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Resolution of inflammation is altered in chronic heart failure and entails a dysfunctional responsiveness of T lymphocytes

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ABSTRACT: Chronic heart failure (CHF) is characterized by an ongoing nonresolving inflammatory status, where T lymphocytes seem critical. It has been recently recognized that transition from acute to chronic inflammation could be caused by defects in resolving inflammation, the resolution of which is mediated by a novel family of ω-3–derived specialized proresolving lipid mediators such as resolvins. We analyzed 27 elderly patients with CHF and 23 healthy age-matched control subjects, and we reported significantly lower levels of D-series resolvin (RvD)1 in plasma of patients with CHF that were associated with a reduced ability of their leukocytes to produce this lipid *via* its biosynthetic enzyme 15-lipoxygenase and that correlated with gas exchange dysfunction. Furthermore, when pretreating *ex vivo* peripheral blood mononuclear cells of patients with CHF with RvD1 or RvD2, we found that neither of them was able to modulate the immune response of $CD8^+$ and $CD4^+$ T cells in terms of proinflammatory cytokine production, namely TNF-α, IFN-γ, IL-17, and IL-2. Such impaired T-cell responsiveness in patients with CHF was associated with a significant reduction in mRNA and protein expression of RvD1 receptor GPR32, suggesting a defective signaling in the proresolving pathway. We conclude that patients with CHF show alterations in producing proresolving mediator RvD1 and a failure of adaptive immune cells in responding to the anti-inflammatory actions of RvDs that may contribute to the progression of chronic inflammation. Thus, the proresolution pathway might be a potential candidate to design better treatments for CHF aimed at reducing T cell–mediated chronic inflammation.—Chiurchiù, V., Leuti, A., Saracini, S., Fontana, D., Finamore, P., Giua, R., Padovini, L., Incalzi, R. A., Maccarrone, M. Resolution of inflammation is altered in chronic heart failure and entails a dysfunctional responsiveness of T lymphocytes. FASEB J. 33, 909–916 (2019). www.fasebj.org

KEY WORDS: resolvins · chronic inflammation · adaptive immunity

Chronic or congestive heart failure (CHF) is one of the most relevant diseases affecting humans and is a common outcome of a variety of cardiovascular diseases in the elderly population (1, 2). Accumulated evidence suggests that inflammation and immune activation are critically involved in the induction of chronicity and the progression of CHF (3–5). Indeed, in CHF activation of the immune system is characterized by increased production and release of several proinflammatory cytokines, and studies have shown that alterations of adaptive immunity are critical for CHF pathophysiology (4), with significant increases in the $CD4^+/CD8^+$ T lymphocyte cell ratio and expression of early activation markers (6, 7). Furthermore, patients with CHF show increased frequencies of proinflammatory $CD4^+$ T-helper (T_h)1 and T_h17 cells and lower frequencies of regulatory T cells, and these are associated with disease severity (8–10). A recent study investigated leukocyte subpopulations and reported that patients with CHF show greater immunosenescence associated with high differentiation in $CD8^+$ and $CD4^+$ T-cell subsets and with worse clinical status (11), suggesting that a compromised and aged adaptive immune system may contribute to CHF. Hence, there is an unmet need for new diagnostic and therapeutic options, especially aimed at reducing the T cell–induced chronic inflammatory responses.

Chronic inflammation can be a consequence of failure to resolve inflammation. The resolution of inflammation is mediated by the newly discovered ω-3–derived essential fatty acids, termed specialized proresolving lipid

ABBREVIATIONS: CHF, chronic heart failure; DHA, docosahexaenoic acid; LOX, lipoxygenase; LX, lipoxin; NYHA, New York Heart Association; PBMC, peripheral blood mononuclear cell; RvD, D-series resolvin; SPM, specialized proresolving mediator; T_h, T helper; T_{reg}, regulatory T

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mediators (SPMs), which include resolvins (12). D-series resolvins (RvDs), synthesized from docosahexaenoic acid (DHA) by the sequential action of lipoxygenases (LOX) 15-LOX and 5-LOX (13), are the most studied among SPMs and act as immunoresolvents, namely as agents that reduce inflammation and promote resolution (12, 14). Recent evidence indicates that altered SPM metabolism and function can contribute to chronicity and to the magnitude of persistent inflammation in several pathologic conditions, such as asthma, chronic obstructive pulmonary disease, and diabetes (14–16). It has been reported that RvDs are reduced in several cardiovascular diseases and exert cardioprotective roles, although documented only in mice and rats, by improving ventricular function and ameliorating heart failure (17–19). In addition, we have recently demonstrated that RvD1 and RvD2 directly target human T lymphocytes, reducing T_h1 and T_h17 cell responses (20), supporting the view that RvDs might prevent T cell–mediated chronicity of inflammation. Hence, in the present investigation we ascertained whether patients with CHF show defects in resolving inflammation as associated with altered production of specific resolvins, and we investigated whether these lipid mediators influence T cell–dependent chronic inflammatory responses in CHF.

