Cell membrane-anchored and tumor-targeted IL-12 T-cell therapy destroys cancer-associated fibroblasts and disrupts extracellular matrix in heterogenous osteosarcoma xenograft models

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ABSTRACT

Background The extracellular matrix (ECM) and cancer-associated fibroblasts (CAFs) play major roles in tumor progression, metastasis, and the poor response of many solid tumors to immunotherapy. CAF-targeted chimeric antigen receptor-T cell therapy cannot infiltrate ECM-rich tumors such as osteosarcoma.

Method In this study, we used RNA sequencing to assess whether the recently invented membrane-anchored and tumor-targeted IL-12-armed (attIL12) T cells, which bind cell-surface vimentin (CSV) on tumor cells, could destroy CAFs to disrupt the ECM. We established an in vitro model of the interaction between osteosarcoma CAFs and attIL12-T cells to uncover the underlying mechanism by which attIL12-T cells penetrate stroma-enriched osteosarcoma tumors.

Results RNA sequencing demonstrated that attIL12-T cell treatment altered ECM-related gene expression. Immunohistochemistry staining revealed disruption or elimination of high-density CAFs and ECM in osteosarcoma xenograft tumors following attIL12-T cell treatment, and CAF/ECM density was inversely correlated with T-cell infiltration. Other IL12-armed T cells, such as wild-type IL-12-targeted or tumor-targeted IL-12-T cells, did not disrupt the ECM because this effect depended on the engagement between CSV on the tumor cell and its ligand on the attIL12-T cells. Mechanistic studies found that attIL12-T cell treatment elevated IFNγ production on interacting with CSV+ tumor cells, suppressing transforming growth factor beta secretion and in turn upregulating FAS-mediated CAF apoptosis. CAF destruction reshaped the tumor stroma to favor T-cell infiltration and tumor inhibition.

Conclusions This study unveiled a novel therapy—attIL12-T cells—for targeting CAFs/ECM. These findings are highly relevant to humans because CAFs are abundant in human osteosarcoma.

INTRODUCTION

Even as therapeutic outcomes for patients with many cancers have dramatically improved over recent decades, those for patients with osteosarcoma, especially those with metastatic disease, have not. The focus of cancer research has been shifting from the tumor cell alone to a more holistic view encompassing the full complexity of tumors...
and the tumor microenvironment (TME). The TME consists of a highly complex network of tumor cells, tumor stroma, vasculature, immune cells, and non-cellular extracellular matrix (ECM).

Osteosarcoma, with its histological hallmark of osteoid production, has a rich ECM. Growing clinical evidence has demonstrated that ECM components in solid tumors are associated with invasiveness, metastasis, and reduced survival. Several ECM-related genes have been linked to poor outcomes among patients with osteosarcoma, such as a COL1A1 polymorphism; high expression of fibronectin and integrin; and high CD44 expression. The major reason for the ECM’s pivotal role in tumor promotion is that dense ECM acts as a tumor-protective shield that impedes immune-cell infiltration into tumors. When immune cells encounter this dense ECM structure, they are diverted from tumors and trapped in tumor stroma. In light of these negative effects of tumor ECM, several approaches to disrupting the ECM have been investigated in preclinical studies, and there is evidence that targeting the ECM can improve responses to anticancer treatments.

Although tumor cells contribute to ECM development, cancer-associated fibroblasts (CAFs) play a bigger role, as they are the source of ECM in tumors. CAFs, derived mainly from tissue-resident fibroblasts, are present in all solid tumors. Although CAFs have heterogeneous phenotypes that are associated with disparate outcomes in patients, a subtype of CAFs that upregulates the markers α-smooth muscle actin (αSMA) and fibroblast activation protein (FAP) is associated with poor prognosis in many cancers. High loads of these CAFs in solid tumors induce production of collagen and other ECM factors, contributing to ECM remodeling that alters the mechanical characteristics of tumor and preventing antitumor T cells from accessing the core regions of tumors. CAFs also highly express immune-suppressive factors such as interleukin 6 (IL-6) and transforming growth factor beta (TGFβ) under the hypoxic conditions prevalent in tumors. These cytokines stimulate cancer stem cell features and impair immune effector cell cytotoxicity. In these ways, CAFs confer resistance to immunotherapies used for cancer treatment.

