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Targeting CD13/Aminopeptidase N as a Novel Therapeutic Approach for Scleroderma Fibrosis

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Objective. Systemic sclerosis (SSc) is an autoimmune multisystem disease with poorly understood pathogenesis and ineffective treatment options. Soluble CD13 (sCD13), generated by the cleavage of cell surface CD13 via matrix metalloproteinase 14 (MMP14), signals through the bradykinin receptor B1 (B1R) to elicit pro-inflammatory, pro-arthritic, and pro-angiogenic responses. In this study, we explored the antifibrotic potential of targeting the sCD13-B1R axis in SSc.

Methods. The expression of CD13, B1R, and MMP14 was examined in SSc skin and explanted dermal fibroblasts. The efficacy of B1R antagonists in the inhibition on fibrosis was determined in vitro and in vivo.

Results. Expression of the genes for CD13, B1R, and MMP14 was elevated in skin biopsies from patients with diffuse cutaneous (dc) SSc. Notably, single-cell analysis of SSc skin biopsies revealed the highest *BDKRB1* expression in *COL8A1*-positive myofibroblasts, a population exclusively seen in SSc. Transforming growth factor beta (TGFβ) induced the expression of *BDKRB1* and production of sCD13 by dcSSc skin fibroblasts. Treatment of dcSSc fibroblasts with sCD13 promoted fibrotic gene expression, signaling, cell proliferation, migration, and gel contraction. The pro-fibrotic responses of sCD13 or TGFβ were prevented by a B1R antagonist. Mice lacking *Cd13* or *Bdkrb1* were resistant to bleomycin-induced skin fibrosis and inflammation. Pharmacological B1R inhibition had a comparable anti-fibrotic effect.

Conclusion. These results are the first to demonstrate a key role for sCD13 in SSc skin fibrosis and suggest that targeting the sCD13-B1R signaling axis is a promising novel therapeutic approach for SSc.

INTRODUCTION

Systemic sclerosis (SSc) is a chronic progressive autoimmune disease characterized by microvascular damage and irreversible fibrosis of organs including the skin, lungs, heart, kidney, and gastrointestinal tract.[1] Skin fibrosis, a hallmark of SSc, is correlated with disease severity and poor quality of life. The etiology of SSc is unknown, and currently no effective disease-modifying treatment exists.[2] Methotrexate, cyclophosphamide, mycophenolate, and tocilizumab have shown only limited efficacy along

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with various safety concerns.[3] There is a need to better elucidate the pathogenesis of SSc to develop new, targeted, safe, and effective therapeutic agents.

Aminopeptidase N (CD13) is a ubiquitously expressed metallopeptidase originally described as a myeloid cell marker that functions as an ectoenzyme with multiple substrates.[4,5] Notably, CD13 can be shed from the cell membrane via its cleavage catalyzed by matrix metalloproteinase 14 (MMP14). Soluble CD13 (sCD13) binds to a G-protein–coupled receptor on activated T cells, monocytes, endothelial cells, and fibroblasts and acts as a chemoattractant for monocyte and T cell migration.[6–8] Moreover, CD13 acts on endothelial cells to promote angiogenesis.[8] Very recently, we identified the bradykinin receptor B1 (B1R) as a surface receptor of sCD13 on a variety of cell types and demonstrated that the sCD13/B1R axis plays a pivotal role in inflammatory arthritis.[9] However, the role of CD13 in other inflammatory diseases remains largely unknown.

Previous studies investigated the expression of CD13 in the skin. In particular, prominent CD13 expression was demonstrated on fibroblasts and blood vessels in the papillary dermis in both healthy and SSc skin.[10] Importantly, Dan et al demonstrated elevated CD13 enzymatic activity in both bronchoalveolar lavage fluid and sera from patients with SSc compared with healthy controls.[11] Moreover, it was shown that blocking CD13 using monoclonal anti-CD13 antibodies inhibited skin fibroblast migration.[12]

We have previously demonstrated that sCD13 signals by binding to a bradykinin receptor.[9] Interestingly, an earlier study showed reduced levels of bradykinin receptors in SSc skin fibroblasts compared with healthy fibroblasts,[13] associated with impaired bradykinin-induced Ca^{2+} release and defective formation of inositol 1,4,5-trisphosphate.[14,15] Despite these intriguing observations, the role of sCD13 and B1R in SSc remains unknown. Notably, inhibiting CD13 using actinonin was shown to reduce chemokine secretion in the lung and mitigate silica-induced lung fibrosis.[16] Moreover, deletion or inactivation of B1R in mice reduced the severity of kidney fibrosis.[17–19] Taken together, these studies suggest that the sCD13/B1R signaling axis might play a pathogenic role in SSc, and its targeting might have the potential to treat SSc fibrosis. In this study, we sought to assess the role of the sCD13-B1R axis in SSc fibrosis, focusing on patient-derived dermal fibroblasts and a mouse model of skin fibrosis. Our results provide compelling evidence for a previously unsuspected pathogenic role for CD13 in SSc and identify the sCD13-B1R axis as a novel target for therapy.

MATERIALS AND METHODS

Animal model, histology, and analysis. All animal protocols were approved by the Institutional Animal Care & Use Committee at the University of Michigan. The *Cd13* and *Bdkrb1* knockout mice were obtained and bred as described in Tsou et al.[9] The bleomycin skin fibrosis model was performed as previously described.[20,21] Briefly, skin fibrosis in both C57BL/6 mice (Jackson Laboratory) and *Cd13* or *Bdkrb1* knockout mice was induced by intracutaneous injection of bleomycin (0.5 mg/mL in phosphate buffered saline [PBS], Hospira) in a defined area on the upper back daily for 2 weeks. PBS injections were used as negative controls. In experiments evaluating the antifibrotic properties of the B1R inhibitor SSR240612 in C57BL/6 mice, one group of mice received intracutaneous PBS injections alone, one group was challenged with bleomycin via intracutaneous injections on the back in conjunction with intraperitoneal (IP) injections of SSR240612 (2 mg/kg, Cayman Chemical), and the third group received bleomycin injections with IP injections of vehicle control (2.2% DMSO/97.8% PBS). After 2 weeks, skin tissues from the defined areas were harvested for histology, hydroxyproline analysis, and messenger RNA (mRNA) extraction. To evaluate the effect of B1R blockade in established fibrosis, in a separate experiment, fibrosis in C57BL/6 mice was induced first by repeated intracutaneous injections of bleomycin for 2 weeks followed by IP injections of SSR240612 (2 mg/kg) for another 2 weeks.

For histology, paraffin-embedded tissues were stained with hemtoxylin and eosin and Masson's trichrome. Dermal thickness was determined using ImageJ.[22] To measure monocytes and macrophages in the skin, immunofluorescence was performed after antigen retrieval using rat antimouse F4/80 (Thermo Fisher Scientific) antibody followed by Alexa Fluor 488–tagged antibodies (Thermo Fisher Scientific). The quantification of F4/80-positive cells was done using ImageJ. To measure hydroxyproline content in the skin, weighed skin sections were hydrolyzed in concentrated HCl at 120°C for 3 hours. A hydroxyproline analysis kit (Abcam) was then used to measure hydroxyproline. The resultant absorbance at 560 nm was assayed using a Synergy HT microplate reader. Tissue weight was used to normalize the hydroxyproline values, which are presented as μg/mg tissue weight.

