Restricted T-Cell Repertoire in the Epicardial Adipose Tissue of Non-ST Segment Elevation Myocardial Infarction Patients

Daniela Pedicino1, Anna Severino1,2†, Gabriele Di Sante3,4†, Maria Cristina De Rosa5, Davide Pirolli2, Ramona Vinci1,2, Vincenzo Pazzano6, Ada F. Giglio7, Francesco Trotta8, Giulio Russo2, Aureliano Ruggio1, Eugenia Pisano2, Alessia d’Aiello1, Francesca Canonico1,2, Pellegrino Cianflone9, Lorenzo Cianfanelli9, Maria Chiara Grimaldi2, Simone Filomia2, Nicola Luciani1,2, Franco Giacie1,9, Piergiorgio Bruno1,2, Massimo Massetti1,2, Francesco Ria3,10‡, Filippo Crea1,2‡ and Giovanna Liuzzo1,2‡

1 Dipartimento di Scienze Cardiovascolari, Fondazione Policlinico Universitario A. Gemelli Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Rome, Italy, 2 Dipartimento di Scienze Cardiovascolari e Pneumologiche, Università Cattolica del Sacro Cuore, Rome, Italy, 3 Dipartimento di Medicina e Chirurgia traslazionale, Università Cattolica del Sacro Cuore, Rome, Italy, 4 Dipartimento di Medicina e Chirurgia, Sezione di Anatomia Umana, Clinica e Forense, Università di Perugia, Perugia, Italy, 5 Istituto di Scienze e Tecnologie Chimiche “Giulio Natta” (SCITEC) - Consiglio Nazionale delle Ricerche (CNR), Rome, Italy, 6 Paediatric Cardiology and Cardiac Arrhythmia/Syncope Unit, Bambino Gesù Children’s Hospital Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Rome, Italy, 7 Cardiology Unit “F. Perinei” Hospital, Bari, Italy, 8 Cardiac Rehabilitation Unit, Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Ospedale San Raffaele, Università Vita-Salute San Raffaele, Milan, Italy, 9 Cardiac Rehabilitation Unit, Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Rome, Italy

Aims: Human epicardial adipose tissue, a dynamic source of multiple bioactive factors, holds a close functional and anatomic relationship with the epicardial coronary arteries and communicates with the coronary artery wall through paracrine and vasocrine secretions. We explored the hypothesis that T-cell recruitment into epicardial adipose tissue (EAT) in patients with non-ST-segment elevation myocardial infarction (NSTEMI) could be part of a specific antigen-driven response implicated in acute coronary syndrome onset and progression.

Methods and Results: We enrolled 32 NSTEMI patients and 34 chronic coronary syndrome (CCS) patients undergoing coronary artery bypass grafting (CABG) and 12 mitral valve disease (MVD) patients undergoing surgery. We performed EAT proteome profiling on pooled specimens from three NSTEMI and three CCS patients. We performed T-cell receptor (TCR) spectratyping and CDR3 sequencing in EAT and peripheral blood mononuclear cells of 29 NSTEMI, 31 CCS, and 12 MVD patients. We then used computational modeling studies to predict interactions of the TCR beta chain variable region (TRBV) and explore sequence alignments. The EAT proteome profiling displayed a...
higher content of pro-inflammatory molecules (CD31, CHI3L1, CRP, EMPRINN, ENG, IL-17, IL-33, MMP-9, MPO, NGAL, RBP-4, RETN, VDB) in NSTEMI as compared to CCS (P < 0.0001). CDR3-beta spectratyping showed a TRBV21 enrichment in EAT of NSTEMI (12/29 patients; 41%) as compared with CCS (1/31 patients; 3%) and MVD (none) (ANOVA for trend P < 0.001). Of note, 11/12 (92%) NSTEMI patients with TRBV21 perturbation were at their first manifestation of ACS. Four patients with the first event shared a distinctive TRBV21-CDR3 sequence of 178 bp length and 2/4 were carriers of the human leukocyte antigen (HLA)-A*03:01 allele. A 3D analysis predicted the most likely epitope able to bind HLA-A3*01 and interact with the TRBV21-CDR3 sequence of 178 bp length, while the alignment results were consistent with microbial DNA sequences.

Conclusions: Our study revealed a unique immune signature of the epicardial adipose tissue, which led to a 3D modeling of the TCRBV/peptide/HLA-A3 complex, in acute coronary syndrome patients at their first event, paving the way for epitope-driven therapeutic strategies.

Keywords: epicardial adipose tissue (EAT), NSTE ACS, T-cell receptor (TCR), immune response, precision medicine, first acute myocardial infarction, antigen-driven immunity, computational modeling

INTRODUCTION

Human epicardial adipose tissue (EAT), a dynamic source of multiple bioactive factors, owns a close functional and anatomic relationship with the epicardial coronary arteries and communicates with the coronary artery wall through paracrine and vasocrine secretions (1). Several studies suggesting the role of EAT in the pathogenesis of atherosclerosis consistently reported that EAT thickness is an independent indicator of cardiovascular risk (2, 3).

Cytokine release and pro-inflammatory cell infiltration of macrophages, lymphocytes, and basophils have been associated with the EAT of patients with established coronary artery disease who underwent elective coronary artery bypass graft (CABG) (4, 5); in these patients, macrophage polarization in EAT is shifted toward the pro-inflammatory M1 phenotype (6, 7).

Although less abundant than macrophages, T cells orchestrate the antigen-specific immune response in the coronary plaque (8–10). This occurs after the T-cell receptor (TCR) has mediated the recognition of short peptides on the human leukocyte antigen (HLA) on presenting cells. The enormous TCR diversity allows for the recognition of a wide range of potential pathogenic molecules and accounts for the difficulties in determining the antigen specificity of each receptor (11, 12).