In contrast to the success of immunotherapy in treating blood cancers, the use of immunotherapy to treat solid tumors has encountered several obstacles. Physical barriers appear to be one major impediment preventing effector immune cells from reaching tumor cells. As the source of the ECM that forms such a physical barrier, CAFs are a promising target to improve immunotherapy responses in solid tumors. Although early studies that depleted the myofibroblast CAF subtype failed, having shown reduced survival in preclinical models, targeting FAP+ cells led to tumor regression after chimeric antigen receptor (CAR)-T cell therapy.

However, FAP+ cell depletion also resulted in severe bone marrow toxicities. Therefore, there is an urgent need for safe approaches to target both FAP+ CAFs and solid tumors.

Given that osteosarcoma tumor cells are encapsulated by rigid ECM barriers, most cytotoxic T cells attracted to tumors via chemotaxis are blocked from accessing the tumor regions. Recently, our group invented membrane-anchored and tumor-targeted IL-12-armed T cells (attIL12-T cells) to eliminate large osteosarcoma patient-derived xenograft (PDX) tumors. The attIL12 protein has three distinct domains: a tumor-targeted ligand, VNTANST, which binds vimentin expressed on the cell surface (cell-surface vimentin, CSV) on most tumor cells; an IL-12 heterodimer; and a transmembrane domain that anchors P40 on the T cells. T cells transduced with attIL12 both target CSV on solid tumor cells and deliver IL-12 into tumors to minimize off-tumor toxicities. In a previous study, we showed that attIL12-T cell treatment markedly increased T-cell infiltration into large solid tumors. In this study, we sought to better understand the mechanisms behind this effect by comparing osteosarcoma PDX models that were sensitive or resistant to attIL12-T cell treatment. We found significant elevation of ECM factors and ECM-associated pathways in the resistant models. Our results further demonstrated that CAFs were abrogated by attIL12-T cells in the treatment-sensitive tumors, leading to destruction of ECM structures and infiltration of T cells. We further deciphered the anti-CAF mechanism of attIL12-T cells, showing that CAFs were deprived of their role in ECM remodeling by FAS upregulation-mediated apoptosis. This FAS upregulation in CAFs was mediated by suppression of TGFβ expression and induction of interferon gamma (IFNγ) in tumors.

RESULTS

High densities of CAFs and collagen are associated with advanced-stage osteosarcoma and less T-cell infiltration

In human osteosarcoma, the mesenchymal stroma induces tumor progression. We therefore assessed the osteosarcoma TME by staining tissue arrays including normal tumor-adjacent tissues and malignant osteosarcoma tumors of different stages. Masson’s trichrome staining showed very low levels of ECM in normal tissue and stage I tumors but significantly higher fibroblast density and collagen deposition in stage IV osteosarcomas (figure 1A), showing that advanced-stage osteosarcoma has a rich ECM. This high ECM density was also found in metastatic human osteosarcoma tumors (online supplemental figure S1).

A growing body of evidence has shown that high ECM density impedes effector T-cell penetration into tumors, so
we evaluated T-cell accumulation on the osteosarcoma tissue array using immunohistochemistry staining. Early-stage osteosarcoma tumors exhibited tumor-infiltrating T cells, whereas stage IV osteosarcoma tumors were almost devoid of immune cells (figure 1B). To determine the association between the osteosarcoma ECM and T-cell infiltration, we used the osteosarcoma tissue array containing 17 stage I, 19 stage II, and 3 stage IV osteosarcomas and assessed the collagen levels and T-cell density. Fiber and collagen levels were both significantly inversely correlated with T-cell density across osteosarcoma stages (Pearson $r=-0.45$; and $p=0.004$, Pearson $r=-0.57$, respectively), with the highest abundance of ECM and the lowest T-cell presence in advanced-stage osteosarcomas (figure 1C,D).