Human subjects. All protocols involving human subjects were approved by the University of Michigan Institutional Review Board and performed upon obtaining written informed consents. Patients fulfilling the American College of Rheumatology classification for SSc[23] and age-, sex-, and ethnicity-matched healthy controls were recruited. Patient characteristics are summarized in Supplementary Table 1.

Cell culture. Punch biopsies were obtained from the dorsal forearm from all enrolled subjects. Dermal fibroblasts were isolated from skin biopsies from healthy volunteers and patients with diffuse cutaneous (dc) SSc as previously described.[20,21] Isolated dermal fibroblasts were cultured in RPMI1640 supplemented with 10% fetal bovine serum (FBS), L-glutamine, and antibiotics. Cells between passages 3 and 6 were used in all experiments. In some experiments, fibroblasts from healthy subjects were treated with 10 ng/mL transforming growth factor (TGF) β1 (Cell Signaling) for up to 72 hours in RPMI1640 with 1% FBS. Dermal fibroblasts from

patients with dcSSc were treated with various concentrations of sCD13 (R&D Systems) for 72 hours in the presence or absence of B1R inhibitor SSR240612 (Cayman Chemical). For signaling experiments, healthy or dcSSc dermal fibroblasts were serum starved overnight in RPMI1640 media with 1% FBS then incubated with 10 ng/mL TGFβ1 for 60 min, or 500 ng/mL of sCD13 for 25 min, with or without pretreatment of the cells with B1R inhibitor SSR240612 for 60 min. Knockdown of MMP14 or B1R in dermal fibroblasts was done by Accell siRNA reagents from Horizon Discovery (Cambridge, United Kingdom).

GEO, RNA, and single-cell transcriptome analysis. To determine the expression of the genes of interest, publicly available data sets (GEO GSE58095 and GSE130955) generated from skin biopsies of healthy controls and patients with SSc were analyzed. To quantitatively evaluate the expression of the genes of interest, real-time polymerase chain reaction was performed, as described.[24]

To examine the expression of ANPEP (gene encoding CD13), MMP14, and BDKRB1 at a single-cell scale, single-cell RNA sequencing (RNA-seq) results previously generated from skin biopsies from 18 healthy controls and 22 patients with early dcSSc[25,26] were analyzed.

Immunolabelling and immunofluorescence. After treatments, fibroblasts were fixed in formalin and blocked. The cells were then immunolabelled with antibodies to p-ERK1/2 (Cell Signaling Technology 4376S; clone 20G11) or phosphorylated SMAD2 (Cell Signaling Technology 18338S; clone E8F3R) followed by Alexa Fluor antibodies (Thermo Fisher Scientific). The slides were mounted with antifade mounting medium with DAPI (Thermo Fisher Scientific) and visualized under an Olympus microscope (Olympus, Tokyo, Japan).

Western blotting. Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis then transferred onto a nitrocellulose membrane. The blots were probed with antibodies against B1R (LS Bio; clone 3A2), collagen I (Abcam ab138492; clone EPR7785), or α smooth muscle actin (SMA) (Abcam ab5694). Antibodies against β-actin (Sigma-Aldrich A2066) or GAPDH (Cell Signaling Technology 2118; clone 14C10) were used as loading controls.

Myofibroblast functional analysis and sCD13 quantification. To evaluate the myofibroblast phenotype of dcSSc fibroblasts, the following endpoints were examined: cell proliferation, migration, and gel contraction, as previously described.[20] The sCD13 in culture media was quantified using the Human Aminopeptidase N/CD13 DuoSet Enzyme-Linked Immunosorbent Assay (R&D Systems).

Skin immunolabelling. Skin biopsies from patients with dcSSc and healthy volunteers were embedded in OCT compound, cryosectioned, and immunolabelled using either anti-human B1R (LSBio clone 3A2), anti–α-SMA (Abcam ab5694), anti-COL8A1 antibody (LSBio), or purified IgG (Thermo Fisher Scientific). Cryosections were incubated with Alexa Fluor secondary antibodies (Thermo Fisher Scientific) for 1 hour at 37°C. Sections were mounted with antifade mounting medium with DAPI (Thermo Fisher Scientific) and visualized under an Olympus microscope (Olympus, Tokyo, Japan).[9]

Statistical analysis. A normality test was first performed to determine the distribution of the data. To determine the differences between groups, Student's 2-tailed *t*-test, Mann-Whitney test, Kruskal-Wallis test, one-way analysis of variance, or two-way analysis of variance were performed using GraphPad Prism version 10 (GraphPad Software, Inc). The Pearson correlation coefficient was used for correlation analysis for gene expression and modified Rodnan skin score (mRSS) or forced vital capacity (percent predicted). *P* values of <0.05 were considered statistically significant. Unless specified, results were expressed as mean ± SD. The values underlying graphed data and reported means in this study are available from the corresponding authors upon request.

RESULTS

Mice lacking *Cd13* are protected from bleomycin-induced skin fibrosis. To test the effect of CD13 on skin fibrosis, we intracutaneously injected bleomycin or PBS into *Cd13*$^{-/-}$ and sex- and age-matched wildtype mice. After 2 weeks, *Cd13*$^{-/-}$ mice injected with bleomycin had reduced dermal thickness ($P < 0.001$) (Figure 1A and B) and skin hydroxyproline content ($P < 0.01$) (Figure 1B) compared with similarly treated wildtype mice. Because sCD13 induces monocyte migration by binding to B1R,[9] we measured monocyte/macrophage infiltration in the lesional skin. We found that *Cd13*$^{-/-}$ mice injected with bleomycin had lower numbers of F4/80-positive cells in the skin compared with wildtype mice ($P < 0.05$) (Figure 1C and Supplementary Figure 1A). We then investigated the effect of *Cd13* loss on the expression of fibrotic and inflammatory genes. In bleomycin-treated *Cd13*$^{-/-}$ mice, *Col1a1, Col8a1*, *Acta2, and Tgfb1* expression were decreased compared with wildtype mice injected with bleomycin, with *Col1a1, Col8a1*, and *Tgfb1* reaching statistical significance (Figure 1D). Moreover, *Ccl2* and *Il6* expression was significantly elevated in bleomycin-treated wildtype but not in *Cd13*$^{-/-}$ mice.

CD13, MMP14, and B1R are abundantly expressed in the skin. To characterize the sCD13 signaling axis in SSc, we analyzed a transcriptome data set (GEO GSE58095)[27] generated from skin biopsies. We found significantly higher expression levels of *ANPEP* ($P < 0.01$) and *MMP14* ($P < 0.05$) in SSc compared with healthy skin biopsies (Figure 2A). Furthermore, the expression of both *ANPEP* and *MMP14* significantly correlated with

