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Original Article

Reg3β from cardiomyocytes regulated macrophage migration, proliferation and functional skewing in experimental autoimmune myocarditis

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Abstract: Macrophages play critical roles in inflammatory initiation, development, resolution and cardiac regeneration of myocarditis. However, Reg3β, as a member of regenerating family of proteins, contributes to dedifferentiation of injury cardiomyocytes as well as cardiac function remodeling. It remains unclear whether Reg3β was associated with macrophages reprogramming during autoimmune myocarditis. Our results showed that Reg3β could effectively recruit macrophages, promoted their proliferation and phagocytosis, and facilitated their polarized into M2 macrophages. Macrophage, especially M1 phenotype contributed to Reg3β production by cardiomyocytes. Our data also indicated that Reg3β was involved in self-protection mechanism following cardiac injury or stress. This suggests that Reg3β might be a critically protective factor of myocardium.

Keywords: Cardiomyocytes, dedifferentiation, macrophage reprogramming, M2 phenotype, phagocytosis, Reg3β

Introduction

The Reg protein family is a group of small secretory proteins and contains Reg1, Reg2, Reg3 and Reg4 based on the primary structures of encoded proteins [1]. Reg3 includes three members: Reg3α, Reg3β, and Reg3γ [2]. Reg3β is also known as human hepatocarcinoma intestine pancreas protein (HIP) or pancreatitis associated protein (PAP), can be upregulated in pancreatic and intestinal tissues during inflammatory disorders [3-5]. Over the past years, it was widely studied in a variety of cells and tissues. Previous investigations had shown that Reg3β, an acute phase secretory protein, act as an anti-inflammatory cytokine during acute pancreatitis [6]. In addition, it can also be detected in intestinal tissues during inflammation. Furthermore, it has been demonstrated that Reg3β is involved in regenerative processes of damaged tissues such as liver regeneration after partial resection [7, 8] neuron nutrition and repair [9]. Due to its diverse physiologic functions, it also has a direct relationship with cancer, such as promoting the transition from chronic pancreatitis to pancreatic cancer [10].

It is widely accepted that macrophages play a crucial role in innate and adaptive immune [11]. The traditional classification of macrophages is divided into two categories, pro-inflammatory (M1) and anti-inflammatory (M2) macrophages [12]. Macrophages exhibit distinct functions both in physiological conditions and during diseases. Accumulated evidence identifies that macrophage can change their function according to their microenvironment to take part in tissue damage and repair [13-15]. Consequently, macrophage, especially alternatively activated macrophages, are considered to benefit for improving tissue inflammation. Conversely, inhibition of inflammatory cell recruitment and proliferation has been suggested as an effective strategy to reduce damage and contribute to maintain tissue homeostasis.

In the present work, we highlight the crosstalk between macrophages and Reg3β in experimental autoimmune myocarditis (EAM). We

demonstrated that Reg3β reprogram macrophage towards M2-like macrophage and facilitated their recruitment. Our data indicated that Reg3β was involved in self-protection mechanism following cardiac injury or stress, suggesting that Reg3β might be a critically protective factor of myocardium.

Materials and methods

Induction of myocarditis

BALB/c Mice were immunized with 100 μg MyHC-$\alpha_{614-629}$ (Ac-SLKLMATLFS TYASAD-OH) according to our protocol [16]. After 21 days, the mice were sacrificed by cervical dislocation. Heart tissues were collected for analysis.

Cardiomyocytes isolation

Mouse cardiomyocytes were isolated and cultured according to previous report [17, 18]. Briefly, Neonatal C57BL/6 mice were immersed in 75% ethyl alcohol and the heart was removed and then cut into pieces. Digest the pieces with digestion solution and gently shaking at 37°C for 10 min and the cell suspension was collected and filtered using a 40-μm cell strainer. The remnant heart tissues were digested with a fresh digestion solution again; the same process is repeated five times. All the cells suspensions were allowed to pellet for about 10 min. The cell pellet was re-suspended in DMEM again after centrifuge at 200 g for 5 min. The cells were seeded on plates pre-plated with 1% gelatin and incubated at 37°C for 1 h. The supernatant containing cardiomyocytes was re-seeded into 24-well plates. After 48 hours, the non-adherent cells were discarded and adherent cells were used to follow-up experiments.