MATERIALS AND METHODS

Patients and cell isolation

We collected peripheral blood from 27 (13 male and 14 female) patients with CHF (average age, 82.61 ± 6.67 yr) admitted to the Bio-Medico Campus Hospital of Rome and 23 age-matched healthy (9 male and 14 male) donors (average age, 78.43 ± 4.78 yr). Numerosity of samples was chosen according to the estimated sample size of 23 samples per group with a power analysis of 80% at a significance level of $P = 0.05$. Patients with CHF and healthy control subjects provided written informed consent according to the Legislative Decree 196/2003. The study was approved by the Ethical Committee of Bio-Medico Campus of Rome (19.15 TS, 09/08/2015) and was conducted according to the ethical principles arising from the Helsinki Declaration. Participation was voluntary and unpaid. Peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation with Ficoll-Hypaque (GE Healthcare Life Sciences, Little Chalfont, United Kingdom), according to standard procedures. PBMCs were suspended in complete RPMI before cell treatments.

Clinical characteristics

The cases of CHF were ischemic, hypertensive, or valvular origin and in various stages of New York Heart Association (NYHA) classification (from II to IV). The exclusion criteria concerned those individuals with at least one of the following characteristics: neoplastic disease, chronic renal insufficiency stage IV and V (classification of the National Kidney Foundation; February 2002), hepatic impairment, immunologic or chronic infections that can affect the immune and cytokine pattern assets, and any condition that may alter the arrangement of the immune system. Patients underwent a complete personal history and physical examination and multidimensional geriatric rating, which included activities of daily living, instrumental activities of daily living, mini-mental state examination, geriatric depression scale, study of body composition by bioimpedentiometry, evaluation of respiratory muscle strength by detection of maximal inspiratory pressure, and maximal expiratory pressure. The clinical details of patients with CHF are shown in **Table 1**.

Detection of resolvins

Plasma from patients with CHF and healthy donors was kept at −80°C. The levels of RvD1 and RvD2 were measured through quantitative competitive ELISA kits and validated in an enzyme immunoassay buffer (EIA; Cayman Chemicals, Ann Arbor, MI, USA), based on the competition between free RvD1/RvD2 Tracers for a limited number of RvD1/RvD2-specific rabbit antiserum binding sites. The number of RvD1/RvD2 able to bind to the rabbit antiserum is inversely proportional to the concentration of free RvD1/RvD2 in the samples. The detection of the rabbit antiserum-RvD1/RvD2 is based on a typical sandwich ELISA, and absorbance was read between 405 and 420 mn (assay sensitivity of 15 pg/ml) on a VarioScan Flash (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

TABLE 1. *Characteristics of patients with CHF*

Characteristic	Total CHF	NYHA II	NYHA III	NYHA IV
N	27	10	9	8
Age (yr)	82	80.5	86	82.5
Gender (% male)	48.1	50	56	38
BMI	26.1	23.1	29.5	26.4
FFM index	19.2	17.7	19.2	19.3
TBW (%)	56.2	56.2	55.5	55.6
pO_2	70.2	70.2	78	58.7
pCO_2	38.3	35.8	36.9	46.1
pH	7.46	7.46	7.47	7.46
HCO_3	27.3	25.8	26.6	32.5
Lactate	12.8	12.8	10.4	10.8
WBCs	6.89	7.15	6.66	8.18
Hb	12.4	12.6	12.6	11
Lymphocytes	1.01	1.03	0.93	1.01
CRP	12.1	17.2	10	9.5
Albumin	3.6	3.8	3.7	3.4
NT-pro BNP	9180	10,999	5911	8107
eGFR (MDRD)	52.1	48.2	52.1	52.6
eGFR (CKD-EPI)	50	45.9	48.7	52.2
EF (%)	46	48	46	33.5
PAPs	45	44	39	52
TAPSE	18	20.5	15	18
E/E′	14.5	11.5	16	15
ADL	6	6	6	4.5
IADL	5	7	6	4
MMSE	26.8	26.5	27.2	26.2
GDS	3	2.5	4	4
Hypertension	81	90	89	62
Ischemic cardiopathy	52	60	56	38
Diabetes	26	40	33	NA

Median values of characteristics and clinical parameters of patients with CHF, also stratified by NYHA class. ADL, activities of daily living; BMI, body mass index; CKD-EPI, chronic kidney disease epidemiology collaboration; CRP, C-reactive protein; EF, ejection fraction; *E/E′*, early filling and early diastolic mitral annular velocity ratio; FFM, fatty free mass; GDS, geriatric depression scale; GFR, glomerular filtration rate; Hb, hemoglobin; IADL, instrumental activities of daily living; MDRD, modification of diet in renal disease; MMSE, mini-mental state examination; NA, not applicable; NT-pro BNP, N-terminal probrain natriuretic peptide; PAPs, systolic pulmonary blood pressure; TAPSE, tricuspid annular plane systolic excursion; TBW, total body water; WBC, white blood cell.

