	1.67[b]
KAIN_HUMAN	Kallistatin	1.54[b]	1.36	1.71[b]
H7C298_HUMAN	Doublecortin domain-containing protein 1	1.80[a]	1.85[b]	1.74[a]

[a] $p < 0.01$.
[b] $p < 0.05$.

diabetes mellitus, dyslipidemia, time between transplant and EMB, immunosuppression, primary graft dysfunction, infection, and use of ventricular assist device before transplantation were included in the model. When the multivariate model was applied, we demonstrated that a *CD5L* value of ≥394 pg/ml (optimum cut-off point obtained from the ROC curve) was the only independent predictor of rejection, with an odds ratio of 14.74 and Nagelkerke $R^2 = 0.539$ (95% CI 3.821−56.815, $p < 0.0001$) for all grades of rejection. The presence of primary graft dysfunction showed a statistical trend but did not reach statistical significance ($p = 0.057$). The remaining variables were not statistically significant. These data are available in the Supplementary Material online (Supplementary Table 1).

Discussion

Although the gold standard of the diagnosis of cardiac allograft rejection is the EMB,[2,3] this procedure has negative consequences for the patient.[4] Its invasive nature causes patient discomfort and carries the risk of potentially serious complications, including cardiac perforation, tamponade, and arrhythmias.[4] In addition, EMB is subject to sampling error and interobserver variability.[5] Because of these important risks and limitations, numerous studies have been focused on finding non-invasive monitoring of acute and chronic rejection after cardiac transplantation.[6–12] Overall, these studies demonstrated insufficient diagnostic accuracy, limited sensitivity, and limited positive predictive value, and some of them still remain incomplete, requiring more work. Thus, it is necessary to continue working in this area to complete the existing tools, getting an optimal diagnostic approach to detect cardiac allograft rejection, even at an early stage.

The incidence of cause-specific mortality changes with time after transplant, and acute rejection accounts for no more than 11% of deaths in the first 3 years, but immune injury is an important contributor to graft failure, which remains a leading cause of death throughout the follow-up.[18] Therefore, improved understanding of the mechanisms leading to rejection and detection in early stages is needed to further enhance survival for cardiac transplant recipients.

To the best of our knowledge, this is the first study using liquid chromatography−tandem mass spectrometry analysis