Isolation of $CD11b^{+}Ly6C^{hi}$ cells

Spleens were extracted from mice (6-8 weeks old) under aseptic conditions, cutting and mincing spleens for single cell suspension and grind with a grinding glass, and passing through nylon mesh. Spleen-derived $CD11b^{+}Ly6C^{hi}$ cells were sorted from mice spleen using BD Biosciences FACS AriaII cell sorter.

Western blot

The cells were collected and washed with ice-cold PBS, lysed in lysis buffer on an oscillator for 30 min 4°C. After centrifuge at 14000 g for 10 min, the supernatant was prepared as a protein extract. Equal concentrations of proteins were separated on a 12% sodium dodecyl sulfate-polyacrylamide electrophoretic gel (SDS-PAGE) and were transferred onto a polyvinylidene fluoride membrane (PVDF). The membranes were blocked with 5% BSA for 1 h at room temperature and then incubated with primary antibodies against inducible nitric oxide synthase (iNOS) (Abcam, Shanghai, China), Arg1, Reg3β (R&D Systems, Shanghai, China) and β-actin (Sigma Aldrich, Shanghai, China) overnight at 4°C. The corresponding secondary antibody was incubated for 1 h at 37°C.

Quantitative RT-PCR (RT-qPCR)

Total RNA was extracted from RAW264.7 cells using the TRIzol method and processed as recommended by the manufacturer's specifications, and then reverse transcribed into cDNA. Equal amounts of cDNA were diluted and amplified by real-time PCR using SYBR Green (Bio-Rad). The primers used were as follows: CCR2 forward; 5'-AGGAGCCATACCTGTAAATGC-3'; CCR2 reverse, 5'-GGCAG GATCCAAGCTCCAAT-3'; GAPDH forward, 5'-ACGGCAAATTCAACGCA CAG-3'; and GAPDH reverse, 5'-AGACTCCACGAC-ATACTCAGCAC-3'. CCR2 mRNA levels were normalized to the level of GAPDH mRNA.

Cell proliferation assay

The effect of Reg3β on macrophage proliferation was measured using a Cell Counting kit-8 (CCK8) assay (Beijing Solarbio Science & Te0 chnology Co., Ltd., Beijing, China). 1×10^5 cells/well were seeded in 96-well plates and allowed adhering for 24 h in DMEM without FBS. Cells were treated with different concentrations of Reg3β (0, 50, 100, 200 and 400 ng/ml) for 24 h in DMEM containing 10% FBS (Hyclone, Logan, UT, USA), eight duplicate wells were set up for each cell group, and then incubated with 10 ul CCK-8 reagent for 2 h. Measure the absorbance at 450 nm with a microplate reader.

Transwell assay

For Transwell migration assays, 1×10^5 cells were suspended in 200 μL DMEM and seeded in the upper chamber of 24 wells Transwell chamber with an 8-μm-pore-size polycarbonate

Table 1. Phagocytotic function of macrophages stimulated with Reg3β (n=6)

Group	Treatments	OD540 nm
	Reg3β (ng/ml)	
1	Control	0.9693±0.02430
2	25	1.094±0.02019*
3	50	1.146±0.01633*
4	100	1.387±0.05019**
5	200	1.280±0.03016*
6	400	1.234±0.03605*

Phagocytotic function of macrophages stimulated with Reg3β (*P<005 and **P<0.01 Vs the group treated with physiological saline alone).

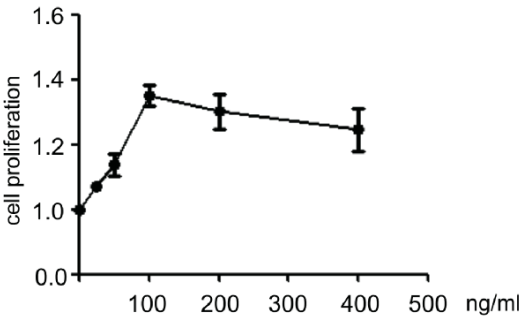

Figure 1. Reg3β promoted proliferation of macrophages. RAW264.7 macrophages were incubation with or without Reg3β for 24 h, cell proliferation was analyzed using CCK8 assay. All the data were obtained from three independent experiments.

filter (Costar, Cambridge, MA). 800 uL complete medium (DMEM contains 10% FBS) with recombinant Reg3β (rReg3β) (R&D Systems, Shanghai, China) was infused to the lower chamber as a chemoattractant. After 24 h, the migrated cells in the lower surfaces were fixed with 4% methanol for 10 min and stained with crystal violet 10 min, then washed three times with PBS. Cells were observed with a microscope (200×) and counted in five randomly selected fields.

