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Letters

RESEARCH LETTER

Macrophages Contribute to Cardiac Fibrosis and Diastolic Dysfunction in Systemic Sclerosis

Systemic sclerosis (SSc) is a multisystem autoimmune disease characterized by fibrosis of the skin and internal organs. Mortality is high, with cardiopulmonary complications as the major cause of death.[1] Diastolic dysfunction is independently associated with increased mortality in SSc patients.[1] Little is known about the fundamental basis of cardiac involvement in SSc. Here, we evaluated cardiac pathology and pathogenesis in a murine model of SSc induced by subcutaneous bleomycin.[2]

For human studies, ethics approval was provided by the Cedars-Sinai Institutional Review Board, and all participants provided their written informed consent. Animal studies were approved by the Cedars-Sinai Institutional Animal Care and Use Committee.

Female mice (for clinical relevance and model consistency, C57BL/6N, 5 to 7 weeks old) were randomized to receive subcutaneous 100-μL injections of bleomycin sulfate dissolved in 0.9% NaCl (1 mg/mL) or vehicle (0.9% NaCl) in a single location in the upper back every other day for 3 weeks (n = 5/group). Data are reported as mean ± SD. Groups were compared by means of Student's *t*-test or analysis of variance with Tukey's post hoc correction for multiple pairwise comparisons. A P value <0.05 was considered to be statistically significant.

Mice injected with bleomycin displayed dermal thickening compared with control mice (**Figure 1A**, Supplemental Figure 1A), along with pulmonary fibrosis (greater Ashcroft scores vs controls, $P < 0.001$) (Supplemental Figure 1B) and congestion (Supplemental Figure 1C). Such findings recapitulate previous reports, but left ventricle (LV) involvement had not been described.[2] Echocardiography revealed diastolic dysfunction (**Figure 1B**) with preserved ejection fraction (**Figure 1C**, Supplemental Figure 1D). There was no cardiac hypertrophy (Supplemental Figure 1E), and blood pressure was unchanged (data not shown) . Pathologically, bleomycin-injected mice exhibited diffuse LV fibrosis (Supplemental Figure 1C).

Gene ontology analyses of RNA sequencing on LV samples implicated immune signaling pathways, especially those relevant to antigen presentation, classical and alternative macrophage activation, and macrophage-stimulating protein signaling, all of which were up-regulated in bleomycin-injected animals (Supplemental Figures 1F and 1G). To probe the role of macrophages in SSc-associated heart disease, we depleted macrophages pharmacologically. After a full 3-week course of bleomycin, mice were randomized to receive biweekly intravenous injections of 0.2 mL clodronate liposomes (Liposoma), or phosphate-buffered saline solution liposomes (as placebo control) for 4 additional weeks (n = 5/group) (**Figure 1D**).[3] Immunofluorescence showed increased numbers of CD68+ cells (ie, macrophages) in the LV of placebo-exposed bleomycin-injected mice ($Bleo^{Placebo}$) compared with control mice (**Figure 1E**). C-C motif chemokine receptor 2 was enriched in LV tissue from $Bleo^{Placebo}$ vs control, indicating recruitment of monocyte-derived macrophages (Supplemental Figure 1H). At that point, immunofluorescence analysis confirmed around 50% depletion of LV macrophages in clodronate-exposed bleomycin-injected mice ($Bleo^{Clod}$) compared with $Bleo^{Placebo}$ (**Figure 1E**). As a consequence of macrophage depletion, $Bleo^{Clod}$ exhibited attenuated diastolic dysfunction (**Figure 1F**) and decreased cardiac fibrosis compared with $Bleo^{Placebo}$ (**Figure 1G**). Finally, to probe the role of macrophages in vitro, human cardiac fibroblasts were exposed to concentrated conditioned media (CM) from human SSc or healthy macrophages for 4 days (**Figure 1H**). At endpoint, only cardiac fibroblasts exposed to SSc-macrophage CM demonstrated overt up-regulation of connective tissue growth factor and α-smooth muscle actin (**Figure 1I**). To normalize the secretome output, monocytes were plated (1×10^6 cells/well) and differentiated for 5 days, and protein concentration was measured in CM.

Many SSc patients (18%-60%) show abnormal diastolic function by echocardiography, which correlates