T cell activation and flow cytometry

PBMCs from patients with CHF were left untreated or were treated with RvD1 or RvD2 (10 nM; Cayman Chemicals) for 30 min. To allow cytokine synthesis, cells were stimulated with a Dynabeads CD3/CD28 T-Cell Expander (1 bead/cell; Thermo Fisher Scientific) for 8 h. To measure the intracellular cytokine levels, secretion was inhibited by adding 1 μg/ml brefeldin A (MilliporeSigma, Burlington, MA, USA) 5 h before the end of stimulation. At the end of the incubation period, cells were stained at cell surface with e780-conjugated anti-CD3 (eBioscience, San Diego CA, USA), anti-CD4 e780 (eBioscience), anti-CD8 v.450, and Pacific Orange Live/Dead Dye. Cells were then made permeable with Cytofix/Cytoperm reagents (BD Biosciences, San Jose, CA, USA) and stained intracellularly with phycoerythrin-Cy7–conjugated anti–TNF-α (eBioscience), allophycocyanin-conjugated anti–IFN-γ (eBioscience), phycoerythrin-conjugated anti–IL-17 (eBioscience), and anti-PercP5.5–conjugated anti–IL-2 (Biolegend, San Diego, CA, USA) at room temperature for 30 min. Intracellular cytokines were analyzed by flow cytometry (FACS-Cyan ADP; Beckman Coulter, Brea, CA, USA). For each analysis, at least 300,000 events were acquired by gating on Pacific Orange–conjugated Live/Dead negative cells, as reported (20).

Quantitative RT-PCR

Total RNA was extracted with an RNeasy Micro Kit (Qiagen, Germantown, MD, USA). A mixture containing random hexamers, oligo(dT)15 (Promega, Madison WI, USA), and SuperScript II Reverse Transcriptase (Thermo Fisher Scientific) was used for cDNA synthesis. Transcripts were quantified by real-time quantitative PCR on an ABI Prism 7900 sequence detector (Applied Biosystems, Foster City, CA, USA) with predesigned TaqMan Gene Expression Assays (Applied Biosystems) and Absolute QPCR Rox mix (Thermo Fisher Scientific) as reported (20). The following Applied Biosystems probes were used: ALOX-5 (Hs00167536_m1), ALOX-15 (Hs009936765_g1), ALX/FPR2 (Hs02759175_s1), and GPR32 (Hs01102536_s1). For each sample, mRNA abundance was normalized to the amount of ribosomal protein L34 (Hs00241560_m1).

Immunoblotting

PBMCs were lysed with RIPA buffer, and cell homogenates were subjected to 10% SDS-PAGE (50 μg/lane) under reducing conditions. Gels were then electroblotted onto 0.45 μm nitrocellulose filters (Bio-Rad, Hercules, CA, USA) and incubated with primary anti-GPR32 polyclonal mouse antibody (1:500, clone GTX71225; GeneTex, Irvine, CA, USA), anti-ALX/FPR2 monoclonal rabbit antibody (1:500, clone FN-1D6-A1; Genovac, Waukesha, WI, USA), or anti–β-actin monoclonal mouse antibody (1:10,000; Bio-Rad) and then with secondary goat anti-rabbit pAb (1:2000; Santa Cruz Biotechnology, Dallas, TX, USA) for GPR32 and goat anti-mouse pAb (1:2000 for ALX and 1:10,000 for β-actin) as reported (20).

Statistical analysis

All data were expressed as means ± SEM. Differences between groups were compared using Student's *t* test (2 groups) or 1-way ANOVA (multiple groups) followed by a *post hoc* Bonferroni test. The criterion for statistical significance was $P < 0.05$. All statistical analyses were performed with GraphPad Prism (La Jolla, CA, USA). FACS analysis was performed using the FlowJo analysis program (Treestar, Ashland, OR, USA).

RESULTS

Resolution of inflammation is altered in patients with CHF

To test our hypothesis that RvD1 and RvD2 could be involved in the failure of the resolution of chronic inflammation in CHF, we measured the levels of these 2 specific resolvins in plasma of patients with CHF compared with age-matched healthy control subjects. Although both RvD1 and RvD2 were detected in plasma, the levels of RvD1 in patients with CHF were significantly lower than in healthy control subjects (2-fold) (**Fig. 1*A***). However, when stratifying patients with CHF by disease class, we observed that the significant decrease in RvD1 was only evident in NYHA class II and class IV patients, who showed a greater reduction in RvD1 (3-fold), but not in NYHA class III patients (Fig. 1*B*). Variations of RvD2 levels, although showing a similar pattern with RvD1, were not reported in total patients (Fig. 1*C*) or in any of the different NYHA classes (Fig. 1*D*). Neither RvD1 nor RvD2 showed a significant association with gender: male and female subjects displayed comparable levels of both resolvins (data not shown).

Because resolvins are generated from DHA through the sequential activity of 2 different forms of lipoxygenase (LOX), namely 15-LOX and 5-LOX (Fig. 1*E*), we asked whether such altered production of RvD1 was associated with the inability of peripheral blood leukocytes to express these biosynthetic enzymes. We found that patients with CHF showed a marked and significant 5-fold down-regulation of the upstream 15-LOX (Fig. 1*F*) and a slight, yet not significant, up-regulation of downstream 5-LOX (Fig. 1H). Stratification of patients showed that the reduction of 15-LOX expression was dependent on disease severity, with NYHA class IV patients showing the lowest 15-LOX expression levels (Fig. 1*G*). On the contrary, the slight up-regulation observed for 5-LOX was attributable only to NYHA class IV patients, who showed a 2.5-fold significant increase; NYHA class II and III patients showed no variation (Fig. 1*I*). These results suggest that patients with CHF have impaired production of RvD1 and that this might be due to defective biosynthesis. Furthermore, because the production of resolvins is indicative of an ability to efficaciously resolve inflammation and to restore tissue homeostasis, we investigated the relationship between the plasma concentration of RvD1 and RvD2 and several clinical parameters of CHF associated with both inflammation and heart function. Surprisingly, we did not find any significant association between resolvin levels and inflammatory markers, such as N-terminal probrain natriuretic peptide and C-reactive protein or markers of heart function, such as ejection fraction, systolic pulmonary blood pressure, and tricuspid annular plane systolic excursion (**Table 2**). However, we found that RvD1 levels positively correlated with pO_2 and negatively correlated with pCO_2 (Fig. 1*J*), suggesting that a dysfunction of this lipid mediator is associated with inadequate gas exchanges in patients with CHF.