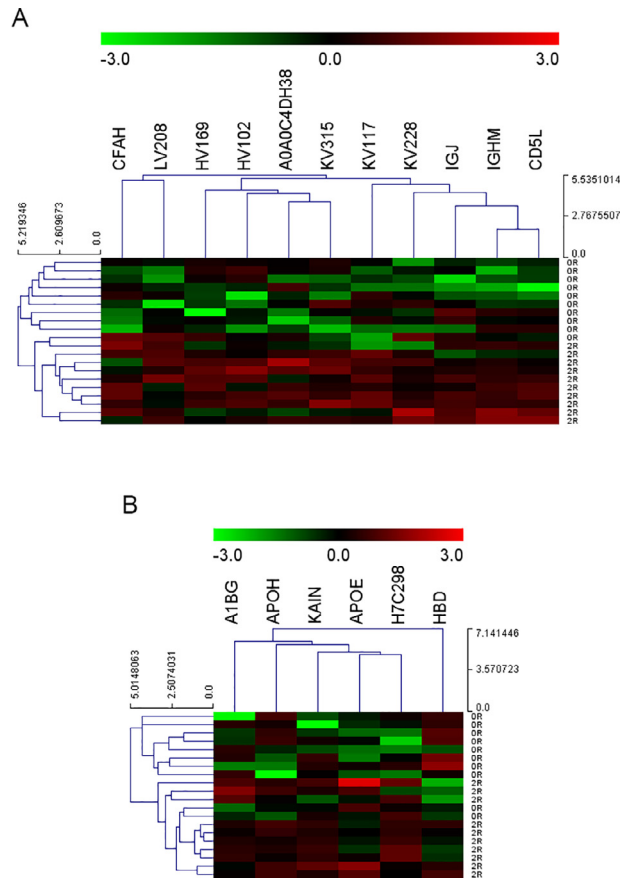

Figure 2 Heat map and hierarchical clustering based on the protein relative expression levels between normal and rejecting heart allografts. The heat map and hierarchical clustering analyses show the separation of the ≥2R rejection grade and non-rejection group for (A) molecules involved in inflammation and immune response and for (B) molecules involved in other processes such as transport of oxygen and lipids. Columns: proteins; rows: samples. Colors depict the relative expression level of each molecule (green: lowest, red: highest).

with isobaric tags (tandem mass tag [TMT] 10-plex) to investigate cardiac allograft rejection. This analysis is based on chemical labeling with identical isobaric TMTs that enable examining up to 10 samples simultaneously in a single run. TMT reagents have the same mass, which results in comigration in chromatographic separations and thus leads to more accurate quantification. During mass spectrometry analysis, each isobaric tag is fragmented to produce a unique reporter ion mass, which allows simultaneous identification and relative quantification of proteins in each sample. Isobaric labeling techniques offer larger channels of quantification in the same experiment. The use of this equipment in routine clinical laboratories has experienced an unprecedented growth during the last 2 decades, not only because of its high specificity, sensitivity, and high-throughput potential, but also because of being more accurate and reliable than classical immunoassays.[14] The reaction to a transplanted graft is an immunologic phenomenon[19] and, as we expected, with this powerful approach we have found significant alterations in different proteins involved in the inflammatory process, corroborating the importance of immune response in the rejection condition. It would be interesting to use a proteome array such as Protoarray platform, the most popular microarray utilized in transplant immunology but only applied to renal transplant patients,[20,21] to associate the results obtained in heart and kidney transplantation studies. However, we have chosen a non-immunoproteomic, non-predetermined, and non-targeted method to discover cardiac rejection markers in an unbiased manner to get non-selective information. In fact, the better marker of rejection found in our study, *CD5L*, is a molecule not included in the protein list of the Protoarray assay.

Identification of biomarkers of cardiac rejection, not only at clinically relevant grades but also at mild rejection grade, could aid the understanding of underlying mechanisms by early indication of damage, when pathological changes are taking place at the molecular level. For this reason, we have focused the study on *CD5L*, a molecule with a great detection power of Grade 2R or greater but maintaining a good detection capability for rejection of Grade 1R. In addition, this protein maintains a close relationship with the rejection condition after adjusting the model with relevant clinical variables, being the only significant independent predictor of rejection as the logistic regression analysis shows. We have obtained a good odds ratio for *CD5L*, but we also must consider that its CI is wide, maybe because of the variability and sample size impact. Moreover, primary graft failure shows a statistical trend as an independent predictor of rejection, and this may have a greater impact on a larger cohort.

CD5L is a soluble protein of approximately 40 kDa, mainly produced in inflamed tissues, that directly inhibits apoptosis of macrophages. This molecule maintains survival and recruits macrophages, and it is associated with pentameric immunoglobulin M, most likely in the ratio of 1:1.[22–25] Therefore, the plasma levels of *CD5L* are highly dependent on that of *IGHM*, being maintained at a relatively high concentration and maintaining a good positive correlation between the levels as previously published,[23,25] and now we have confirmed this in the transplanted patients studied. Different proteomic studies have identified *CD5L* as a recognized biomarker of disease, giving it a crucial role in the modulation of inflammatory responses.[23] In fact, this is not the first time that *CD5L* appears altered in graft rejection; previously, Sigdel et al. reported that *CD5L* was more abundant in the exosomal compartment of urine collected from kidney transplant patients with acute rejection than in patients without rejection.[26] In addition, the involvement of this apoptosis inhibitor of macrophages in cardiovascular diseases has been demonstrated, specifically in atherosclerosis[27] and also in myocardial infarction[28,29] where the deletion of *CD5L* reduced the inflammatory response and infarct size, improving survival. All these data provide strong evidence that *CD5L* plays an important role in the inflammatory responses after myocardial injury.