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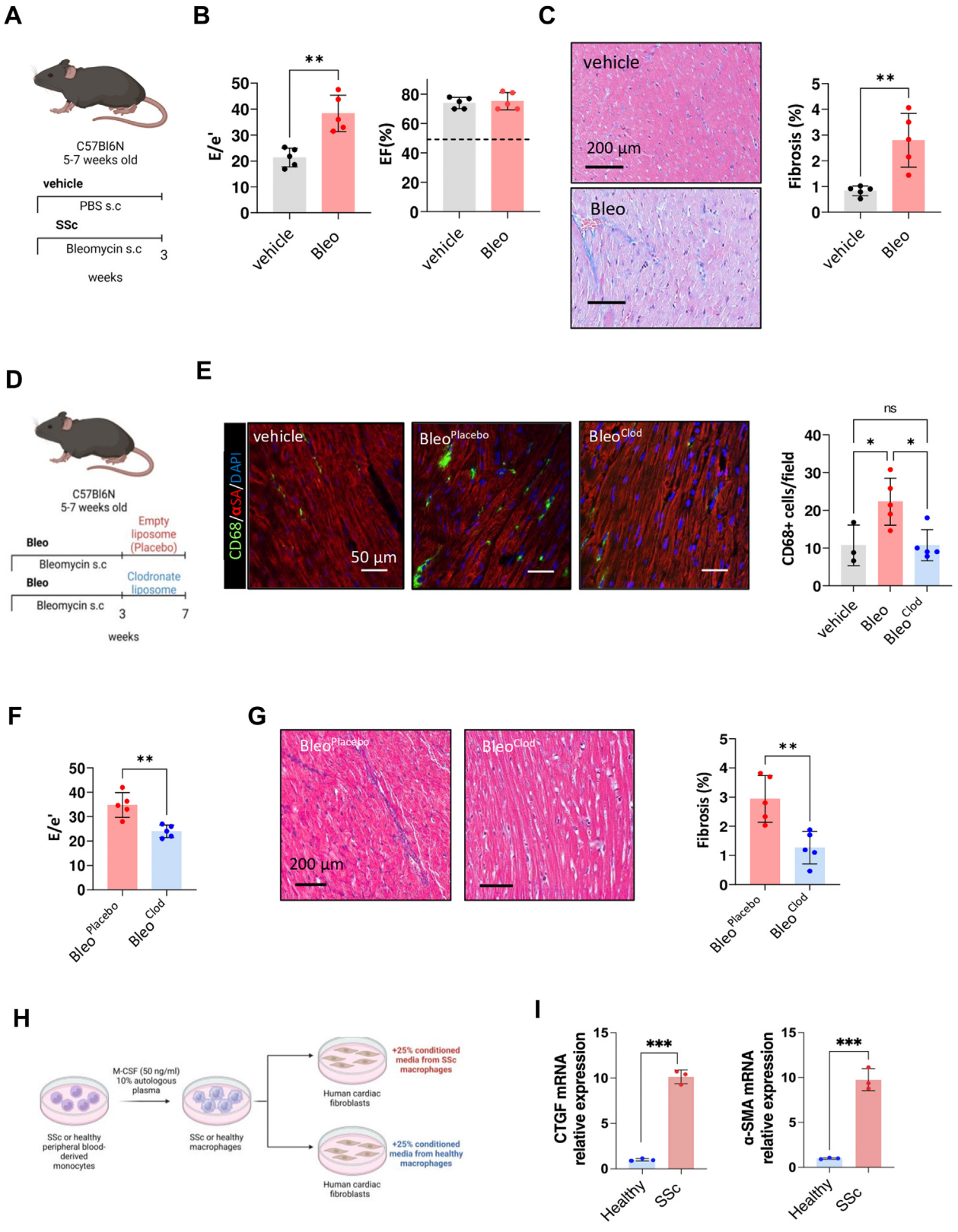
FIGURE 1 Macrophage-Dependent Modulation of Cardiac Systemic Sclerosis



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with higher mortality.[1] Interstitial myocardial fibrosis has been recognized as a hallmark of SSc cardiac involvement, which is thought to occur because of coronary microvascular dysfunction and/or myocardial inflammation.[4] Circulating monocytes and infiltrating macrophages are increased in SSc patients and have been shown to be pathogenic.[5] However, their role in LV dysfunction was previously unexplored. Here, we found an enrichment of immune pathways at the transcriptomic tissue level, and a higher number of macrophages in cardiac tissue, after bleomycin injection. Furthermore, macrophage depletion improved cardiac function and structure, whereas cardiac fibroblasts exposed to SSc macrophage-conditioned media resulted in profibrotic activation. Our observations motivate further studies on immune-mediated cardiac phenotypes in SSc and macrophages as therapeutic targets.

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The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the Author Center.

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APPENDIX For supplemental methods, tables, and figure, please see the online version of this paper.

FIGURE 1 Continued

Cardiac manifestations in bleomycin-induced scleroderma model. (A) Study scheme for the bleomycin-induced model of systemic sclerosis (n = 5 animals/group). (B) Echocardiographic ratio of transmitral Doppler early filling velocity to tissue Doppler early diastolic mitral annular velocity (E/e′), and ejection fraction (EF). (C) Representative Masson trichrome cardiac images and pooled analysis of % fibrosis. (D) Study scheme for macrophage depletion studies in bleomycin-injected mice (separate cohort; n = 5 animals/group). (E) Representative CD68-immunostained images of macrophages in left ventricular tissue of bleomycin-injected and vehicle-injected from study A and clodronate-exposed bleomycin-injected hearts, and pooled data. (F) Pooled data for ratio of transmitral Doppler early filling velocity to tissue Doppler early diastolic mitral annular velocity (E/e′). (G) Representative Masson trichrome cardiac images and pooled analysis of % fibrosis and vehicle-injected hearts from study A. (H) Study scheme for the cardiac fibroblast challenge study in vitro. (I) Pooled data for relative mRNA expression of connective tissue growth factor and α-smooth muscle actin (SMA) genes according to quantitative polymerase chain reaction. Data are reported as mean ± SD. Statistical analysis by means of Student's *t*-test or repeated-measures analysis of variance (ANOVA) (E) followed by Tukey post hoc test with 95% CI: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Bleo = bleomycin; Clod = clodronate; CSF = colony-stimulating factor; PBS = phosphate-buffered saline solution; SSc = systemic sclerosis.