In the present study, we sought to investigate if T-cell recruitment within EAT in acute coronary syndrome (ACS) patients with non-ST segment elevation myocardial infarction (NSTEMI) might be part of a specific antigen-driven response potentially implicated in ACS onset and progression. To this aim, we performed the EAT proteome profiling and an extended analysis of the TCR beta chain variable region (TRBV) in EAT using the T-cell repertoire of peripheral blood mononuclear cells (PBMCs) as a reference for selective EAT enrichment which led to the design of a 3D model of the cognate/specific peptide-major histocompatibility complex (MHC) target, associated with the first acute coronary event.

METHODS

For a detailed description of the methods, see the Online Supplementary Material.

Study Population Design

We enrolled 1) 32 patients admitted to our Coronary Care Unit with a diagnosis of NSTEMI, who underwent CABG within 14 days of symptom onset, either at their first manifestation (n = 19) or with previous acute coronary events (n = 13); 2) 34 patients with a history of chronic stable effort angina (CCS) lasting more than 12 months, severe coronary artery disease (CAD) requiring CABG, and no clinically evident effort or rest ischemic episodes during the previous 2 weeks; and 3) 12 patients presenting with mitral valve disease (MVD) undergoing cardiac surgery for mitral valve regurgitation due to degenerative disease, with angiographically normal coronary arteries. Figure 1 displays a schematic allocation of the study population in each experimental setting. For a detailed description of the inclusion and exclusion criteria, see the Online Supplementary Material. The Clinical and Research Ethics Committee of Fondazione Policlinico A. Gemelli-IRCCS and the Catholic University of the Sacred Heart of Rome approved the study protocol (protocol no. 2047) that has been conducted in accordance with the principles of the Declaration of Helsinki. All participants provided their written informed consent.

Peripheral Blood Sampling

Venous blood samples were taken a day before the surgery. PBMCs were obtained from whole blood by density gradient
centrifugation over Ficoll-Hypaque (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) and stored at −80°C for RNA extraction. Coded serum samples were stored at −80°C and analyzed for high-sensitivity C-reactive protein (hs-CRP) in a single batch at the end of the study. In NSTEMI patients, serum cardiac troponin I (cTnI) was determined at the time of hospital admission as a routine measurement. All categorization and management of patients were independent from these results.

**EAT Sampling and Profiling**

EAT biopsy (average 0.9 g) was collected before surgery in all cases. In CCS and MVD patients, EAT was collected near the proximal right coronary artery, while in NSTEMI patients, it was collected near the culprit coronary artery, i.e., left coronary artery (LCA) in 20 cases and right coronary artery (RCA) in 12 cases. Liquid nitrogen EAT specimens were stored at −80°C and subsequently used for RNA isolation. Isolated and stimulated EAT T cells were stained with fluorochrome-conjugated mAbs anti-CD4-FITC and anti-CD8-PE-Cy5 (all from Beckman Coulter, Brea, CA, USA). A total of 100,000 events have been acquired. Non-specific staining with isotype-matched control mAb was <1%; the intra- and interassay variability was <10%. Flow cytometry analysis was conducted with FC 500 (Beckman Coulter, Brea, CA, USA) and the data were analyzed with Kaluza software (Beckman Coulter, Brea, CA, USA). EAT biopsies (average weight = 0.2 g) from CCS (n = 3) and NSTEMI (n = 3), collected as described above, were pooled to evaluate the inflammatory proteome profile (Proteome Profiler Array, R&D, USA). Details of EAT T-cell isolation and stimulation and profiling are described in the **Online Supplementary Material**.

**TCR Repertoire Analysis and CDR3 Sequencing**

TCR repertoire analysis and CDR3 sequencing were performed as previously published (13–15). The method for quantifying TCR repertoire perturbation in EAT as compared with PBMCs was adapted from Gorochov et al. (16) (see the **Online Supplementary Material** and Figure S1).

**DNA Extraction and HLA-A Genotyping**

Genomic DNA from peripheral whole blood was extracted by QIAamp DNA Mini kits (Qiagen GmbH, Hilden, Germany) and 0.1 μg of purified genomic DNA was used for HLA-DRB1 exon PCR amplification. After PCR amplification, HLA-A molecular typing was performed by a reverse hybridization method using the INNO-LiPA HLA-A kit (Fujirebio, Tokyo, Japan), following the manufacturer’s instructions. Interpretation of hybridization of HLA-A probes was made using the LiRAS software (Fujirebio, Tokyo, Japan) to predict one-digit HLA.

**In-Silico 3D Modeling of the TRBV/Peptide/HLA-A3 Complex**

We employed a computational modeling study to predict TRBV/peptide/MHC interactions (17, 18). The homology modeling algorithm MODELLER v9.10 as implemented in Discovery Studio 4.0 (Dassault Systèmes, San Diego, CA, USA) was used to generate the computational model structure of TRBV21* (19). The 3D structure of the human anti-pre-pro insulin (PPI) protein T-cell receptor (1E6) bound to an HLA-A*0201-restricted glucose-sensitive PPI peptide (PDB code: 3UTT REF 10.1038/ni.2206), showing 70.0% sequence identity, was used as template (Online Supplementary Material).
Supplementary Material Figure S2). The best-ranked model based on the probability density function (PDF) was selected, and the quality of the structure was assessed by PROCHECK and VERIFY3D (Online Supplementary Material Figures S3, S4). The structure of the human MHC class I molecule HLA-A*0301 (HLA-A3), in complex with a peptide (KLIETYFSK) from proteolipid protein (PDB code: 2XPG REF 10.1107/S090744911007888), was used as the interaction partner for the modeled TRBV21*. The quaternary complex 1BD2 was used as a reference for the relative orientation of the interacting structures (20). Following the replacement of each residue by glycine (glycine), the peptide backbone was used to build putative epitope peptides by side chain construction and CHARMM’s energy minimization. This process was automatically performed by the Grow Scaffold module in Discovery Studio 4.0 by identifying the top-ranking residue in each position. After calculating and scoring, the best peptide to act as a ligand was selected for further analyses (see the Online Supplementary Material for references).