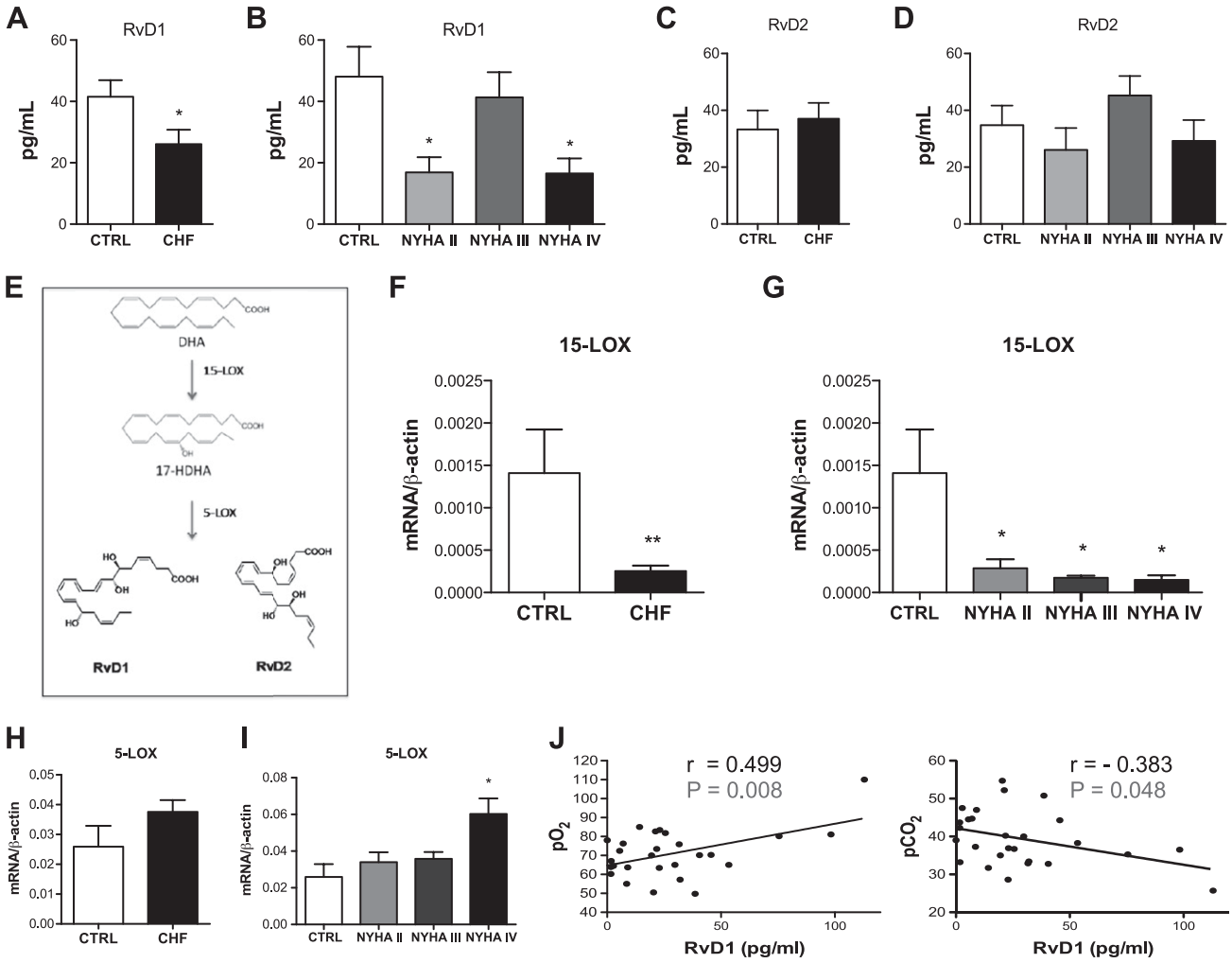

Figure 1. Plasma levels of RvD1 and RvD2 and their biosynthetic enzymes in leukocytes of patients with CHF and healthy control (ctrl) subjects. *A–D*) Quantification of RvD1 and RvD2 in plasma of healthy (ctrl) subjects and patients with CHF (total patients and stratified according to NYHA classes II–IV). Data are shown as means ± SEM of 23 ctrl and 27 CHF independent experiments. $*P < 0.05$ *vs.* ctrl by Student's *t* test or 1-way ANOVA. *E*) Schematic representation of RvD1 and RvD2 biosynthetic pathway. *F–I*) mRNA expression by quantitative RT-PCR of 15-LOX and 5-LOX in leukocytes of healthy (ctrl) subjects and patients with CHF (total patients and stratified according to NYHA classes II–IV). Data are shown as means ± SEM of 10 (ctrl) and 24 (CHF) independent experiments. $*P < 0.05$ (1-way ANOVA), $**P < 0.01$ (Student's *t* test). *J*) Correlation between RvD1 levels and percentage change of pO_2 or pCO_2 in patients with CHF ($n = 27$). Data were compared by Pearson's correlation coefficient ($P < 0.05$).