Considering all of this, our findings demonstrate the direct involvement of apoptosis regulation in macrophages

Table 3 ROC Curve of Circulating Altered Proteins for Detecting Heart Transplant Rejection

Protein name	AUC Rejection all grades	95% CI	AUC Rejection Grade 1R	95% CI	AUC Rejection Grade ≥2R	95% CI
Immune response						
CD5L	0.850[a]	0.711–0.989	0.790[b]	0.562–1.000	0.910[a]	0.783–1.000
IGHM	0.830[a]	0.683–0.977	0.740	0.505–0.975	0.920[a]	0.803–1.000
IGJ	0.765[b]	0.568–0.962	0.760[b]	0.541–0.979	0.770[b]	0.547–0.993
KV228	0.770[b]	0.580–0.960	0.750	0.525–0.975	0.790[b]	0.570–1.000
HV169	0.775[b]	0.605–0.945	0.760[b]	0.549–0.971	0.790[b]	0.588–0.992
A0A0C4DH38	0.670	0.472–0.868	0.550	0.285–0.815	0.790[b]	0.586–0.994
LV208	0.725[b]	0.541–0.909	0.650	0.386–0.914	0.800[b]	0.600–1.000
HV102	0.730[b]	0.548–0.912	0.650	0.398–0.902	0.810[b]	0.621–0.999
KV315	0.795[a]	0.618–0.972	0.740	0.518–0.962	0.850[a]	0.678–1.000
KV117	0.795[a]	0.707–0.983	0.830[b]	0.644–1.000	0.760[b]	0.546–0.984
CFAH	0.590	0.372–0.808	0.400	0.142–0.658	0.780[b]	0.568–0.992
Others						
HBD	0.775[b]	0.598–0.952	0.780[b]	0.573–0.987	0.770[b]	0.561–0.979
APOE	0.740[b]	0.550–0.930	0.690	0.446–0.934	0.790[b]	0.591–0.989
APOH	0.735[b]	0.522–0.948	0.710	0.476–0.944	0.760[b]	0.533–0.987
A1BG	0.775[b]	0.591–0.959	0.830[b]	0.648–1.000	0.780[b]	0.569–0.991
KAIN	0.735[b]	0.550–0.920	0.650	0.405–0.895	0.720	0.475–0.965
H7C298	0.805[a]	0.647–0.963	0.780[b]	0.560–1.000	0.830[b]	0.650–1.000

AUC, area under the curve; CI, confidence interval; ROC, receiver operating characteristic.
[a]$p < 0.01$.
[b]$p < 0.05$.