Sequence Alignment and Similarity Analysis

The Basic Local Alignment Search Tool (BLAST) (21) was used to compare the predicted peptide sequence to specific microbial databases and to calculate the statistical significance of matches (see the Online Supplementary Material).

Statistical Analysis

Categorical variables were described as numbers and percentages (%), and they were analyzed using the chi-square (χ²) test or Fisher’s test, depending on sample size restrictions. The continuous variables that were normally distributed, as assessed by the Shapiro–Wilk test, were described as mean ± SD and analyzed with parametric tests. For comparisons among the three groups, we used one-way (or two-way) analysis of variance (ANOVA) with Bonferroni or Sidak correction. For between-group comparisons, we used unpaired or paired Student’s t-test. Data that did not follow a normal distribution were described as median and interquartile range and analyzed by using a non-parametric test. We used the Kruskal–Wallis non-parametric ANOVA and the Dunn’s test for comparisons among groups. For between-group comparisons, we used the Mann–Whitney U-test. To compare two related samples within groups, we used the Wilcoxon test. A two-tailed P-value < 0.05 was considered statistically significant. Statistical analysis was performed by using SPSS Statistics 20.0 (IBM Corp., Armonk, NY, USA) and Prism software 8.02 (GraphPad, San Diego, CA 92121, USA).

RESULTS

The baseline characteristics of the patients are presented in Table S1. The study design is described in Figure 1.

EAT T-Cell Infiltration and Proteome Profiling

In order to characterize the presence of immune infiltrates in EAT, we analyzed the cell suspensions obtained from EAT specimens of NSTEMI (n = 10) and CCS (n = 10) patients by flow cytometry. We observed that 50% of EAT-infiltrating T cells were CD4⁺, while the 30% were CD8⁺ without differences between groups (Figure 2A).

To determine the relative amount of inflammatory mediators at the local level, proteome profiling of pooled EAT specimens from NSTEMI (n = 3) and CCS (n = 3) patients was performed. The results displayed highly significant differences between the two groups for multiple molecules involved in the pro-inflammatory response, cell recruitment and adhesion to the arterial wall, and vascular remodeling (Figures 2B, C; Table S2), thus highlighting the unique composition of the EAT in patients with NSTEMI.

T Cells in EAT Display a Broad TCR Repertoire

To deepen the characterization of T lymphocyte infiltrates, a TRBV-TRBJ spectratyping was performed. We examined the size distribution of the TCR CDR3 region for 25 BV families by immunoscope spectratyping (Table S3). A total of 3,600 spectra were analyzed in PBMC and EAT samples from 72 patients (NSTEMI, n = 29; CCS, n = 31; MVD, n = 12). PBMCs and EAT T cells displayed no difference in TCR BV usage pattern. EAT obtained from MVD patients showed a comparable width of T-cell repertoire. The repertoire used by each individual is highly variable, although among the 25 BV families (Table S3) analyzed, 14 were used more consistently among SA and NSTEMI patients, while only 6 BV among MVD patients (Figure 3 and Figure S5). Despite the high variability of the TCR repertoire used by each individual, it was possible to detect several TCR signatures characterizing specifically NSTEMI, CCS, or MVD patients.

Perturbation of the TCR Repertoire (TRBV21) in EAT Is Associated With the First Manifestation of NSTEMI

Focusing on the most shared TCRs, we calculated using algorithms the threshold values and determined the cutoffs to identify specific disease-related TCRs as biomarkers. The perturbation (D) degree (%) of the TCR repertoire was calculated for each patient in PBMC and EAT specimens as a function of the difference between the P distribution of the EAT sample and the P distribution of the PBMC sample (reference sample). This approach provides a quantitative determination of repertoire perturbations with a D value carrying from 0% to 100% (Online Supplementary Material, Figure S1). As illustrated in Figure 3 and Figure S5, the perturbations of the TCR repertoire in EAT are distributed differently in NSTEMI and CCS patients: in NSTEMI patients, alterations of TRBV21 were strongly prevalent, while perturbations of TRBV28 were observed in CCS and MVD patients, although not statistically significant. Indeed, TRBV21 perturbation was significantly higher in NSTEMI (median, range: 7.4, 0.3–56) as compared to CCS (1.4, 0.02–11.6) and MVD (1.2, 0.2–1.7; ANOVA by Kruskal–Wallis P < 0.001; Dunn’s multiple comparisons test: both P = 0.002) (Figure 4A). A TRBV21* perturbation (D >
10%) was observed in 12/29 (41%) of NSTEMI patients (compared with an expected 1%, in a random use of TCR gene segments) (22). TRBV21 was not enriched in EAT of CCS and MVD patients (3% and 0%, respectively; ANOVA for trend \( P < 0.001 \)). Of note, 11/12 (92%) NSTEMI patients with TRBV21* perturbation (\( D > 10\% \)) were at their first manifestation and only one patient (8%) had previous acute coronary events (\( P = 0.008 \)) (Figure 4B). Figure S6 shows the receiver-operating characteristic (ROC) curves for TRBV21* perturbation. Furthermore, most perturbations of the TRBV21 distribution in NSTEMI patients focused on one single CDR3 of 178 bp length (Figure 4C and Figure S7). The same analysis was performed for TRBV28 (Online Supplementary Material Figure S8), demonstrating the clonal peculiarity of EAT T cells.