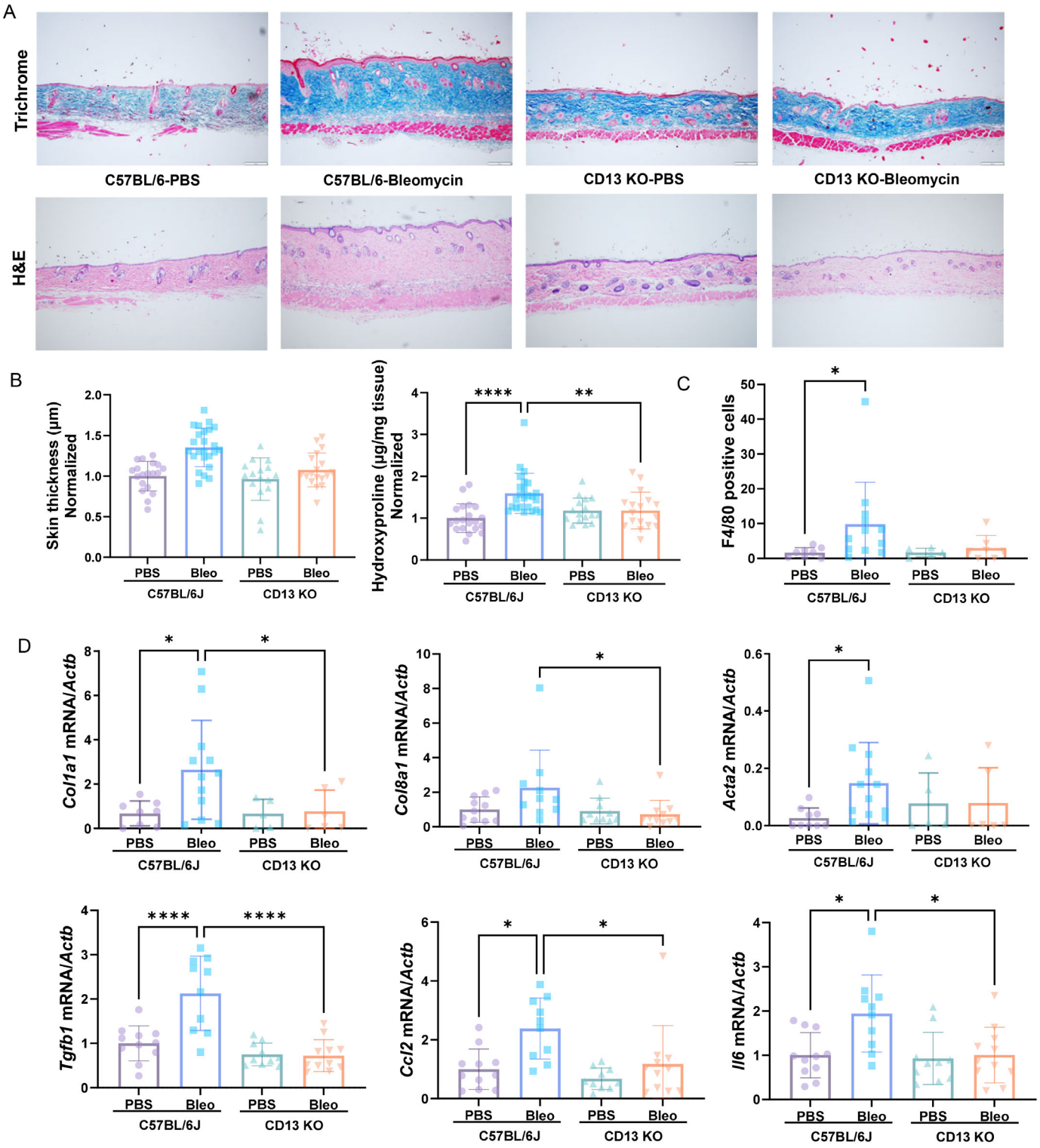

Figure 1. *Cd13* knockout mice are protected from bleomycin-induced skin fibrosis. (A and B) *Cd13*$^{-/-}$ mice injected with bleomycin showed less skin thickness than wildtype C57BL/6 mice injected with bleomycin ($P < 0.001$). Hydroxyproline content in skin tissues was significantly reduced in bleomycin-injected *Cd13*$^{-/-}$ mice compared with bleomycin-injected wildtype mice ($P < 0.01$). Original magnification ×10. (C) Significantly higher number of F4/80-positive monocytes/macrophages were present in skin sections of bleomycin-injected wildtype mice compared with PBS-injected mice ($P < 0.05$); this difference was not observed in *Cd13*$^{-/-}$ mice. (D) Bleomycin induced significant upregulation of fibrotic and pro-inflammatory genes in wildtype mice ($P < 0.05$), whereas in *Cd13*$^{-/-}$ mice bleomycin failed to increase these genes. Results are expressed as mean ± SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. Significance was determined by (B-D) one-way analysis of variance or (D) Kruskal-Wallis test. Bleo, bleomycin; H&E, hemtoxylin and eosin; KO, knockout; mRNA, messenger RNA; PBS, phosphate buffered saline.

mRSS (Figure 2B). In contrast, *BDKRB1* expression was comparable between healthy and dcSSc skin biopsies and showed no significant correlation with mRSS (Figure 2A and B). In an independent SSc skin biopsy data set (GEO GSE130955),[28] we observed significant elevation of *MMP14* and *BDKRB1* expression in patients with SSc compared with healthy controls (Supplementary Figure 2A). However, the expression levels for the three genes examined did not corelate with mRSS. We noted that *BDKRB1* expression levels trended with mRSS at the site of the biopsy (Supplementary Figure 2B). Importantly, expression levels significantly correlated with forced vital capacity (percentage predicted).

To gain further insights into cell type–specific gene expression, single-cell analysis on skin biopsies from 22 patients with