CD8$^+$ and CD4$^+$ T cells of patients with CHF are unresponsive to RvD1 and RvD2

Recent evidence suggests that T cells are critically involved in the pathogenesis and progression of CHF (4, 11). Given the physiologic role of resolvins in modulating T cells (20) and the observed alteration of the RvD1 pathway in plasma and leukocytes of patients with CHF, we performed pharmacological treatment of adaptive immune cells with these proresolving lipids. We investigated the potential response of CD8$^+$ and CD4$^+$ T cells of patients with CHF to RvD1 and RvD2 (**Fig. 2*A***). Both T cell subsets of patients with CHF, when activated with anti-CD3 and anti-CD28, a specific and physiologic polyclonal stimulus of the T-cell receptor, produced significantly higher amounts of intracellular cytokines compared with unstimulated cells (Fig. 2*B*, *C*). In particular, cytotoxic CD8$^+$ T cells produced higher levels of TNF-α, IFN-γ, and IL-2 (Fig. 2*B*), whereas CD4$^+$ T cells produced higher levels of TNF-α, IL-2, IFN-γ, and IL-17 (Fig. 2*C*). Surprisingly, the pharmacological pretreatment of both activated T-cell subsets with RvD1 or RvD2 had no effect on their cytokine production, with the levels of TNF-α, IFN-γ, and IL-2 from CD8$^+$ and of TNF-α, IFN-γ, IL-2, and IL-17 from CD4$^+$ cells being almost identical to those of untreated cells (Fig. 2*B*, *C*). Next, to rule out the possibility that T-cell responsiveness to resolvins could be associated with CHF severity, we stratified patients with CHF according to their NYHA class and analyzed their cytokine production. Our results showed that CD8$^+$ and CD4$^+$ T cells of NYHA class II (Supplemental Fig. 1), NYHA class III (Supplemental Fig. 2), and NYHA class IV (Supplemental Fig. 3) were unresponsive to either RvD1 or RvD2, suggesting that these proresolving mediators are completely inefficient at reducing T-cell inflammatory responses in patients with CHF independently of their level of severity.

TABLE 2. *Correlations between resolvin levels and clinical parameters*

Parameter	RvD1	RvD2
Age (yr)	0.119 (0.553)	0.23 (0.248)
BMI	−0.219 (0.293)	−0.291 (0.159)
FFM index	−0.118 (0.576)	−0.126 (0.549)
pH	−0.112 (0.579)	−0.341 (0.082)
CRP	−0.171 (0.424)	−0.294 (0.163)
GFR (MDRD)	0.058 (0.777)	−0.022 (0.917)
GFR (CKD-EPI)	0.093 (0.65)	−0.035 (0.864)
NT-proBNP	−0.017 (0.933)	0.013 (0.952)
EF	0.119 (0.597)	0.209 (0.35)
TAPSE	0.362 (0.116)	0.23 (0.329)
PAPs	−0.324 (0.151)	0.031 (0.893)
E/E′	0.243 (0.403)	0.028 (0.923)

Values are Pearson's correlation coefficients with *P* values in parentheses. BMI, body mass index; CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration; CRP, C-reactive protein; *E/E′*, early filling and early diastolic mitral annular velocity ratio; EF, ejection fraction; FFM, fatty free mass; GFR, glomerular filtration rate; MDRD, Modification of Diet in Renal Disease; NT-pro BNP, N-terminal pro-brain natriuretic peptide; PAPs, systolic pulmonary blood pressure; TAPSE, tricuspid annular plane systolic excursion.

Patients with CHF show defects in the RvD1 proresolution pathway

Having observed a defective production of RvD1 in patients with CHF, we next sought to verify the molecular nature underlying the inefficacy of their T-cell subsets to respond to this lipid mediator. Accordingly, we analyzed the expression of both GPR32 and ALX/FPR2, the only known receptors for RvD1, at mRNA and protein levels in leukocytes of healthy (control) subjects and patients with CHF (**Fig. 3**). Quantitative RT-PCR analysis showed that both receptors were present and almost equally expressed in healthy subjects. Only the levels of GPR32 expression were significantly reduced in patients with CHF compared with control subjects, whereas those of ALX/FPR2 were only slightly reduced (Fig. 3*A*, *D*). When stratifying patients with CHF, we observed that such a significant GPR32 mRNA reduction was associated with disease severity, with NYHA IV patients displaying the lowest expression (Fig. 3*B*). In contrast, a reduction in ALX/FPR2 expression was more evident, yet not significant, in both NYHA II and NYHA IV patients (Fig. 3*E*). Furthermore, because mRNA expression is not indicative of the actual presence of a molecular target, we confirmed the protein expression of GPR32 and ALX/FPR2. Immunoblotting analysis not demonstrated the presence of both proteins in control subjects and patients with CHF and partly confirmed our mRNA data. In particular, all NYHA patients showed a significant reduction of GPR32 expression compared with control subjects, with class II and III patients showing the greatest reduction in protein level (Fig. 3*C*), whereas the expression ALX/FPR2 showed no significant variation compared with controls or between different NYHA classes (Fig. 3*F*). These results suggest that the unresponsiveness of T lymphocytes to the immunomodulatory activity of RvD1 might be due to a reduced expression of their specific receptors, mainly GPR32, as schematized in Fig. 3*G*.