**The NSTEMI-Associated TRBV21 Family Shares a Common CDR3 Sequence**

To understand whether the enriched NSTEMI-associated TCR shared the same hypervariable region, several sequencing analyses were performed. Each CDR3 BV peak contains thousands of different sequences, each coding for a unique TCR with a particular antigen specificity. To determine whether the perturbation observed for EAT CDR3 profiles reflected a clonal expansion of T cells, CDR3 sequences of EAT samples were analyzed. Two hundred and eighty-eight sequences were obtained from nine NSTEMI patients, after a screening of about 50 plasmids per sample. Surprisingly, the same sequence (TRBV21 CASSKA ETDE ETQYFGP GTRL) was obtained in four out of the nine NSTEMI patients, all at their first manifestation. This observation supported the idea that T cells carrying this TCR were selectively enriched or expanded in EAT, at the onset of NSTEMI.

A similar CDR3 sequence analysis was carried out with the BV28 family for which several perturbations were also observed in CDR3 spectratyping profiles of CCS patients. In this case, however, we could not find sequences frequently recurring among samples. The resulting sequences and the expected length for TRBV21 and TRBV28 are reported in the Online Supplementary Material Tables S4–S6.

**HLA-A Genotyping**

In Caucasians, the most frequent HLA alleles belong to the HLA-A2 family, which collectively has an allelic frequency of approximately 30%, leading to an \( \approx 50\% \) of individuals being HLA-A2 positive in the population (23). However, two of four NSTEMI patients at their first manifestation of disease with EAT samples displaying the usage of sequence TRBV21 CASSKA ETDE ETQYFGP GTRL were HLA-A*03+ (Figure 4D). These observations led us to propose HLA-A*03 as the candidate restricting element for the public TCR (TRBV21 CASSKA ETDE ETQYFGP GTRL) in our NSTEMI patients, for further studies.

**In-Silico 3D Modeling**

We decided to design a 3D molecular modeling strategy aimed at identifying putative antigen peptide sequence and conformation in the TCRBV/HLA-class I/epitope complex. A scoring algorithm was used to rank sequence candidates, and the top-
ranking sequence KVFLHFRVK was selected as the most likely epitope able to bind HLA-A*01 and interact with the TRBV21* CASSKA ETDE ETQYFGPTRL. The 3D model structures of the TCRBV/HLA-class I/epitope complex and the interacting residues are shown in Figure 5 and Table 1.

Proteins of EAT Microbiota Contain Sequences Homologous to the Putative Epitope Sequence

Our group has recently demonstrated the existence of a local microbiome signature in EAT (24). Based on this scenario, a sequence similarity analysis between the putative antigen sequence (K¹V²F³L⁴H⁵F⁶R⁷V⁸K⁹) and the EAT microbiota genome was performed by BLASTp (21). We find that several sequences from these bacteria display homology higher than 80% with our candidate epitope sequence (Table 2).

Among the Firmicutes-derived sequences, two display the RV residues in the appropriate position, with one having an R for the H in the query. Since R (arginine) and H (histidine) have distinct chemical properties, we are not certain that this peptide would be able to interact properly with the HLA-A*0301 molecule. In the sequences obtained from Cyanobacteria (VFLHYRVK), Y substitutes for F⁶. Although this is not a conservative substitution, in our model, F⁶...
interacts only with an A (alanine) of HLA-A*0301, an interaction that may occur also for a Y (tyrosine) residue in the same position.

Overall, we propose that sequences from Firmicutes/Ruminococcus (LHFRVK) and Cyanobacteria (VFLHYRVK) display most of the characteristics required for the interaction with HLA-A*0301 and TRBV21* and can be considered good candidates as epitopes triggering the activation of T cells at the first episode of NSTEMI.

DISCUSSION

The close anatomical relationship between EAT and coronary arteries has always suggested a likely involvement of the adipose tissue in CAD (1). However, the functional role of the adipose tissue surrounding the heart is still barely elucidated. Several clues indicate that EAT is one of the key characteristics of CAD pathophysiology (25–27). Therefore, in addition to its storage and protective functions, EAT must be considered by all accounts a lymphatic organ, characterized by leukocyte trafficking and cytokine and adipokine release. Our NSTEMI EAT specimens effectively hold a peculiar proteome profile displaying an increased content of a bunch of pro-inflammatory molecules (CRP, IL-17, IL-33, CDF, RETN, RBP-4, CHI3L1) as well as proteins involved in cell recruitment and adhesion to the arterial wall and vascular remodeling (CD14, NGAL, CD31, MMP-9, VCAM1, MPO, ENG, ANG), thereby strengthening the EAT multifaced nature.

Indeed, the EAT of patients with CAD undergoing CABG shows high levels of pro-inflammatory cytokines and cell infiltration with lymphocytes, basophils, and macrophages (4, 5), mostly displaying the pro-inflammatory M1 phenotype (6, 7). The total amount of T lymphocytes is increased in EAT of CAD patients as compared with subcutaneous adipose tissue and EAT of non-CAD patients (28).

A sizeable proportion of patients presenting with ACS shows a unique adaptive immune system profile, characterized by higher levels of effector T cells and reduced levels and/or function of circulating T regulatory cells, together with a disproportionate TCR activation (29–31). T-cell clonal restriction has been demonstrated both in peripheral blood and in coronary thrombi of ACS patients (9, 10), suggesting a specific antigen-driven response. Given the role of adaptive immune dysregulation in the pathogenesis of ACS, EAT might likely contribute, as immunologically active tissue, to the immune unbalance leading to the unstable plaque (4).