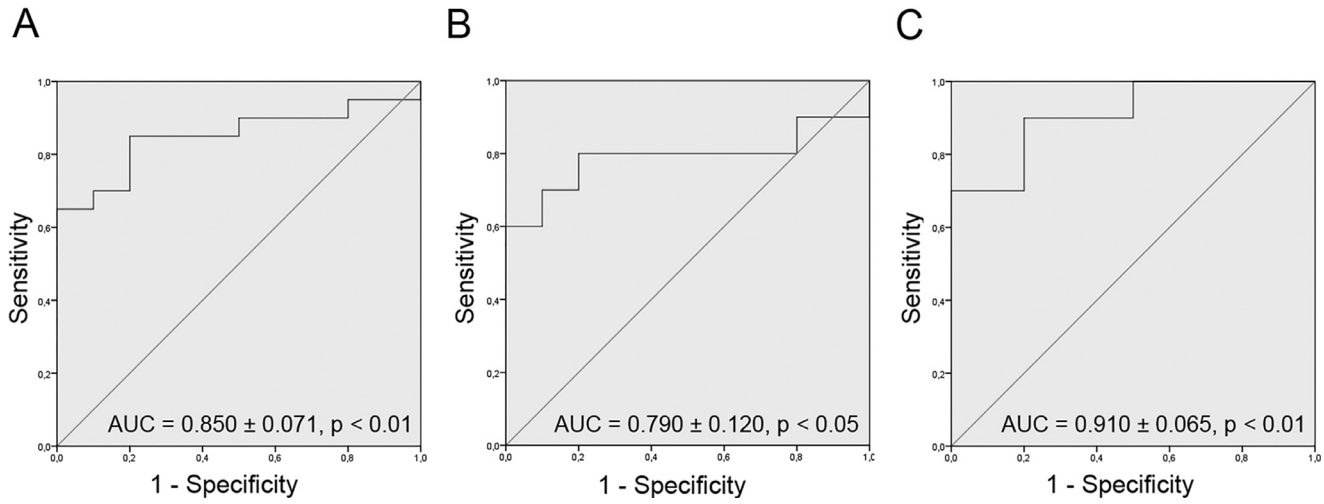

Figure 3 ROC curves based on the primary analysis of circulating levels of *CD5L* between non-rejection and rejection heart allografts. ROC curve of circulating *CD5L* for the detection of cardiac allograft rejection: (A) All grades, (B) Grade 1R, (C) Grade ≥2R. AUC, area under the curve; ROC, receiver operating characteristic.

on the physiopathological mechanism leading to cardiac rejection. This disorder is reflected in peripheral blood and discriminates with excellent accuracy between patients with allograft rejection and those without. It also shows a solid capability for detection that improves in the most clinically relevant rejection degrees but preserves the detection capability in mild rejection. Despite these good results, we believe that because of the heterogeneity of the patients and study populations, a panel of molecules rather than a single biomarker is essential to maximize the sensitivity, specificity, positive predictive value, and negative predictive value required for detecting cardiac rejection. In fact, although both populations of study, primary analysis and validation cohort, presented similar clinical characteristics, we found higher prevalence of hypertension and infection in the rejection group only in the primary cohort. However, these variables were not independent predictors of cardiac rejection in our validation cohort, with *CD5L* the only independent predictor. In this sense, the discovery of precise markers of cardiac

Table 4 Sensitivities, Specificities, and Predictive Values for the Diagnosis of Cardiac Rejection (Cut-Off Point 394 pg/ml)

Grade	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
All grades combined	75	82	83	73
Grade 1R	70	82	76	76
Grade ≥2R	87	82	65	94

NPV, negative predictive value; PPV, positive predictive value.