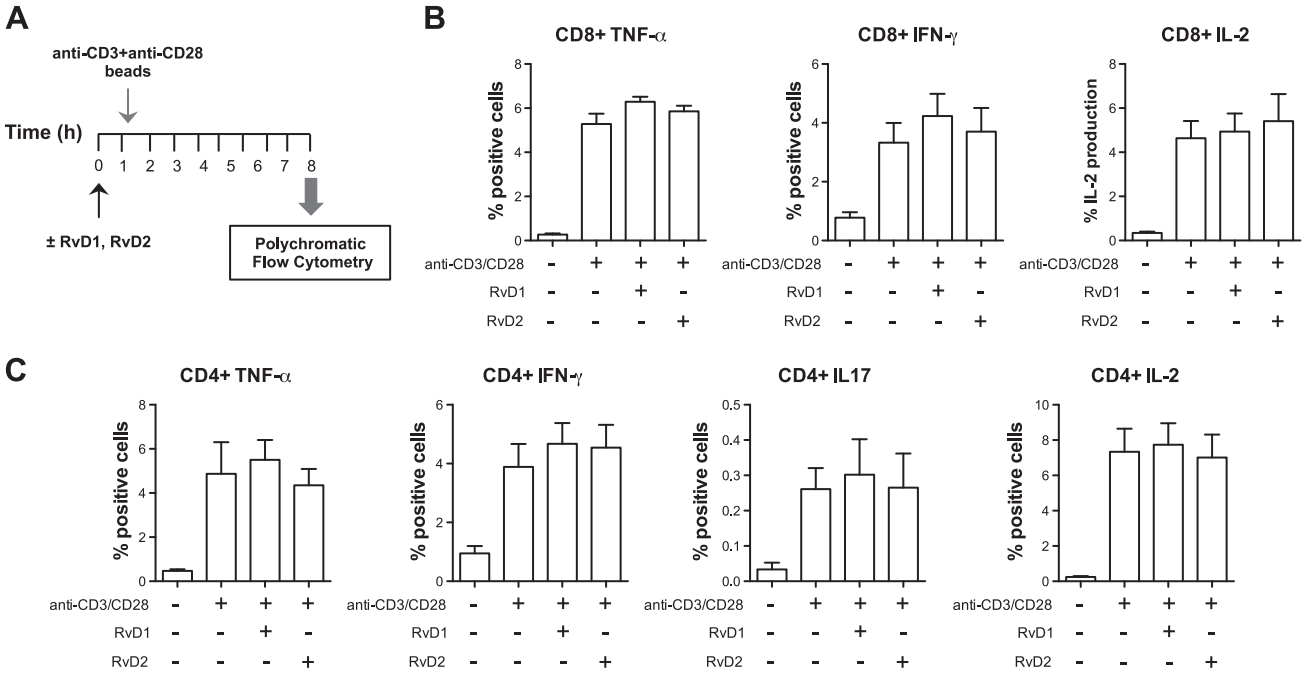

Figure 2. CD8$^+$ and CD4$^+$ T-cell responsiveness to RvD1 and RvD2 in patients with CHF. *A*) PBMCs (1×10^6 cells/well) were left untreated or treated with 10 nM of RvD1 or RvD2 for 30 min. Cells were then stimulated with a Dynabeads CD3/CD28 T-Cell Expander for 8 h, stained at the cell surface and intracellularly, and analyzed by flow cytometry. *B*) Percentages of intracellular production of TNF-α, IFN-γ, and IL-2 from CD8$^+$ T cells. *C*) Percentages of intracellular production of TNF-α, IFN-γ, IL-17, and IL-2 from CD4$^+$ T cells. Data are shown as means ± SEM of 14 independent experiments. $P < 0.05$ by 1-way ANOVA.