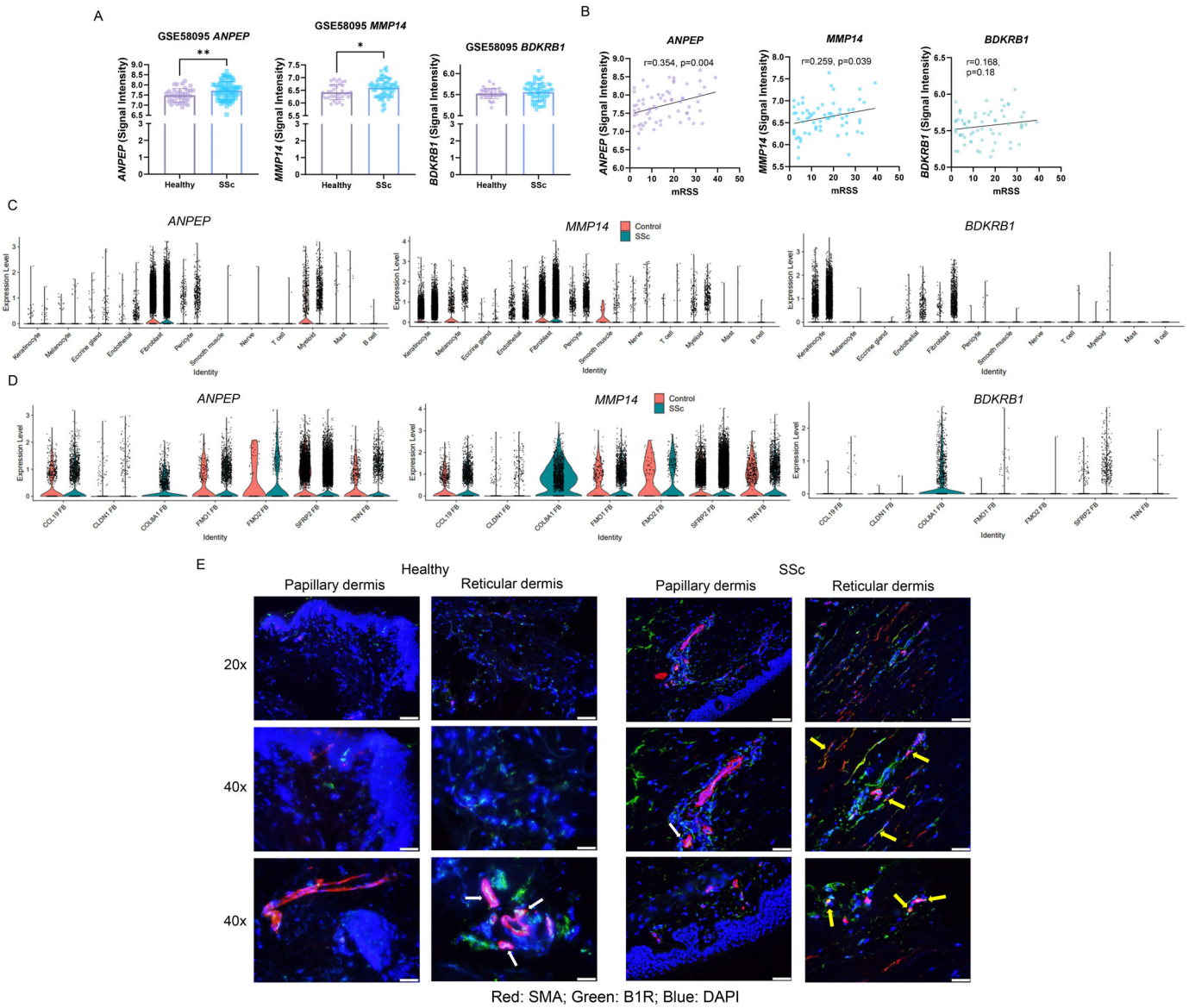

Figure 2. CD13, MMP14, and B1R are abundantly expressed in the skin. (A) Increased expression of *ANPEP* ($P < 0.01$) and *MMP14* ($P < 0.05$) was observed in skin biopsies from patients with SSc compared with healthy controls, and *BDKRB1* levels were similar in the two groups. Data were extracted from GEO GSE58095. (B) Analyzing the same GEO dataset, *ANPEP* and *MMP14* expression correlated significantly with modified Rodnan skin score (mRSS), and *BDKRB1* did not. (C) The expression of *ANPEP*, *MMP14*, and *BDKRB1* was observed in various cell types in the skin, analyzing single-cell RNA-seq results generated in 18 healthy controls and 22 patients with diffuse cutaneous SSc. (D) The expression of *ANPEP*, *MMP14*, and *BDKRB1* was observed in various fibroblast subsets. (E) Co-expression of B1R (green) and α-SMA (red) was observed in SSc skin mainly in the reticular dermis, whereas in healthy skin, B1R expression was low, with some staining in the blood vessels. White arrows point to blood vessels, and yellow arrows point to myofibroblasts. Original magnification ×20–40. Results are expressed as mean ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$. Significance was determined by (A) unpaired *t*-test and (B) Pearson correlation. B1R, bradykinin receptor B1; MMP14, matrix metalloproteinase 14; SMA, smooth muscle actin; SSc, systemic sclerosis.

SSc and 18 healthy adults was performed. In this analysis, 12 distinct cell types were identified in the skin.[25,26] Remarkably, the expression of all three genes involved in the sCD13 signaling axis was elevated in multiple cell populations in SSc skin biopsies compared with healthy controls (Figure 2C). *ANPEP* was abundantly expressed on fibroblasts and myeloid cells and was also expressed on endothelial cells and pericytes. *MMP14* was expressed on various cell types, most abundantly on fibroblasts, keratinocytes, endothelial cells, and pericytes. *BDKRB1* was mainly expressed in keratinocytes, fibroblasts, and endothelial cells. Because we had previously noted multiple distinct subpopulations of fibroblasts in the skin,[26] we next sought to identify the expression of *ANPEP*, *MMP14*, and *BDKRB1* in fibroblast subpopulations (Figure 2D). *ANPEP* and *MMP14* were expressed in all seven fibroblast subpopulations identified in the skin, whereas the expression of *BDKRB1* was most abundantly expressed in *COL8A1*+ fibroblasts, a subpopulation exclusively seen in SSc biopsies,[26] and a low-level expression on the *SFRP2*+ subset. Furthermore, expression of all the genes in the identified fibroblast subsets was elevated in patients with SSc compared with controls. Importantly, *ANPEP*, *MMP14*, and *BDKRB1* were all highly expressed on SSc-exclusive myofibroblast *COL8A1*+ fibroblasts.

To validate the findings of the single-cell RNA-seq analysis, we immunolabelled B1R in skin biopsies. Because *BDKRB1* was abundantly expressed on myofibroblasts (Figure 2D), α-SMA, which is expressed by vascular smooth muscle cells and myofibroblasts, was also included. In skin biopsies from healthy controls, B1R expression was overall low and predominantly seen in dermal blood vessels (Figure 2E, white arrows). In sharp contrast, B1R expression was remarkably abundant in SSc skin compared with healthy skin, specifically in the reticular dermis (Figure 2E, yellow arrows). Although B1R was expressed in blood vessels, it was also frequently observed in α-SMA–positive myofibroblasts. Here we found that COL8A1 was expressed mainly in the blood vessels in normal skin but was abundantly expressed in SSc skin, in both blood vessels (white arrows) and myofibroblasts (yellow arrows, Supplementary Figure 3). Co-expression of B1R with α-SMA and COL8A1 in SSc skin biopsies suggests a functional relationship between myofibroblast differentiation and B1R expression.

We have previously demonstrated that MMP14 generates the soluble form of CD13 in fibroblast-like synoviocytes.[7] To determine whether similar CD13 cleavage also occurs in dermal fibroblasts, we performed MMP14 knockdown in dcSSc dermal fibroblasts and measured sCD13 levels in the culture media. The results showed that knocking down MMP14 in dcSSc fibroblasts significantly reduced sCD13 in the culture media (Supplementary Figure 4A). These results therefore indicate that MMP14 acts as the sheddase for sCD13 in skin fibroblasts similar to fibroblast-like synoviocytes.

To determine the expression of *ANPEP*, *MMP14*, and *BDKRB1* in explanted fibroblasts, we performed quantitative polymerase chain reaction and immunoblotting using fibroblasts isolated from skin biopsies of healthy controls and patients with dcSSc. We found that the expression of *BDKRB1* was significantly elevated in dcSSc fibroblasts compared with those of healthy controls ($P < 0.05$) (Figure 3A), whereas the expression of *ANPEP* and *MMP14* was comparable. Elevated protein levels of B1R in dcSSc dermal fibroblasts were also observed (Figure 3B).

Because TGFβ plays a key role in SSc fibrosis, we next sought to determine the effect of TGFβ1 on *ANPEP*, *MMP14*, and *BDKRB1* expression in normal fibroblasts. We found that *ANPEP* expression was significantly down-regulated by TGFβ1 (Figure 3C). In contrast, *MMP14* and *BDKRB1* were up-regulated by TGFβ1, as early as 6 hours for *BDKRB1*. Next, we determined the effect of TGFβ1 on the secretion of CD13. sCD13 released from TGFβ1-treated dcSSc fibroblasts, as measured in the culture media, was significantly higher compared with cells from healthy controls (Figure 3D). These results suggest that TGFβ1, an essential cytokine in this disease, upregulates sCD13 and B1R in SSc dermal fibroblasts.