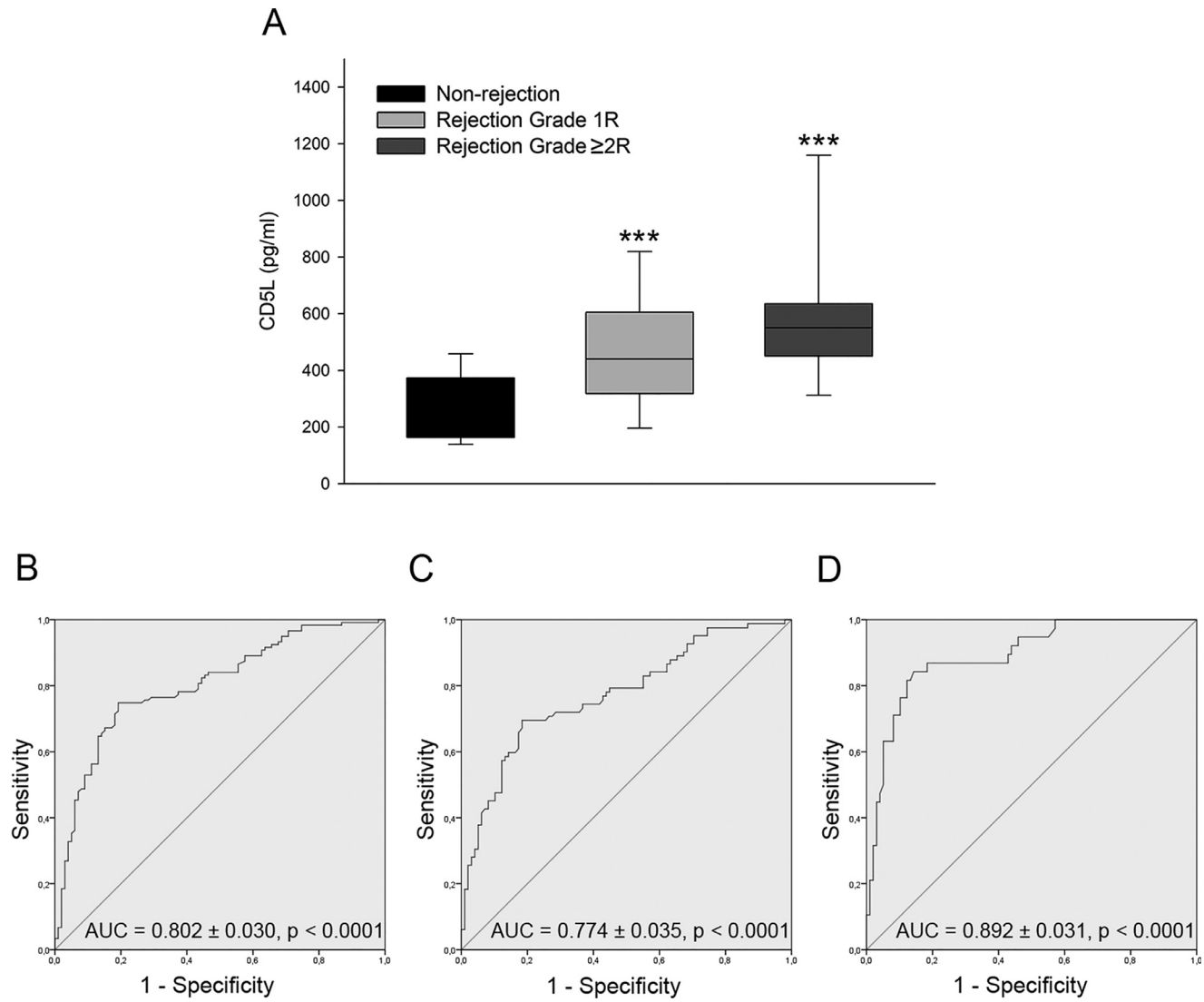

Figure 4 Validation of circulating levels of *CD5L* between non-rejection and rejection heart allografts and ROC curves. (A) Comparison between non-rejection and all grades of rejection heart allografts. The middle line in boxplots represents the median, the lower box bound the first quartile, the upper box bound the third quartile, and the whiskers the 95% confidence interval of the fourth mean. ROC curve of circulating *CD5L* for the detection of cardiac allograft rejection: (B) All grades, (C) Grade 1R, (D) Grade ≥2R. *** $p < 0.0001$ versus the non-rejection group. AUC, area under the curve; ROC, receiver operating characteristic.

rejection like SERCA2a[11] and now *CD5L* could allow the possibility of establishing improved panels and advancing personalized regimens that could improve the survival of patients while diminishing risks associated with immunosuppression. Furthermore, it would be suitable to take other identified proteins such as *IGHM* into account in future studies, because they potentially could have a relevant role in combination with *CD5L*.

Our work is limited on several points and the results must be interpreted in this context. The study population was limited because of its monocentric nature and the relative rarity of enrolled patients with the most clinically relevant grades of rejection. Nevertheless, we believe that the current analyses provide valuable information, and our findings represent a crucial first step for future investigations in which these limiting factors could be the goals. We show a

preliminary study that may be validated in larger patient cohorts to contribute to a better investigation on cardiac rejection and lead to the use of this relatively simple non-invasive determination as a complement to other analytic methods and also as an alternative to EMB. In addition, this study is focused on cellular rejection and has not specifically evaluated antibody-mediated rejection, a relevant clinical entity of rejection associated with worse graft survival and characterized for intravascular macrophage accumulation.[16] Thus, *CD5L* could be studied as a potential marker of this clinical-pathological entity.