Co-culturing system

Co-culture system was carried out in a Transwell system. Firstly, RAW264.7 cells were induced into M1 or M2 by LPS or IL-4, respectively. Then, polarized macrophage cells were seeded in the upper compartments and the isolated cardiomyocytes cells were seeded in the lower compartment. After co-culturing for 24 h, Reg3β levels were detected by western blotting.

ELISA

RAW264.7 cell line or isolated PBMC were cultured in a 24-well plate and treated with Reg3β (100 ng/mL) in DMEM media containing 10% FBS for 24 h at 37°C, respectively. According to the manufacturer's instructions we harvested cell culturing supernatant to detect IL-4 and IL-10 by ELISA kits (MultiSciences (Lianke) Biotech Co., Ltd, Hangzhou, China).

Flow cytometry

Macrophages were cultured in a 24-well plate and treated with Reg3β (100 ng/mL) 24 h at 37°C. Then cells were harvest and samples were re-suspended in PBS and stained with fluorescence-conjugated antibody for 20 minutes at 4°C. Cell populations were washed and re-suspended with fresh PBS. Flow cytometry was used to detect the expression of cell-surface molecules on macrophages.

Macrophage phagocytosis in vitro

4×10^4 macrophages were seeded into a 96-well plate, cells were attached overnight in medium with 10% FBS and then pretreated with Reg3β for 24 h. 100 μL 0.05% Neutral Red were added in every well. After 4 h, the cells were washed third times with PBS. The acicular neutral red crystals should not appear in the field of view. Neutral red was extracted by addition of acid ethanol (containing 50% ethanol, 50% acetic acid). Measure the absorbance at 540 nm.

Statistical analysis

All data were presented as the mean ± standard deviation (SD). Comparisons between groups were performed using the paired t-test or one-way ANOVA with Bonferroni correction. A p value of <0.05 was considered statistically significant.

Results

Reg3β promoted phagocytosis and proliferation of macrophages

The phagocytosis of macrophages plays an important role for immune tolerance, preventing autoimmune and chronic inflammatory disease [19, 20]. Therefore, phagocytosis of macrophage was assessed, as **Table 1** shown. Following Reg3β treatment, the phagocytosis of macrophage was obviously enhanced at 100