Blockade of sCD13 by B1R inhibition or knockdown alleviates myofibroblast phenotypes and signaling in dcSSc fibroblasts. Next, we examined how sCD13 and B1R affect the phenotype of SSc fibroblasts. We investigated whether sCD13 and the B1R inhibitor SSR240612 affected fibrotic genes, including *COL1A1* and *ACTA2*. sCD13 increased *COL1A1* and *ACTA2* expression and protein production in dcSSc fibroblasts in a dose-dependent manner. The inhibition of B1R by SSR240612 down-regulated both gene expression and protein levels (Figure 4A and B). Furthermore, we observed that cell proliferation was promoted by sCD13 in a dose-dependent manner and significantly suppressed by the addition of higher concentrations of SSR240612 (Figure 4C). Gel contraction by myofibroblasts was enhanced in dcSSc compared with healthy controls.[29] We determined that gel contraction was enhanced by sCD13 in dcSSc fibroblasts and inhibited by SSR240612 (Figure 4D). Consistent with these results, fibroblast migration was enhanced by sCD13 and inhibited by SSR240612 (Figure 4E).

We had previously shown that sCD13 induces the phosphorylation of ERK1/2 in various cell types.[9] Therefore, we next investigated whether sCD13 similarly activates this signaling pathway in SSc dermal fibroblasts. Immunolabelling and immunofluorescence showed that sCD13 stimulation of SSc fibroblasts led to significant phosphorylation of ERK1/2, which could be blocked by pretreatment with the B1R inhibitor SSR240612 (Figure 4F). Notably, sCD13 treatment also induced phosphorylation of SMAD2 in dcSSc fibroblasts, and this effect was also inhibited by B1R blockade (Figure 4G). Furthermore, in a separate series of experiments, we noted that sCD13 failed to stimulate expression of *COL1A1* and *ACTA2* in dcSSc dermal fibroblasts with

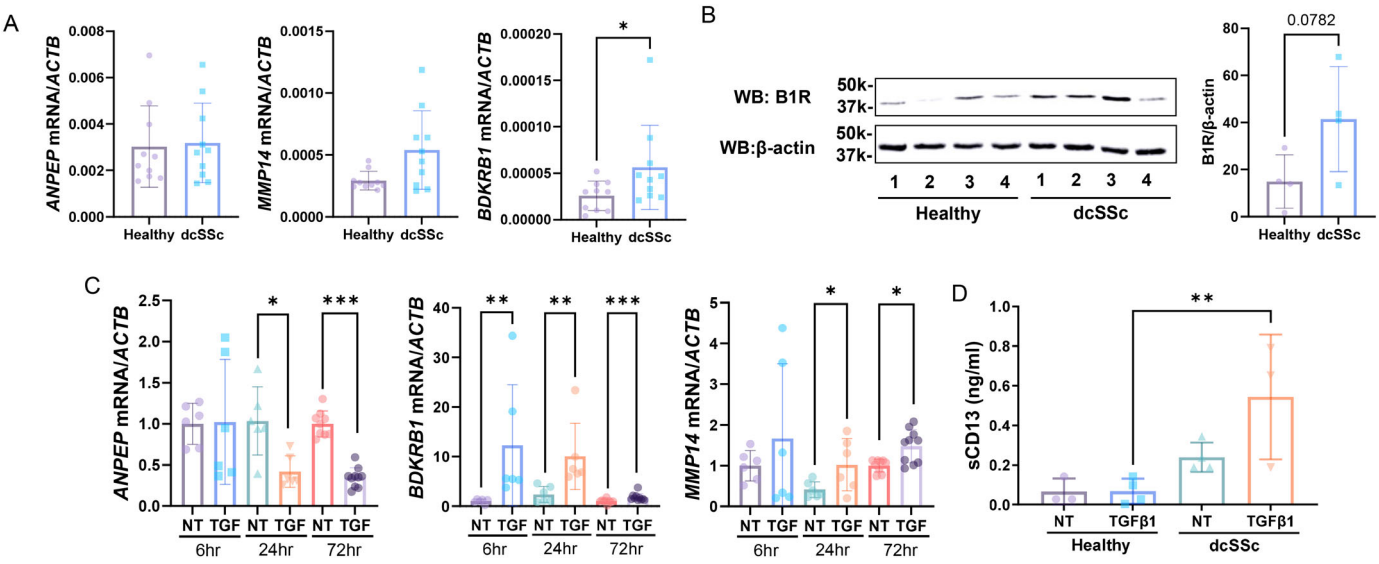

Figure 3. CD13, MMP14, and B1R are increased in dermal fibroblasts isolated from patients with dcSSc. (A) In cultured dcSSc dermal fibroblasts, increased expression of *BDKRB1* was detected compared with that from healthy controls ($P < 0.05$), whereas *ANPEP* and *MMP14* levels were similar. (B) Increased B1R levels were shown in dermal fibroblasts from patients with dcSSc compared with healthy controls. (C) TGFβ suppressed the expression of *ANPEP* and increased *MMP14* and *BDKRB1* mRNA in fibroblasts isolated from healthy controls. (D) TGFβ increased sCD13 levels in culture media from dcSSc dermal fibroblasts compared with those of healthy controls. Results are expressed as mean ± SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Significance was determined by (A-B) unpaired *t*-test, (C) Mann-Whitney test, and (D) one-way analysis of variance. B1R, bradykinin receptor B1; dcSSc, diffuse cutaneous systemic sclerosis; MMP14, matrix metalloproteinase 14; mRNA, messenger RNA; NT, Not-treated; sCD13, soluble CD13; TGFβ, transforming growth factor β; WB, Western blot.

B1R knockdown (Figure 4H and Supplementary Figure 4B), and this effect was consistent at the protein level as well (Figure 4I). These results suggest that the B1R blockade ameliorates a myofibroblast phenotype that is exacerbated by sCD13.

B1R inhibition mitigates the pro-fibrotic effects of TGFβ1 in healthy dermal fibroblasts. Treatment with TGFβ1 stimulates the expression of both sCD13 and its receptor B1R in SSc dermal fibroblasts. Therefore, we next sought to determine if B1R inhibition could counteract the pro-fibrotic effects of TGFβ1. Treatment of healthy dermal fibroblasts with TGFβ1 induced robust fibrotic gene expression at both the mRNA and protein levels; however, in the presence of the B1R inhibitor SSR240612, the TGFβ1 induction of these genes was substantially attenuated (Figure 5A and B). Notably, SSR240612 significantly blocked TGFβ1-induced sCD13 secretion from healthy dermal fibroblasts (Figure 5C). Moreover, B1R inhibition significantly prevented TGFβ1-induced cell proliferation and migration in these fibroblasts (Figure 5D and E). B1R inhibition with SSR240612 also blocked the TGFβ1-induced phosphorylation of SMAD2 (Figure 5F).

B1R blockade in mice attenuates bleomycin-induced skin fibrosis. To further determine whether B1R is a potential therapeutic target for SSc fibrosis, we evaluated the anti-fibrotic efficacy of B1R inhibitor SSR240612 in a bleomycin skin fibrosis mice model. In the prevention model in which B1R inhibition was initiated concurrently with the start of bleomycin injections, we noted that SSR240612 significantly attenuated bleomycin-induced skin thickness, including fibrotic area, and reduced skin hydroxyproline content (Figure 6A and B). We also observed that the number of F4/80-positive monocytes/macrophages in the skin was promoted by injection of bleomycin and significantly suppressed by SSR240612 (Figure 6C and Supplementary Figure 1B). The expression of fibrotic genes, including *Col1a1*, *Col8a1*, *Acta2*, and *Tgfb1* was up-regulated by bleomycin and significantly down-regulated by treatment with SSR240612 (Figure 6D). Similar results were observed for inflammatory genes *Ccl2* and *Il6*. Comparable results were observed in the therapeutic model, in which fibrosis was first established before administering the B1R inhibitor, with reductions in dermal thickness and hydroxyproline content (Figure 6E and F).