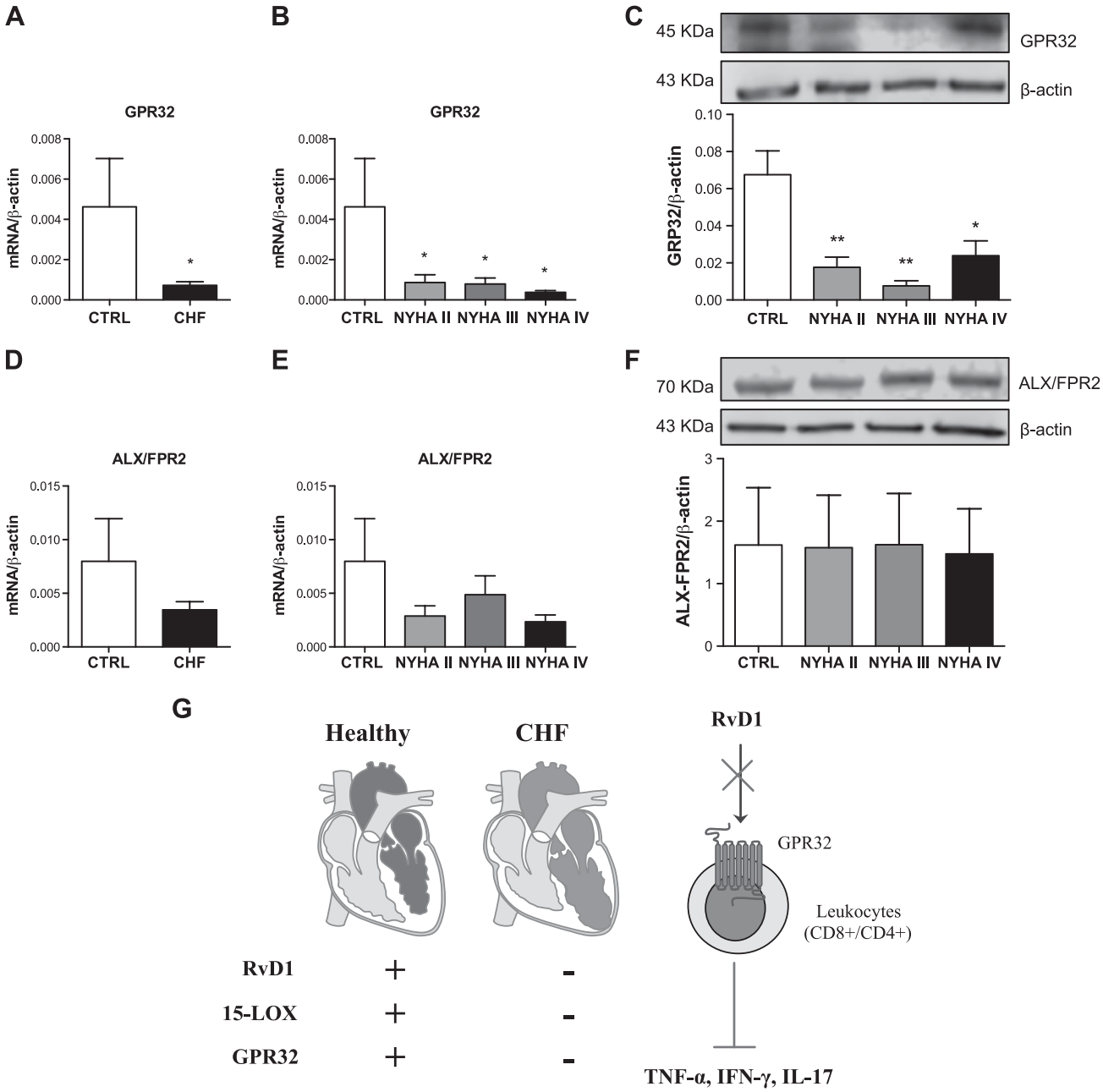

Figure 3. Expression of RvD1 receptors in leukocytes from patients with CHF and healthy control (ctrl) subjects. *A*, *B*, *D*, *E*) mRNA expression by quantitative RT-PCR of GPR32 and ALX/FPR2 in leukocytes of healthy (ctrl) subjects and patients with CHF (total patients and stratified according to NYHA classes II–IV). Data are shown as means ± SEM of 6 (ctrl) and 16 (CHF) independent experiments. $*P < 0.05$ *vs.* ctrl by Student's *t* test or 1-way ANOVA. *C*, *F*) Immunoblotting of GPR32 and ALX/FPR2 in leukocytes of healthy (ctrl) subjects and patients with CHF (total patients and stratified according to NYHA classes II–IV). Data are shown as means ± SEM of 5 independent experiments. $*P < 0.05$, $**P < 0.01$ *vs.* ctrl by 1-way ANOVA. *G*) Schematic representation of alteration of resolution of inflammation in CHF.

DISCUSSION

In recent years many studies have shown that resolvins are able to control the transition from acute to chronic inflammation and that unresolved inflammation plays a pivotal role in the development of several chronic conditions, including cardiovascular diseases (16–19). Indeed, inflammation not only contributes to the impairment of cardiac performance and to the reduction of systolic output by promoting endothelial dysfunction and thromboembolic phenomena but is also essential for the propagation and magnification of the overall immune response (21, 22). Attempts to determine the immune component of CHF and to target specific cytokines yielded poor results in clinical trials (3, 23); therefore, a novel approach in counteracting inflammation is needed. In the present study, we have reported the existence of an alteration in the pathway of resolution of inflammation in CHF in terms of production of specific proresolving lipid mediators and lack of responsiveness of adaptive immune cell populations that are actively involved in the chronic inflammatory processes underlying CHF pathophysiology.

Our finding that the marked decrease of RvD1 plasma levels in patients with CHF compared with healthy subjects suggests a defect in the biosynthetic pathway of this resolvin. This decrease, which is mainly observed in NYHA II and IV classes, is also associated with a reduction in the expression levels of 15-LOX, the limiting enzyme responsible for resolvin biosynthesis. This finding is consistent with results from a recent study showing that levels of lipoxin (LX)A4, another proresolving lipid mediator generated by 15-LOX activity, has been found to be depressed in plasma of patients with CHF in all NYHA classes (24). In the latest study, the decrease in LXA4 levels occurred consistently with the increase of CHF severity, whereas in our study class III patients showed a reestablishment in RvD1 levels, accounting for a compensatory mechanism during this phase of the disease. Nevertheless, the key role of LXA4 biosynthetic enzyme has been supported by a recent study reporting that the inhibition of 15-LOX in rats attenuated an RvD1-dependent cardioprotective effect (25).