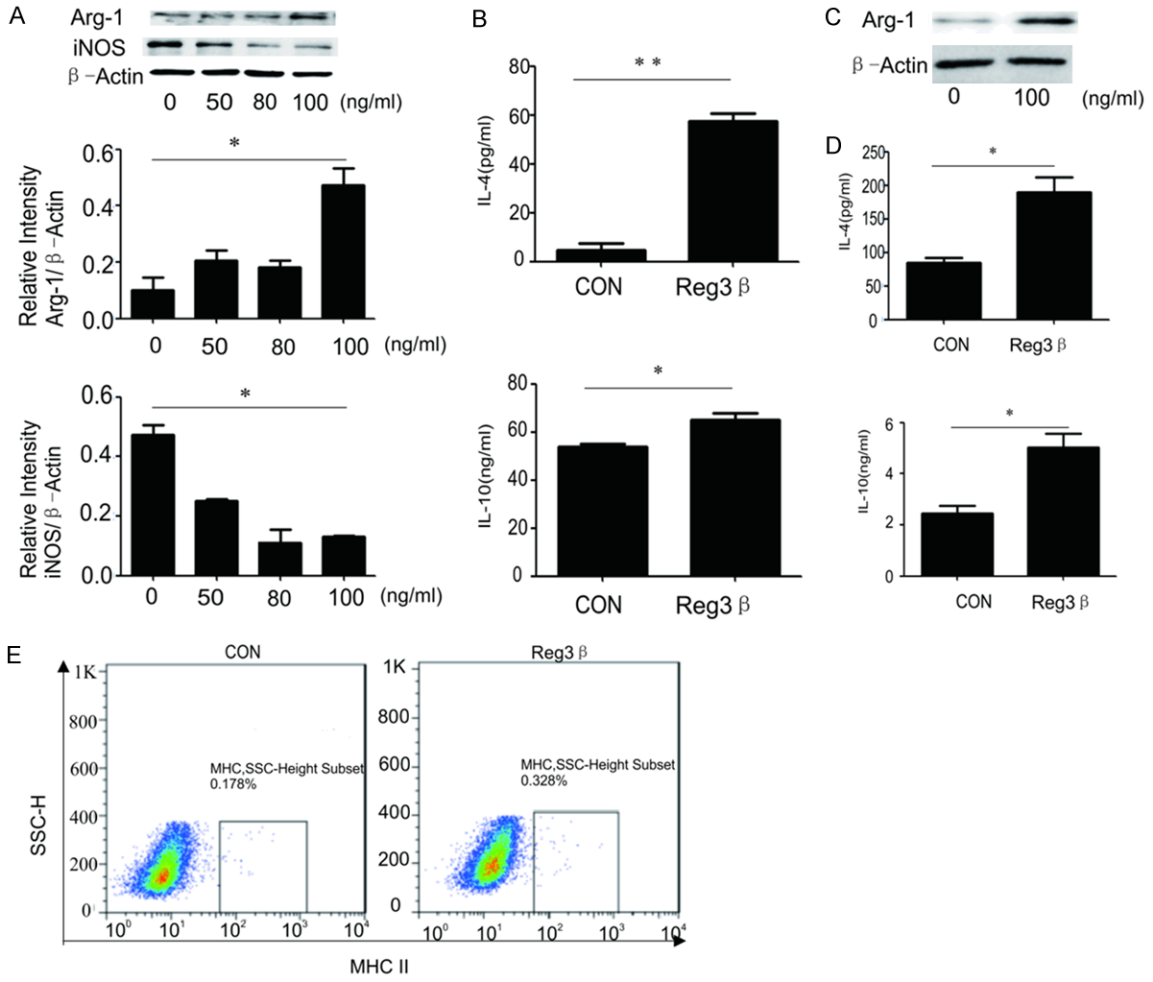

Figure 2. Reg3β could induce macrophage polarization into M2. RAW264.7 cells were treated by 0, 50, 80, 100 ng/mL Reg3β for 24 h, the cells and supernatant were collected used to the next analysis. A. Western blot was used to analysis the expression of iNOS and Arg-1. The upper showed the representative blots and the lower showed the densitometric analysis. B. IL-4 and IL-10 levels in culturing supernatant were assessed by ELISA. C. Mouse primary monocytes were isolated from mouse spleen to analysis the expression of Arg-1 expression after treatment with Reg3β for 24 h by western blot (n=4). D. Data represent IL-4 and IL-10 released by mouse primary monocytes. E. MHC II expression was analyzed by flow cytometry. All the data were obtained from three independent experiments. Quantitative data were mean ± SD. $P<0.05$ was considered statistically significant. *$P<0.05$ and **$P<0.01$. CON means control.

ng/mL. Furthermore, Reg3β also contributed to the proliferation of macrophage (**Figure 1**).

Reg3β could induce macrophage polarization into M2

It is well known that macrophage, a highly heterogeneous population, can polarize into classically activated, proinflammatory M1 and alternatively activated, anti-inflammatory M2 macrophage following different stimulus [21-23]. To determine the effect of Reg3β on macrophage polarization, Reg3β was employed to treat RAW264.7 cells, as **Figure 2A** shown, iNOS expression was obviously down-regulated. However, Arg-1 expression was significantly up-regulated at 100 ng/mL. IL-4 and IL-10 levels in supernatant were 57.60±3.03 pg/mL and 6.51±0.27 ng/mL, respectively. The same phenomenon can be observed in primary monocytes (**Figure 2C**, **2D**). Furthermore, Reg3β couldn't up-regulate MHC II expression as shown in **Figure 2E**. All these data indicated that Reg3β facilitated macrophage polarized into M2 phenotype.