In conclusion, we have demonstrated that episodes of acute cardiac allograft rejection are related to significant changes in a key inhibitor of apoptosis in macrophages, *CD5L*. Because of the strong potential and precision of this protein to detect all grades of acute cellular rejection, we propose *CD5L* as an excellent candidate to be included in the studies of molecule combination panel assays. This finding could contribute to improving the diagnostic and preventive methods for the surveillance of cardiac transplanted patients.

Disclosure statement

The authors have no conflicts of interest to disclose.

The authors are grateful to Paula Signes for their technical support and the Hemodynamic Unit (University and Polytechnic La Fe Hospital) for their help in obtaining the samples. The authors are grateful for the professional English language editing to Mr. Arash Javadinejad, English Instructor and Publication Editor at the Health Research Institute La Fe, Valencia, Spain.

This work was supported by the National Institute of Health "Fondo de Investigaciones Sanitarias del Instituto de Salud Carlos III" [PI16/01627, PI17/01925, PI17/01232, CP18/00145], "Consorcio Centro de Investigación Biomédica en Red, M.P." (CIBERCV, under Grant CB16/11/00261), and the European Regional Development Fund (FEDER).

Supplementary data

Supplementary data associated with this article can be found in the online version at www.jhltonline.org.

Supplementary materials

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.healun.2019.11.004.

References

1. McMurray JJ, Pfeffer MA. Heart failure. Lancet 2005;365:1877-89.
2. Gradek WQ, D'Amico C, Smith AL, Vega D, Book WM. Routine surveillance endomyocardial biopsy continues to detect significant rejection late after heart transplantation. J Heart Lung Transplant 2001;20:497-502.
3. Costanzo MR, Dipchand A, Starling R, et al. The International Society of Heart and Lung Transplantation Guidelines for the care of heart transplant recipients. J Heart Lung Transplant 2010;29:914-56.
4. Cooper LT, Baughman KL, Feldman AM, et al. The role of endomyocardial biopsy in the management of cardiovascular disease: a scientific statement from the American Heart Association, the American College of Cardiology, and the European Society of Cardiology. Endorsed by the Heart Failure Society of America and the Heart Failure Association of the European Society of Cardiology. J Am Coll Cardiol 2007;50:1914-31.
5. Nielsen H, Sørensen FB, Nielsen B, Bagger JP, Thayssen P, Baandrup U. Reproducibility of the acute rejection diagnosis in human cardiac allografts. The Stanford Classification and the International Grading System. J Heart Lung Transplant 1993;12:239-43.
6. Di Francesco A, Fedrigo M, Santovito D, et al. MicroRNA signatures in cardiac biopsies and detection of allograft rejection. J Heart Lung Transplant 2018;37:1329-40.
7. Duong Van Huyen JP, Tible M, Gay A, et al. MicroRNAs as non-invasive biomarkers of heart transplant rejection. Eur Heart J 2014;35:3194-202.
8. Sukma Dewi I, Torngren K, Gidlöf O, Kornhall B, Ohman J. Altered serum miRNA profiles during acute rejection after heart transplantation: potential for non-invasive allograft surveillance. J Heart Lung Transplant 2013;32:463-6.
9. Sukma Dewi I, Hollander Z, Lam KK, et al. Association of serum MiR-142-3p and MiR-101-3p levels with acute cellular rejection after heart transplantation. PLOS ONE 2017;12:e0170842.
10. Crespo-Leiro MG, Stypmann J, Schulz U, et al. Clinical usefulness of gene-expression profile to rule out acute rejection after heart transplantation: CARGO II. Eur Heart J 2016;37:2591-601.
11. Tarazón E, Ortega A, Gil-Cayuela C, et al. SERCA2a: a potential non-invasive biomarker of cardiac allograft rejection. J Heart Lung Transplant 2017;36:1322-8.
12. Kennel PJ, Saha A, Maldonado DA, et al. Serum exosomal protein profiling for the non-invasive detection of cardiac allograft rejection. J Heart Lung Transplant 2018;37:409-17.
13. Roedder S, Sigdel T, Salomonis N, et al. The kSORT assay to detect renal transplant patients at high risk for acute rejection: results of the multicenter AART study. PLOS Med 2014;11:e1001759.
14. Lindsey ML, Mayr M, Gomes AV, et al. Transformative impact of proteomics on cardiovascular health and disease: A scientific statement From the American Heart Association. Circulation 2015;132:852-72.
15. Martin-Rojas T, Mourino-Alvarez L, Gil-Dones F, et al. A clinical perspective on the utility of alpha 1 antichymotrypsin for the early diagnosis of calcific aortic stenosis. Clin Proteomics 2017;14:12.
16. Stewart S, Winters GL, Fishbein MC, et al. Revision of the 1990 working formulation for the standardization of nomenclature in the diagnosis of heart rejection. J Heart Lung Transplant 2005;24:1710-20.
17. Macrae DJ. The Council for International Organizations and Medical Sciences (CIOMS) guidelines on ethics of clinical trials. Proc Am Thorac Soc 2007;4:176-8. discussion 178-9.
18. Lund LH, Edwards LB, Kucheryavaya AY, et al. The Registry of the International Society for Heart and Lung Transplantation: thirtieth official adult heart transplant report—2013; focus theme: age. J Heart Lung Transplant 2013;32:951-64.
19. Madsen JC. Advances in the immunology of heart transplantation. J Heart Lung Transplant 2017;36:1299-305.
20. Porcheray F, DeVito J, Yeap BY, et al. Chronic humoral rejection of human kidney allografts associates with broad autoantibody responses. Transplantation 2010;89:1239-46.
21. Li L, Wadia P, Chen R, et al. Identifying compartment-specific non-HLA targets after renal transplantation by integrating transcriptome and "antibodyome" measures. Proc Natl Acad Sci U S A 2009;106:4148-53.
22. Gebe JA, Llewellyn M, Hoggatt H, Aruffo A. Molecular cloning, genomic organization and cell-binding characteristics of mouse Spalpha. Immunology 2000;99:78-86.