To confirm the role of B1R in bleomycin-induced fibrosis, we used *Bdkrb1* knockout mice. Consistent with the findings from SSR240612 treatment, *Bdkrb1* knockout mice exhibited reduced bleomycin-induced skin thickness and lower hydroxyproline levels (Figure 6G and H). In addition, *Bdkrb1* knockout mice injected with bleomycin had lower numbers of F4/80-positive cells in the skin compared with wildtype mice injected with bleomycin (Figure 6I and Supplementary Figure 1C). And finally, fibrotic genes, including *Col1a1*, *Col8a1*, *Acta2*, and *Tgfb1*, as well as inflammatory genes *Ccl2* and *Il6* were significantly lower in

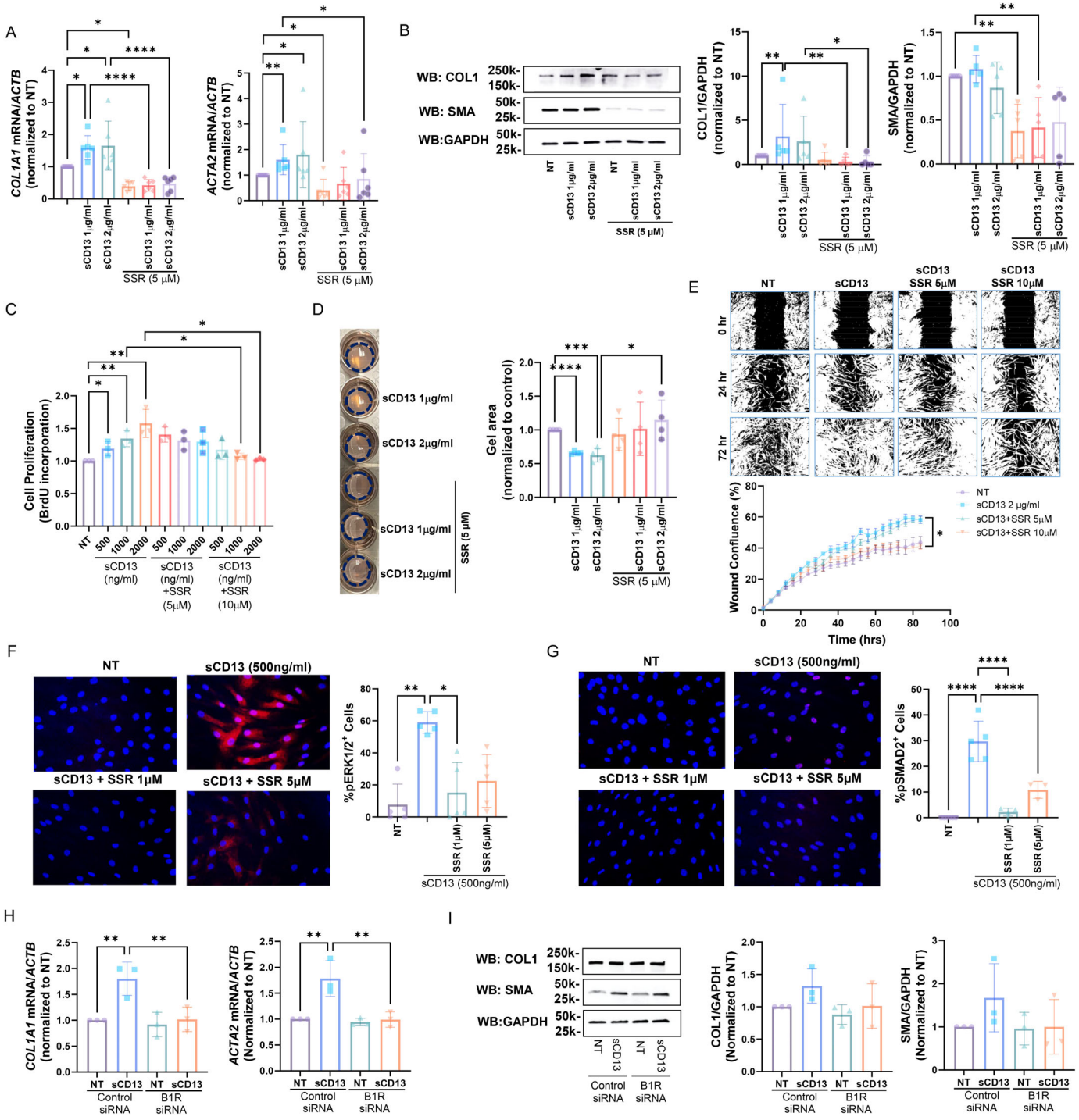

Figure 4. sCD13 and B1R promote myofibroblast phenotypes and signaling in dcSSc fibroblasts. (A and B) B1R inhibitor SSR240612 suppressed sCD13-induced both mRNA and protein levels of COL1 and α-SMA in dcSSc fibroblasts. (C–E) SSR240612 inhibited sCD13-induced cell proliferation, gel contraction, and migration of dcSSc fibroblasts ($P < 0.05$). (F–G) sCD13 induced phosphorylation of ERK1/2 (red) or SMAD2 (red) in dcSSc fibroblasts, which were blocked by SSR240612 ($P < 0.05$). Results were presented as the percentage of red-positive cells/DAPI-stained nucleus. (H and I) B1R knockdown suppressed sCD13-induced fibrotic genes in dcSSc fibroblasts. Results are expressed as mean ± SD or (E) mean ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. Significance was determined by (A–D, F) Kruskal-Wallis test, (G–I) one-way analysis of variance, and (E) two-way analysis of variance. B1R, bradykinin receptor B1; dcSSc, diffuse cutaneous systemic sclerosis; mRNA, messenger RNA; NT, not-treated; sCD13, soluble CD13; siRNA, small interfering RNA; SMA, smooth muscle actin; SSR, SSR240612; WB, Western blot.