The direct correlation of RvD1 serum level with PaO_2 and the inverse correlation with $PaCO_2$ suggest a role for RvD1 as a marker of CHF severity. Indeed, abnormal gas exchanges are the hallmark of the pulmonary damage, which is variably related to fluid overload and fibrotic changes in CHF (26). Thus, it is unlikely that hypoxemia and hypercapnia, which deeply affect cell metabolism (27), are causally related to the depressed RvD1 serum level; it is more likely that they reflect CHF severity. However, the possibility of concurrent effects of low tissue perfusion and hypoxia/hypercapnia on cell metabolism cannot be excluded.

The activity of RvD1 in cardiovascular diseases has been reported in recent studies in animal models, where RvD1 has been shown to improve ventricular function and to delay the onset of CHF after myocardial infarction in mice (18), to reduce atheroprogression and increase efferocytosis in mice (28), to reduce infarct size *via* PI3-K/Akt pathway (26), and to decrease postmyocardial infarct depression in rats (29). In addition, aspirin-triggered RvD1 modulated the immune response and potentially reduced further heart damage in Chagas' heart disease (30). These findings suggest that our observed marked decrease in plasma RvD1 in patients with CHF may significantly contribute to its inefficiency in the syndrome.

Yet, a dysfunctional activity of the adaptive branch of the immunity is arguably of the most recently investigated pathogenic factor of CHF, with both $CD8^+$ and $CD4^+$ T lymphocytes being recruited in the left ventricular endothelium and with a reported alteration of the T_h/regulatory T (T_{reg}) or T_h17/T_{reg} balance toward T_h1 and T_h17. The dysfunction of $CD4^+$-derived T_h cells seems to be related to the type, severity, and prognosis of CHF, suggesting that these cells contribute to tissue damage and disease progression (31, 32). Thus, on the basis of our previous finding that resolvins are able to critically and directly modulate T cells by dampening their inflammatory responses (20), we assessed the response to resolvins of $CD8^+$ and $CD4^+$ T cells of patients with CHF. Our study revealed that, contrary to results seen in healthy subjects, RvD1 and RvD2 are incapable of modulating the immune response of T cells on all the proinflammatory cytokines analyzed (*i.e.*, TNF-α, IFN-γ, and IL-17) and on mitogenic cytokine IL-2. Thus, not only the synthesis but also the proresolving and anti-inflammatory effects of resolvins on T cells seem to be defective in CHF. We hypothesized that this lack of immunomodulatory effect could be due to an impaired T-cell responsiveness caused by a defect in their mechanism of action. This was confirmed by the altered expression in leukocytes of patients with CHF of 1 of the 2 receptors of RvD1 (*i.e.*, GPR32), which progressively decreased with disease severity, at least for the mRNA expression, whereas its protein showed its greatest reduction in NYHA II patients. These findings, coupled with the lack of significant variations in the expression of the other RvD1 receptor (ALX/FPR2), suggest that GPR32 receptor is likely responsible for the unresponsiveness of T lymphocytes to RvD1. Thus, although directly administering this specific lipid or providing a diet rich in its ω-3 fatty acid DHA precursor could potentially reverse the defect of patients with CHF to produce RvD1, this strategy would not prevent the reduced expression of its receptor on leukocytes of patients with CHF.

Limitations of this study deserve consideration. First, although we sized the study according to a well-defined working hypothesis, we cannot exclude the possibility that selected immunologic traits would have required a larger sample to become fully evident. Second, we classified CHF severity according to the universally used and recommended NYHA staging system. However, we are aware of limitations intrinsic to NYHA classifications (33), and this might smoothen the discriminative capacity of the immunologic variables. Finally, we excluded comorbidities likely or known to affect the immune system, some of which, like severe chronic renal failure, are highly prevalent populations with CHF. Accordingly, present data apply to an immunologically pure CHF population and, once confirmed in larger series, should be assessed in the multimorbid patient with CHF.

Overall, this study shows that CHF is associated with impaired levels of RvD1 and that, in chronic inflammatory disease, T cells are unresponsive to the proresolving actions of RvDs, probably due to defects of their molecular target receptors, especially regarding RvD1. These findings suggest that a failure to resolve inflammation could be of pathophysiological importance for CHF and that the proresolution pathway might be a potential candidate to design better treatments for this disease and other cardiovascular diseases, with the aim of reducing chronic inflammation. Because these endogenous mediators lack a cytotoxic effect, new therapies based on resolvins are expected to be well tolerated because devoid of immunosuppression. FJ

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R.A.I. and M.M. share senior authorship. The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

V. Chiurchiù, R. A. Incalzi, and M. Maccarrone designed the research; V. Chiurchiù, A. Leuti, and S. Saracini performed the research and analyzed the data; D. Fontana,

P. Finamore, R. Giua, L. Padovini, and R. A. Incalzi recruited patients and control subjects and performed the clinical analyses; V. Chiurchiù, R. A. Incalzi, and M. Maccarrone contributed with reagents or analytic tools; V. Chiurchiù wrote the paper; and M. Maccarrone and R. A. Incalzi revised the paper.

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