Microbial DNA has been found in the EAT environment of ACS patients in association with the NOD-like receptor P3/ inflammasome activation (24), suggesting, along with other evidence (32), that the gut-resident microbiome might directly or indirectly influence the progression toward plaque instability through an antigen-driven response. These data have brought...
FIGURE 5 | In-silico 3D modeling. Molecular modeling of the TCRBV/HLA-class I/epitope complex. Overall 3D structure of the quaternary complex (A). The backbone structures of TCRBV21 (yellow), HLA-A*0301 α-chain (blue), and β2-microglobulin (green) are displayed in ribbon and solvent-accessible surface representations. The epitope residues are in stick representation color-coded by atom types. A zoom view of the contact interface showing the residues important for the stabilization of the complex (B). Sketch of the predicted interactions at the interface (C); epitope residues are shown as blue circles, and HLA and TCR residues as green and violet rectangles, respectively.

TABLE 1 | Intermolecular non-bond interactions established by the predicted epitope with HLA-A*0301 and TCR.

| Epitope | HLA-A*0301 | TCR | Non-bond interactions |
|---------|-------------|-----|-----------------------|
| LYS1 (N) | TYR7 (OH)   |     | H-bond                |
| LYS1 (N) | TYR171 (OH) |     | H-bond                |
| LYS1 (NZ) | GLN82 (OE1) |     | H-bond                |
| LYS1 (NZ) | TYR159 (OH) |     | H-bond                |
| LYS1 (NZ) | GLU83 (OE2) |     | Salt bridge           |
| LYS1 (NZ) | TRP167 (AR) |     | Pi-cation             |
| VAL2 (CG1) | TYR7       |     | Hydrophobic           |
| VAL2 (N)  | GLU83 (OE1) |     | H-bond                |
| PHE3 (AR) | TYR159 (AR) |     | Hydrophobic           |
| PHE3 (AR) | TYR99 (AR)  |     | Hydrophobic           |
| PHE3 (N)  | TYR99 (OH)  |     | H-bond                |
| PHE3 (OH) | TYR159 (OH) |     | Hydrophobic           |
| HIS5 (AR) | LEU156 (AR) |     | Hydrophobic           |
| HIS5 (ND1) | GLN155 (OE1) |   | H-bond                |
| PHE6 (AR) | ALA69 (AR)  |     | Hydrophobic           |
| ARG7 (NH1) | GLU152 (OE2) | | Salt bridge           |
| ARG7 (NH2) | GLU152 (OE2) | | H-bond                |
| VAL8 (CG1) | ALA99 (CB)  |     | Hydrophobic           |
| VAL8 (O)  | LYS146 (N2) |     | H-bond                |
| VAL8 (OG2) | VAL16 (OG1) |     | Hydrophobic           |
| LYS9 (OXT) | LYS146 (NZ) |     | Salt bridge           |
| LYS9 (N)  | ASP77 (OD2) |     | Salt bridge           |
| LYS9 (NZ) | ASP116 (OD2) |   | Salt bridge           |
| LYS9 (NZ) | ASP77 (OD1) |     | H-bond                |
| LYS9 (O)  | THR143 (OG1) | | H-bond                |
| LYS9 (O)  | TYR84 (OH)  |     | H-bond                |

Intermolecular non-bond interactions established by the predicted epitope with HLA-A*0301 and TCR as identified by Discovery Studio 4.0 (Dassault Systèmes 2018). A three-letter amino acid code followed by position number is used. Atoms involved in the interaction are reported in standard PDB atom names.
back the “infection hypothesis,” according to which an infectious event, or even just an altered composition of gut microbiome without clinical signs of infection, could act as a trigger for ACS (33). Given this, our missing piece in the history was to understand whether and how the EAT milieu might contribute to the immune alterations leading to coronary plaque instability. The goal of this work was to prove that an immune response to specific antigens might occur in the EAT as one of the steps toward ACS.

T-cell accumulation in non-lymphoid tissues (EAT in our study) is shaped by several mechanisms including migration and retention of circulating T cells as well as expansion of clones specific for tissue-specific antigens. The local chemokine and cytokine milieu and the expression of specific antigens in the tissue promote chemotaxis and clonal expansion of T cells (10, 12). Here, we report for the first time that EAT of NSTEMI patients at their first clinical manifestation showed the enrichment of an exclusive TRBV21* public T-cell receptor, demonstrating a consistent pattern of clonal restriction in EAT T cells. We could also observe that the presence of TRBV21* was co-occurring with an HLA-A*03+ haplotype that in turn was more frequent in our NSTEMI patients when compared with CCS patients and the expected frequency in the general population. These observations reinforced the hypothesis of a specific, antigen-driven, T-cell expansion in EAT along with the first presentation of ACS.

We previously reported in human (14) and experimental (34) autoimmune diseases that the first wave of T cells specific for a given antigen during the immune response is often characterized by the use of public TCRs, leading to a skewed TCR repertoire. At later times, during chronic disease, the early T-cell repertoire can be modified by exhaustion of some of the activated antigen-specific T cells expanded by the first event (34), in tandem with epitope spreading and TCR repertoire enlargement. Thus, the memory repertoire generated following primary immunization and expanded upon secondary encounter(s) with the antigen changes its clonal composition over time, at least in part to address a presumably larger epitope repertoire. This might account for the differences observed in the TCR repertoire composition between NSTEMI patients with and without previous events.