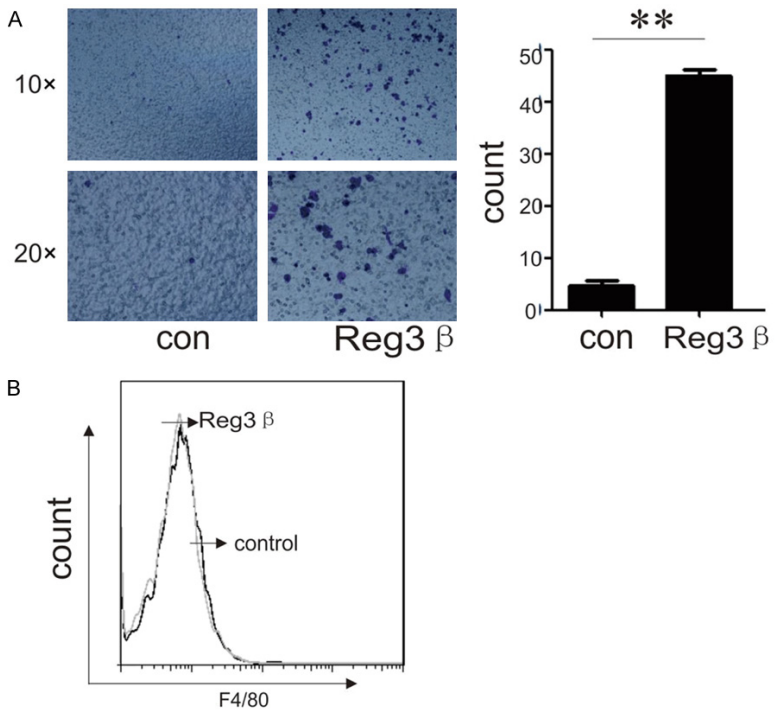

Figure 3. Reg3β promoted macrophages migration *in vitro*. A. RAW264.7 macrophages were co-cultured with or without Reg3β for 24 h, then stained and calculated the cells that migrated to the undersurface of the membrane, scale bar: upper: 10×, lower: 20×. B. Reg3β couldn't up-regulate F4/80 expression. Treatment Ly6C^{hi} cells were isolated from spleen and treated by 100 ng/mL Reg3β for 24 h. F4/80 expression was analyzed by Flow cytometry. All the data were obtained from three independent experiments. CON means control.

Reg3β promoted macrophages migration in vitro

To evaluate the function of Reg3β on monocytes/macrophages recruitment from circulation to inflammatory site, Transwell system was used to measure the macrophage chemtaxis with or without Reg3β stimulus. As **Figure 3A** shown, the number of migrated was significantly increased compared with untreated group (P<0.05). Reported data have shown that inflammatory monocytes CD11b^{+}Ly6C^{hi} cells can be recruited to sites of chronic inflammation [24]. To determine the effects of Reg3β on Ly6c^{hi} monocytes, Ly6c^{hi} monocytes were isolated from mouse spleen. Flow cytometry was used to analysis the phenotype of macrophage F4/80 expression, and there is no difference between Reg3β treated and untreated group (**Figure 3B**).

Reg3β expression was regulated by macrophage

Reg3β can regulate macrophages phenotype and function. And published data also indicated that Reg3β contributed to the dedifferentiation of injury cardiomyocytes [25]. Then we also want to know whether polarized macrophage could contribute to Reg3β production by cardiomyocytes. Firstly, we detected the Reg3β expression in cardiomyocytes, as **Figure 4A** shown, cardiomyocytes didn't express Reg3β. And then we isolated cardiomyocytes from neonatal mouse and co-cultured with polarized M1 and M2 for 24 h, the expression of Reg3β was detected by western blot. As **Figure 4B** shown, macrophage and M1 macrophage both promoted Reg3β expression in cardiomyocytes, however, M2 macrophage couldn't promote Reg3β expression. The Reg3β expression in adult mice heart suffering from autoimmune myocarditis was also detected. As **Figure 4C** shown, Reg3β expression was significantly up-regulated at day 7 and 14, but it was almost undetectable at day 21; which congruent with the inflammatory development and a lot of monocytes/macrophages infiltrated into injury heart and polarized into M1 phenotype at day 7 and 14, however, at day 21, the inflammation was resolved and injury cardiac began remodeling [16, 26]. All these results indicated that macrophage, especially M1 not M2 significantly up-regulated Reg3β production by cardiomyocytes.