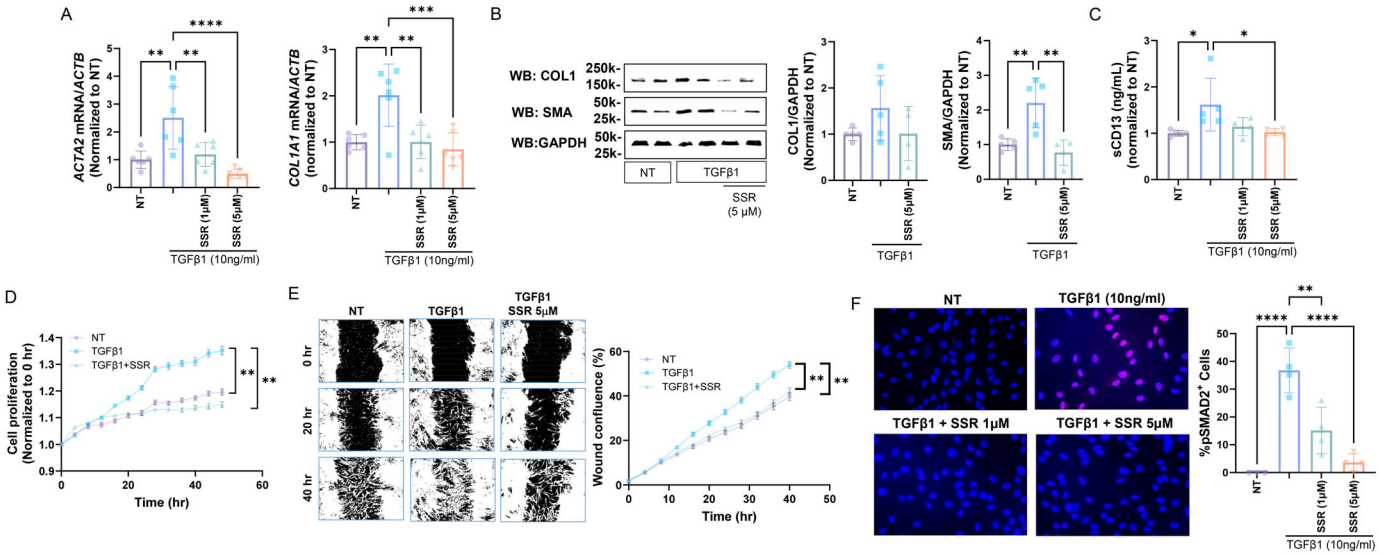

Figure 5. B1R inhibition alleviates the pro-fibrotic effects of TGFβ1 in dcSSc fibroblasts. (A and B) B1R inhibitor SSR240612 suppressed TGFβ1 induced both mRNA and protein levels of COL1 and α-SMA in dcSSc fibroblasts. (C) SSR240612 inhibited TGFβ-induced sCD13 shedding in dcSSc fibroblasts ($P < 0.05$). (D and E) TGFβ1-induced cell proliferation and migration was blocked by B1R inhibitor SSR240612 ($P < 0.05$). (F) TGFβ1-induced phosphorylation of SMAD2 (red) in dcSSc fibroblasts was blocked by SSR240612 ($P < 0.05$). Results are expressed as mean ± SD or (D and E) mean ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. Significance was determined by (A–C, F) one-way analysis of variance and (D and E) two-way analysis of variance. B1R, bradykinin receptor B1; dcSSc, diffuse cutaneous systemic sclerosis; mRNA, messenger RNA; NT, not-treated; sCD13, soluble CD13; SMA, smooth muscle actin; SSR, SSR240612; TGFβ, transforming growth factor β; WB, Western blot.

bleomycin-treated *Bdkrb1* knockout mice compared with bleomycin-treated wildtype mice (Figure 6J).

DISCUSSION

In this study, we sought to determine the expression and role of the widely expressed aminopeptidase CD13 in SSc. CD13 is increasingly implicated in inflammatory diseases and promotes inflammatory cell adhesion, migration, and aggregation.[4] Previous studies have demonstrated important roles for CD13 in rheumatoid arthritis, psoriasis, and connective tissue diseases.[11,30,31] We showed that MMP14 cleaves off the soluble form of CD13 from the cell surface.[7] Significantly, using a series of orthogonal experimental approaches, we recently identified B1R as the cellular receptor for sCD13.[9] These observations highlight the significance of the sCD13-B1R signaling axis in the pathogenesis of multiple inflammatory conditions. In the present study, we demonstrated, for the first time, the importance of this pathway in SSc by showing the antifibrotic efficacy of B1R blockade in vitro and in vivo, complemented with the demonstration that mice lacking either *Cd13* or *Bdkrb1* were protected from bleomycin-induced skin fibrosis. We believe that these results identify a novel and promising therapeutic target for SSc fibrosis.

We found abundant expression of *ANPEP*, *MMP14*, and *BDKRB1* in SSc skin as well as in isolated dermal fibroblasts. According to our single-cell RNA-seq analysis, *ANPEP* was highly expressed on fibroblasts and myeloid cells, whereas *MMP14* was widely expressed on various cell types (Figures 2 and 3). Interestingly, *BDKRB1* was highly expressed on *COL8A1*+ fibroblasts. This subset is the myofibroblast cluster observed exclusively in dcSSc skin.[25,26] Indeed, we confirmed in SSc skin that B1R is expressed mainly on myofibroblasts in the deep dermis and that its expression is also significantly elevated in isolated SSc dermal fibroblasts. This could possibly explain the discrepancy we found regarding *BDKRB1* expression in whole-skin biopsies and dermal fibroblasts (Figure 2 and 3). It could also explain why *BDKRB1* expression does not correlate with skin score, whereas *ANPEP* and *MMP14* correlate significantly in the GEO data analysis (Figure 2B). Nonetheless, the consistent observation that B1R is highly expressed on SSc-specific myofibroblasts, and that CD13 and MMP14 are expressed in the skin, suggest that sCD13, which is shed by MMP14 in SSc skin, acts on myofibroblasts via B1R locally. This notion supports our findings that sCD13 is significantly more abundant in supernatants from SSc fibroblast cultures compared with healthy fibroblasts (Figure 3D).

We further demonstrated that sCD13-treated SSc fibroblasts exhibited characteristic myofibroblast features, and myofibroblast transition by sCD13 could be suppressed by a B1R antagonist (Figure 4). Notably, B1R inhibition also blocked the pro-fibrotic effects of TGFβ1 in healthy dermal fibroblasts (Figure 5). Furthermore, mice null of Cd13 or B1R, or wildtype mice treated with SSR240612, were protected from bleomycin-