TCRs recognize short peptides presented on the HLA. To date, several strategies have been used to determine the antigen specificities of T cells knowing the TCR sequence and the restricting element (35). In this study, we used in-silico molecular modeling to describe the TCRBV/HLA-class I/epitope quaternary complex and predict a putative sequence of the target epitope, starting with an unbiased interrogation of TCR specificity. Finally, this computational modeling allowed us to highlight a similarity between the putative epitope sequence and the sequences found in bacterial phyla associated with ACS and found in the gut microbiota (24). Overall, this scenario is in line with recent demonstrations of cross-reactive CD4+ T cells, primed by epitopes derived from microbes colonizing different mucosal tissues, able to infiltrate target organs, causing or exacerbating both autoimmune (36) and autoimmune diseases (37). Moreover, in agreement with our findings, another study has recently described the involvement and the activation of heart-specific Th cells by bacterial peptide mimics derived from the intestinal microbiota, able to enter the myocardium, enhancing the damage caused by infection during lethal inflammatory cardiomyopathy (38).

Thanks to its privileged position of close proximity to the coronary arteries, EAT represents the ideal environment for a specific T-cell clonal expansion in response to antigen exposure. Whether the enriched T cells and the antigens detected in the EAT result from direct microbial colonization or represent the consequence of previous peripheral immune responses needs to be proven in further studies. However, the evidence for an antigen-driven immune response as a molecular and cellular marker of the first coronary event represents the first step toward a personalized approach in cardiovascular medicine for the ideation of epitope-based vaccines in the treatment of ACS. In the future, more
advanced methods that integrate computational biology and structural modeling might be used to design highly specific and powerful TCRs for use in T-cell therapies (Figure 6).

CONCLUSIONS

The identification of a specific T-cell enrichment in the EAT of patients presenting at their first coronary event represents the clue of a specific antigen-driven immune response in the pathogenesis of ACS. Together with the available evidence on the role of dysbiosis in plaque instability, our data pave the way for the fascinating hypothesis of microbiome-derived antigens as triggers for plaque instability. These observations represent a significant step toward the perspective of engineered T-cell or epitope-based vaccine therapies (Figure 6).

LIMITATIONS AND PERSPECTIVES

Our study is more hypothesis-generating than hypothesis-proving by its nature. Some limitations should be recognized. First, T cells may have encountered bacteria somewhere else, and we cannot exclude that the restricted TCR diversity observed in the EAT surrounding diseased coronary arteries may reflect differential entrapment of antigen primed T cells from the circulating T-cell pool. One possibility is the cross-reactivity of T cells exposed to peptide motifs shared by the human proteome and gastrointestinal microbiota (39) or, as it happens for other autoimmune/autoinflammatory mechanisms, a TCR-independent pathway or a bystander effect (40). Second, in a previous study, we have documented the presence of bacterial DNA of gut microbiota phyla in the EAT of NSTEMI patients (24). However, we did not demonstrate a direct bacterial colonization in EAT, since bacterial DNA in EAT might represent both the clue of direct bacterial colonization and the result of antigen-presenting cell translocation following phagocytosis that occurred elsewhere. In the current study, we did not have the opportunity to directly search bacterial DNA in the EAT samples showing the related TRBV21 sequences because of the scarcity of tissue. Third, we could not directly prove that induction of pro-inflammatory signaling by the related T cells in EAT plays a role in the generation of unstable plaques. However, taking together the present work and our previous data, the demonstration of T-cell enrichment in EAT of NSTEMI patients agrees with the already demonstrated upregulation of NLRP3 inflammasome (24) and with the available evidence of an altered immune response as a trigger for plaque instability. Whether or not microbial colonization of EAT antecedes this immune response, the demonstration of enriched T cells in the EAT of NSTEMI patients at their first manifestation represents sufficient proof of an antigen-driven immune response taking place in the adipose tissue surrounding the coronary artery.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The name of the repository and link to the data can be found below: Figshare; 10.6084/m9.figshare.19932182.
ETHICS STATEMENT
The studies involving human participants were reviewed and approved by the Clinical and Research Ethics Committee of Fondatazione Policlinico A. Gemelli-IRCCS and the Catholic University of the Sacred Heart of Rome. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS
GL, DPe, AS, FR and GDS designed the research. GL provided funding support. FT, AFG, Ad’A, PC, AR, and PB collected the biological materials. AS, VP, GDS, RV, FCa, and EP processed the biological materials and performed the statistical analyses. DC, LC, DPe, GR, NL, FG, PB, MCG, and SF performed the screening and selection of patients MCDR and DPi performed computational modelling. AS, DPe, FR, and GDS investigated and analyzed the data and wrote the original manuscript. DPe, AS, RV, GL, GDS, and FR reviewed and edited the final manuscript. GL, FCr, MM, and FR provided suggestions and performed critical reading of the manuscript. All authors read and approved the submitted version.