Discussion

Reg3β, as an up-regulated protein, was firstly discovered in a rat model of pancreatitis [27] and was later found to promote closure of the epidermis during wound repair [28] and to contribute to neuronal regeneration [9]. However, very little reports are focused on the role of Reg3β in heart disease, despite several data suggest that Reg3β protein was up-regulated in the ischemic heart [25] and Reg2 gene expressions were remarkably increased in myocarditis [29]. The secreted Reg3 contributes to dedif-

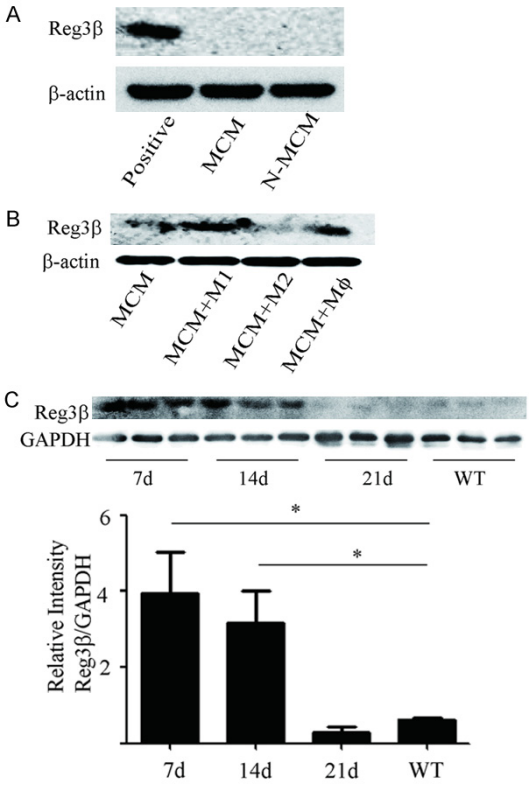

Figure 4. Reg3β expression was regulated by macrophage. A. Reg3β expression in cardiomyocytes. B. M1 macrophage promoted Reg3β expression in cardiomyocytes. 500 ng/mL LPS and 20 ng/mL IL-4 were used to induce M1 and M2, respectively, then polarized macrophages co-cultured with cardiomyocytes. Western blot was used to detect Reg3β expression. C. Western blot analyzed Reg3β expression in heart of myocarditis at day 7, 14, 21. The upper showed the representative blots and the lower showed the densitometric analysis. MCM and N-MCM mean cardiomyocytes cell line and neonatal mouse cardiomyocytes. All the data were obtained from three independent experiments. **P*<0.05 was considered statistically significant.

ferentiation of injury cardiomyocytes as well as cardiac function remodeling [25]. Published data also indicated that macrophages play critical roles in inflammatory initiation, development, resolution and cardiac regeneration of myocarditis [30-33]. It remains unclear whether Reg3β was associated with macrophages reprogramming during myocarditis.

Our results demonstrated that Reg3β could effectively recruit macrophages, promoted their proliferation and phagocytosis, and facilitated their polarized into M2 macrophages, which indicated that Reg3β contributed to myocardial injury repair partially by monocyte/macrophage recruitment and polarize into M2. Furthermore, our data also suggested that macrophage, especially M1 phenotype contributed to Reg3β production by cardiomyocytes. It is well known that proinflammatory M1 phenotype could promote cardiac injury [34], in other words, only injury cardiomyocytes could produce Reg3β. Our speculation was further confirmed *in vivo*, we found that the Reg3β was upregulated during initiation and development stage of myocarditis.

In conclusion, our results demonstrated that Reg3β is an important inducer of macrophage recruitment and macrophage reprogramming toward M2 and we investigated that Reg3β was a self-protection mechanism following cardiac injury or stress, suggesting that Reg3β might be a critically protective factor of myocardium.

Acknowledgements

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Disclosure of conflict of interest

None.

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