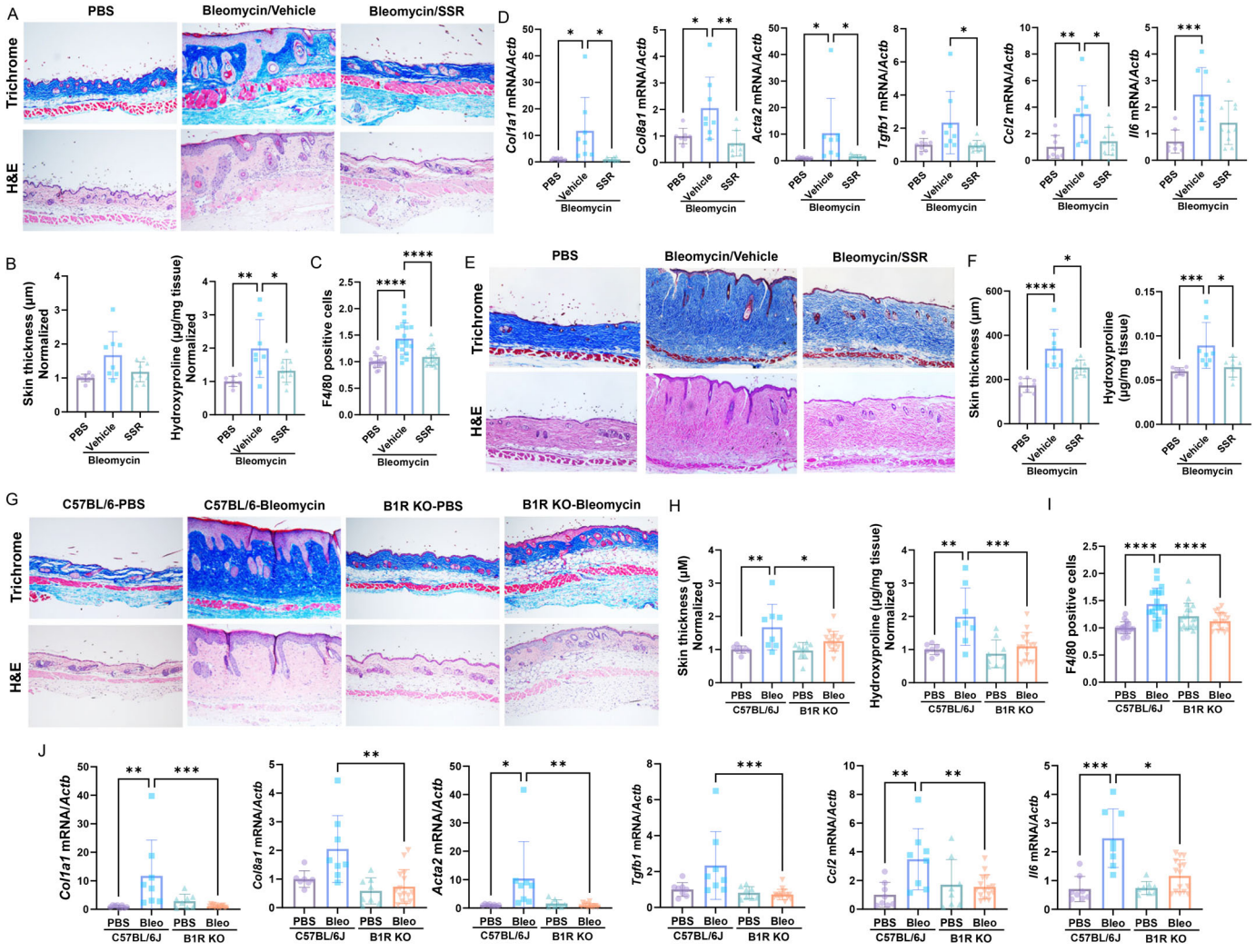

Figure 6. B1R blockade in mice attenuates bleomycin-induced skin fibrosis. (A and B) B1R inhibitor SSR240612 attenuated bleomycin-induced skin thickness and hydroxyproline content ($P < 0.05$) in wildtype C57BL/6 mice in the prevention model. (C) SSR240612 reduced bleomycin-induced F4/80 positive cells in skin tissue ($P < 0.0001$). (D) SSR240612 decreased bleomycin-induced expression of pro-fibrotic and pro-inflammatory genes in mice skin ($P < 0.05$). (E and F) Significant fibrosis was established by bleomycin injection in mice for 2 weeks, and SSR240612 treatment for the following 2 weeks blocked skin fibrosis significantly. (G and H) Bleomycin-induced skin fibrosis was less prominent in B1R knockout mice compared with wildtype mice as shown by significant reduction in skin thickness and hydroxyproline content ($P < 0.05$). (I) B1R knockout mice showed fewer F4/80-positive cells in the skin compared with wildtype mice injected with bleomycin ($P < 0.0001$). (J) B1R knockout mice showed lower levels of bleomycin-induced fibrotic and inflammatory genes in the skin compared with wildtype mice. Original magnification ×10. Results are expressed as mean ± SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. Significance was determined by (B–D, F, H–J) one-way analysis of variance and (D, F, J) Kruskal-Wallis test. B1R, bradykinin receptor B1; Bleo, bleomycin; H&E, hemtoxylin and eosin; KO, knockout; mRNA, messenger RNA; PBS, phosphate buffered saline; SSR, SSR240612.

induced skin fibrosis, including attenuated induction of *Col8a1* and pro-inflammatory genes, indicating that the sCD13-B1R axis plays a pivotal role in SSc fibrosis and could be a potential therapeutic target.

In the present study, we showed that B1R is abundant in dcSSc skin, specifically in myofibroblasts. We further demonstrated that this elevation is mediated, at least in part, by TGFβ1. Although TGFβ1 down-regulated CD13 at the transcriptional level (Figure 3C), we showed that TGFβ1 significantly promoted sCD13 release from dcSSc fibroblasts. This could be mediated by the significant increase of *MMP14* observed in various stromal cell populations in dcSSc skin, including various fibroblast subsets (Figure 2D). The upregulation of MMP14 also appears to be TGFβ1-dependent (Figure 3C). We also confirmed that MMP14 is the sheddase for generating sCD13 in dermal fibroblasts (Supplementary Figure 4A).

Because sCD13 activates the ERK signaling pathway via B1R,[8,9] we posit that the pro-fibrotic effect of the sCD13-B1R axis could also be mediated via the ERK pathway. Indeed, we showed that sCD13 induced significant ERK1/2 phosphorylation

in dcSSc dermal fibroblasts (Figure 4F). There is growing evidence that ERK1/2 is involved in fibrosis in SSc. In fact, ERK1/2 has been documented to be involved in fibroblast proliferation,[32] and IL11-dependent ERK1/2 signaling is required for TGFβ to induce a pro-fibrotic response in dermal fibroblasts.[33] Interestingly, PTP4A1 is reported to be a key promoter of TGFβ signaling in primary skin fibroblasts and bleomycin-induced fibrosis in vivo.[34] PTP4A1 promotes TGFβ signaling in human fibroblasts through enhanced ERK1/2 activity, which stimulates SMAD3 expression and nuclear migration. These findings support the possibility that the pro-fibrotic effect of sCD13 is mediated in part by activating ERK1/2 in fibroblasts.

There are some limitations of this study. Although our goal is to treat systemic fibrosis in SSc, we focused exclusively on skin fibrosis in this study. Interestingly, B1R expression in various lung diseases has been reported.[35,36] Further investigation is needed to assess the effect of inhibition of sCD13-B1R in other organs or fibrotic conditions. Second, we only focused on specimens from patients with dcSSc. Because B1R expression depends highly on stimuli such as TGFβ1, the expression pattern of this receptor on other SSc disease subtypes requires further examination. B1R could be a potential target for precision medicine approaches for this disease.

Based on our findings, the role of the sCD13-B1R axis in dcSSc skin lesions is depicted in Supplementary Figure 5. CD13 is distributed mainly in myeloid cells and fibroblasts in SSc skin. MMP14 is widely expressed in the skin, including keratinocytes, endothelial cells, and fibroblasts, and serves to cleave CD13 off from cell membranes. B1R is specifically expressed on both *SFRP2*+ and *COL8A1*+ fibroblasts. Interestingly, from our pseudotime analysis there is evidence that the *SFRP2*+ fibroblasts differentiate into the *COL8A1*+ fibroblasts.[26] Therefore, it is possible that the sCD13-B1R pathway on these cells promotes and maintains myofibroblast differentiation in the deep dermis. Furthermore, suppression of B1R may indirectly inhibit myofibroblast transformation by suppressing the migration of monocytes or macrophages to the cutaneous loci. We have previously shown that sCD13 induces angiogenesis in endothelial cells.[8] Because B1R is expressed on SSc endothelial cells, yet angiogenesis is impaired in SSc, this apparent disconnection requires further investigation and may reflect abnormal responses to angiogenic stimuli in SSc endothelial cells. Taken together, our results provide evidence that sCD13 is involved in skin fibrosis via B1R by promoting myofibroblast transformation and that the blockade of the sCD13-B1R axis warrants evaluation as a potential therapeutic option for SSc fibrosis.

AUTHOR CONTRIBUTIONS

All authors contributed to at least one of the following manuscript preparation roles: conceptualization AND/OR methodology, software, investigation, formal analysis, data curation, visualization, and validation AND drafting or reviewing/editing the final draft. As corresponding authors, Drs Fox and Tsou confirm that all authors have provided the final approval of the version to be published, and take responsibility for the affirmations regarding article submission (eg, not under consideration by another journal), the integrity of the data presented, and the statements regarding compliance with institutional review board/Helsinki Declaration requirements.

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