REFERENCES
1. Chaowalit N, Lopez-Jimenez F. Epicardial Adipose Tissue: Friendly Companion or Hazardous Neighbour for Adjacent Coronary Arteries? Eur Heart J (2008) 29:989–7. doi: 10.1093/eurheartj/ehm463
2. Maimaituxun G, Shimabukuro M, Fukuda D, Yagi S, Hirata Y, Iwase T, et al. Local Thickness of Epicardial Adipose Tissue Surrounding the Left Anterior Descending Artery Is a Simple Predictor of Coronary Artery Disease - New Prediction Model in Combination With Framingham Risk Score. Circ J (2018) 82:1369–78. doi: 10.1253/circj.CJ-17-1289
3. Hassan M, Said K, Rizk H, ELMogy F, Donya M, Houseni M, et al. Segmental Peri-Coronary Epicardial Adipose Tissue Volume and Coronary Plaque Characteristics. Eur Heart J Cardiovasc Imaging (2016) 17:1169–77. doi: 10.1093/ehjci/jev298
4. Mazurek T, Zhang L, Zaleski A, Mannion JD, Diehl JT, Arafat H, et al. Human Epicardial Adipose Tissue Is a Source of Inflammatory Mediators. Circulation (2003) 108:2460–6. doi: 10.1161/01.CIR.0000099542.57313.C5
5. Karastergiou K, Evans I, Ogston N, Miheisi N, Nair D, Kaski JC, et al. Epicardial Adipokines in Obesity and Coronary Artery Disease Induce Atherogenic Changes in Monocytes and Endothelial Cells. Arterioscler Thromb Vasc Biol (2010) 30:1340–6. doi: 10.1161/ATVBAHA.110.204719
6. Hirata Y, Tabata M, Kurobe H, Motoki T, Akaishi M, Nishio C, et al. Coronary Atherosclerosis Is Associated With Macrophage Polarization in Epicardial Adipose Tissue: J Am Coll Cardiol (2011) 58:248–55. doi: 10.1016/j.jacc.2011.01.048
7. Hirata Y, Kurobe H, Akaishi M, Chikugo F, Horii T, Bando Y, et al. Enhanced Inflammation in Epicardial Fat in Patients With Coronary Artery Disease. Int Heart J (2011) 52:139–42. doi: 10.1536/ihj.12.139
8. Liuzzo G, Goronyz JJ, Yang H, Kopecky SL, Holmes DR, Frye RL, et al. Monoclonal T-Cell Proliferation and Plaque Instability in Acute Coronary Syndromes. Circulation (2000) 101(25):2883–8. doi: 10.1161/01.CIRC.101.25.2883
9. De Palma R, Del Galdo F, Abbate G, Chiarleto M, Calabro R, Forte L, et al. Patients With Acute Coronary Syndrome Show Oligoclonal T-Cell Recruitment Within Unstable Plaque: Evidence for a Local, Intraocular Immunologic Mechanism. Circulation (2006) 113:640–6. doi: 10.1161/CIRCULATIONAHA.105.537712
10. Klingenberg R, Brokopp CE, Grivès A, Courtier A, Jaguszewski M, Pasqual N, et al. Clonal Restriction and Predominance of Regulatory T Cells in Coronary Thrombi of Patients With Acute Coronary Syndromes. Eur Heart J (2015) 36:1041–8. doi: 10.1093/eurheartj/ehu543
11. Swansson SJ, Rensenzweig A, Seidman JG, Libby P. Diversity of T-Cell Antigen Receptor V Beta Gene Utilization in Advanced Human Atheroma. Arterioscler Thromb (1994) 14:1210–4. doi: 10.1161/01.ATV.14.7.1210
12. Glanzville J, Huang H, Nau A, Hatton O, Wagener LE, Rubelt F, et al. Identifying Specificity Groups in the T Cell Receptor Repertoire. Nature (2017) 547:94–8. doi: 10.1038/nature22976
13. Ria F, Gallard A, Gabaglia CR, Guery J-C, Sercaz EE, Adorini L. Selection of Similar Naive T Cell Repertoires But Induction of Distinct T Cell Responses by Native and Modified Antigen. J Immunol (2004) 172:3447–53. doi: 10.4049/jimmunol.172.6.3447
14. Ria F, Penitente R, De Santis M, Nicolò C, Di Sante G, Orsini M, et al. Collagen-Specific T-Cell Repertoire in Blood and Synovial Fluid Varies With Disease Activity in Early Rheumatoid Arthritis. Arthritis Res Ther (2008) 10: R135. doi: 10.1186/ar2533
15. Di Sante G, Tolusso B, Fedele AL, Gremses E, Alvernini S, Nicolò C, et al. Collagen-Specific T Cell Repertoire and HLA-DR Alleles: Biomarkers of Active Refractory Rheumatoid Arthritis. EBioMedicine (2015) 2:2037–45. doi: 10.1016/j.ebiom.2015.11.019
16. Gorochov G, Neumann AU, Kereveur A, Parizot C, Li T, Katlama C, et al. Perturbation of CD4+ and CD8+ T-Cell Repertoires During Progression to AIDS and Regulation of the CD4+ Repertoire During Antiviral Therapy. Nat Med (1998) 4:215–21. doi: 10.1016/S1074-7613(00)80546-4
17. De Rosa MC, Giardina B, Bianchi C, Carelli Alinovi C, Pirolli D, Ferraccioli G, et al. Selective Inhibitors of T Cell Receptor Recognition of Antigen-MHC Complexes for Rheumatoid Arthritis. ACS Med Chem Lett (2019) 10:644–9. doi: 10.1021/acsmedchemlett.8b00601
18. Eswar N, Webb B, Marti-Renom MA, Madhusudhan MS, Eramian D, Shen MY, et al. Comparative Protein Structure Modeling Using Modeller. Curr Protoc Bioinformatics (2006) 5:5.6. doi: 10.1002/0471250953.bi0506s15
19. Ding YH, Smith KJ, Garboczi DN, Utz U, Buddison WE, Wiley DC. Two Human T Cell Receptors Bind in a Similar Diagonal Mode to the HLA-A2/Tax Peptide Complex Using Different TCR Amino Acids. Immunity (1998) 8:403–11. doi: 10.1016/S1074-7613(00)80546-4

FUNDING
The present study was partially supported by the Catholic University of the Sacred Heart Linea D1 2016 Grant and by the Italian National Project Grant PRIN 2017, Protocol 2017WJBKKW_001.