23. Sanjurjo L, Amézaga N, Aran G, et al. The human CD5L/AIM-CD36 axis: A novel autophagy inducer in macrophages that modulates inflammatory responses. Autophagy 2015;11:487-502.
24. Miyazaki T, Yamazaki T, Sugisawa R, Gershwin ME, Arai S. AIM associated with the IgM pentamer: attackers on stand-by at aircraft carrier. Cell Mol Immunol 2018;15:563-74.
25. Arai S, Maehara N, Iwamura Y, et al. Obesity-associated autoantibody production requires AIM to retain the immunoglobulin M immune complex on follicular dendritic cells. Cell Rep 2013;3:1187-98.
26. Sigdel TK, Ng YW, Lee S, et al. Perturbations in the urinary exosome in transplant rejection. Front Med (Lausanne) 2015;1:57.
27. Arai S, Shelton JM, Chen M, et al. A role for the apoptosis inhibitory factor AIM/Spalpha/Api6 in atherosclerosis development. Cell Metab 2005;1:201-13.
28. Nishikido T, Oyama J, Shiraki A, Komoda H, Node K. Deletion of apoptosis inhibitor of macrophage (AIM)/CD5L attenuates the inflammatory response and infarct size in acute myocardial infarction. J Am Heart Assoc 2016;5:e002863.
29. Ishikawa S, Noma T, Fu HY, et al. Apoptosis inhibitor of macrophage depletion decreased M1 macrophage accumulation and the incidence of cardiac rupture after myocardial infarction in mice. PLOS ONE 2017;12:e0187894.