ACKNOWLEDGMENTS
The authors thank the Minister of Health Ricerca Corrente 2021 for the support. The authors thank all their colleagues, technicians, and nurses for their technical and scientific support. The authors strongly believe in translational research involving clinicians, interventional cardiologists, surgeons, and molecular biologists to promote personalized medicine.

SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.845526/full#supplementary-material
21. Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, et al. BLAST+: Architecture and Applications. *BMC Bioinformatics* (2009) 10:421. doi: 10.1186/1471-2105-10-421

22. Freeman JD, Warren RL, Webb JR, Nelson BH, Holt RA. Profiling the T-Cell Receptor Beta-Chain Repertoire by Massively Parallel Sequencing. *Genome Res* (2009) 19:1817–24. doi: 10.1101/gr.092924.109

23. González-Galarza FF, Takeda LY, Santos EJ, Kempson F, Maia MH, da Silva AL, et al. Allele Frequency Net 2015 Update: New Features for HLA Epitopes, KIR and Disease and HLA Adverse Drug Reaction Associations. *Nucleic Acids Res* (2015) 43(Database issue):D784–8. doi: 10.1093/nar/gku1166

24. Pedicino D, Severino A, Ucci S, Bugli F, Flego D, Giglio AF, et al. Epicardial Adipose Tissue Microbial Colonization and Inflammation Activation in Acute Coronary Syndrome. *Int J Cardiol* (2017) 236:95–9. doi: 10.1016/j.ijcard.2017.02.040

25. Baker AR, Silva NF, Quinn DW, Harte AL, Pagano D, Bonser RS, et al. Human Epicardial Adipose Tissue Expresses a Pathogenic Profile of Adipokines in Patients With Cardiovascular Disease. *Cardiovasc Diabetol* (2006) 5:1. doi: 10.1186/1471-2458-5-1

26. Oikonomou EK, Marwan M, Desai MY, Mancio J, Alashi A, Hutt Centeno E, et al. Allele Frequency Net 2015 Update: New Features for HLA Epitopes, KIR and Disease and HLA Adverse Drug Reaction Associations. *Nucleic Acids Res* (2015) 43(Database issue):D784–8. doi: 10.1093/nar/gku1166

27. Bellmúñez V, Durán P, Rojas E, Díaz MP, Rivas J, Nava M, et al. The Sick Heart Is a Suitable Environment for Defective Signaling and Crosstalk With the Myocardium. *Front Endocrinol (Lausanne)* (2021) 12. doi: 10.3389/fendo.2021.735070

28. Mráz M, Cinkajzlова A, Kloučková J, Lacinová Z, Kratochvílova H, Lipsí P, et al. Coronary Artery Disease Is Associated With an Increased Amount of T Lymphocytes in Human Epicardial Adipose Tissue. *Mediators Inflamm* (2019) 2019:4075086. doi: 10.1155/2019/4075086

29. Flego D, Severino A, Trotta F, Previtero M, Ucci S, Zara C, et al. Increased Metalloproteinase-9 Might Affect Adaptive Immunity in non-ST-Segment Elevation Acute Coronary Syndromes: A Prognostic Marker for Incident Cardiovascular Events Beyond Traditional Risk Factors. *Eur Heart J* (2017) 38:814–24. doi: 10.1093/eurheartj/ehw582

30. Flego D, Liuzzo G, Weyand CM, Crea F. Adaptive Immunity Dysregulation in Acute Coronary Syndromes: From Cellular and Molecular Basis to Clinical Implications. *J Am Coll Cardiol* (2015) 65:1175–86. doi: 10.1016/j.jacc.2015.01.027

31. Gil-Cruz C, Perez-Shibayama C, De Martin A, Ronchi F, van der Borgt K, Niederer R, et al. Microbiota-Derived Peptide Mics Drive Lethal Inflammatory Cardiomyopathy. *Science* (2019) 366(6467):881–6. doi: 10.1126/science.aav3487

32. Birnbaum ME, Hong S, Garcia KC. Diversity-Oriented Approaches for Interrogating T-Cell Receptor Repertoire, Ligand Recognition, and Function. *Immune Rev* (2012) 250:82–101. doi: 10.1111/imr.12006

33. Freeman JD, Warren RL, Webb JR, Nelson BH, Holt RA. Profiling the T-Cell Receptor Beta-Chain Repertoire by Massively Parallel Sequencing. *Genome Res* (2009) 19:1817–24. doi: 10.1101/gr.092924.109

34. Pedicino D, Giglio AF, Galiffa VA, Cialdella P, Trotta F, Graziani F, et al. Infections, Immunity and Atherosclerotic Pathogenic Mechanisms and Unsolved Questions. *Int J Cardiol* (2013) 166:572–83. doi: 10.1016/j.ijcard.2012.05.098

35. Freeman JD, Warren RL, Webb JR, Nelson BH, Holt RA. Profiling the T-Cell Receptor Beta-Chain Repertoire by Massively Parallel Sequencing. *Genome Res* (2009) 19:1817–24. doi: 10.1101/gr.092924.109

36. Oikonomou EK, Marwan M, Desai MY, Mancio J, Alashi A, Hutt Centeno E, et al. Allele Frequency Net 2015 Update: New Features for HLA Epitopes, KIR and Disease and HLA Adverse Drug Reaction Associations. *Nucleic Acids Res* (2015) 43(Database issue):D784–8. doi: 10.1093/nar/gku1166

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher’s Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Pedicino, Severino, Di Sante, Di Rosa, Pirolli, Vinci, Pazzano, Giglio, Trotta, Russo, Ruggio, Pisano, d’Aiello, Canonico, Ciampi, Cianfalone, Cianfanielli, Grimadli, Filomia, Luciani, Glieca, Bruno, Mussatti, Ria, Crea and Liuzzo. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.