**ECM destruction is vital for the effectiveness of attIL12-T cell treatment in osteosarcoma PDX models**

The association between T-cell infiltration and ECM abundance in human osteosarcoma tumors provided a clear clue that ECM blocks T-cell infiltration, but this required experimental validation. Our group invented a novel attIL12-T-cell therapy approach in which a tumor-targeted IL-12 (ttIl12, previously developed by us) is anchored to the T-cell membrane through a transmembrane polypeptide. In previous publications, we showed that attIL12-T cells can effectively inhibit large and heterogeneous osteosarcoma PDX tumors and induce T-cell infiltration into tumors. We postulated that this treatment could be used to experimentally validate the hypothesis that ECM impedes T-cell infiltration into osteosarcoma tumors. Because sensitivity and resistance to attIL12-T cell treatment are directly associated with T-cell infiltration, we first compared the transcriptome profiles of sensitive and resistant tumors. Previously, we demonstrated that attIL12-T cell transfer inhibited large osteosarcoma tumor growth in several PDX models, which we refer to as sensitive models. By contrast, other PDX models were either insensitive or less sensitive to the attIL12-T cell treatment; these are referred to here as resistant models (figure 2A). We performed total RNA sequencing (RNA-seq) of three sensitive (OS1, OS2, and OS31) and three resistant PDX models (OS33, OS34, and OS9). A total of 465 genes were differentially expressed between the

**Figure 1** High densities of fibroblasts and collagen are associated with advanced-stage osteosarcoma and less T-cell infiltration. (A) Tissue arrays of human osteosarcoma of the indicated stages and adjacent normal tissue were purchased from US Biobank and subjected to Masson’s trichrome staining. Blue staining indicates fiber, and red staining indicates collagen. Graphs show staining intensity (individual value±SEM) of fiber or collagen. (B) Immunohistochemical staining of human CD3 (T-cell marker) on human osteosarcoma tissue arrays of the indicated stages. The graph shows the percentages (individual value±SEM) of CD3-stained cells per high-power field. (C, D) The correlation of fiber (C) and collagen (D) levels with CD3 expression in human osteosarcoma tissue arrays, determined by the Pearson product test. *$p<0.05$; ***$p<0.001$; NS, not significant.
sensitive and resistant tumors (fold change>2 and q<0.05) (figure 2B, online supplemental table S1). The two most significantly differentially expressed genes (DEGs) were PROM1 (also known as CD133) and CCN4 (also known as WISP1), both of which were downregulated in the sensitive models (figure 2B). PROM1 is a marker of cancer stem cells, and its expression is associated with poor prognosis in osteosarcoma patients.28 CCN4, a matricellular protein, promotes collagen linearization in response to TGFβ1 in breast carcinoma.29 Gene Ontology (GO) analysis identified differential gene enrichment in collagen-containing ECM, ECM, ECM region, ECM organization, and ECM structure between the two groups (figure 2C). The pathway most strongly associated with sensitivity to attIL12-T cell treatment was ECM-receptor interaction (figure 2D). These results clearly showed that the antitumor efficacy of attIL12-T cell treatment likely depends on changes in ECM abundance, composition, and architecture.

**High-density CAFs in OS PDX tumors**

Since ECM factors are mainly produced by CAFs in solid tumors, we sought to determine how CAFs regulate ECM in osteosarcoma. We analyzed mRNA expression (z-scores) in an osteosarcoma dataset from The Cancer Genome Atlas (Pediatric Preclinical Testing Consortium) which contained the same patient samples we used to establish the PDX tumor models in mice. Pearson correlation analysis suggested that expression of the hallmark genes for CAFs (FAP, ACTA2, THY1, and PDPN) was significantly correlated with expression of an ECM gene signature (COL1A1, SPARC, LAMB1, ITGB1, FN1, and CD44) (p=0.048; Pearson r=0.371) and that of CCN4 (p=0.012; Pearson r=0.462) in osteosarcoma (figure 3A,B). We then detected the protein levels of these genes to validate the sequencing results. Immunoblots of ECM markers confirmed the results of the RNA-seq analysis, showing that attIL12-T cell treatment suppressed FAP, pan-collagens, integrin β1, laminin, and fibronectin expression in two sensitive PDX models (OS1...
Figure 3 Membrane-anchored and tumor-targeted IL-12 (attIL12)-T cells disrupt extracellular matrix (ECM) in osteosarcoma tumors. (A, B) Pearson correlation analysis of expression of hallmark genes for cancer-associated fibroblasts (CAF) (FAP, ACTA2, THY1, and PDPN) with expression of an ECM gene signature (COL1A1, SPARC, LAMB1, ITGB1, FN1, and CD44) (A) or CCN4 (B); data obtained from TCGA. (C) Immunoblots of ECM markers fibroblast activation protein (FAP), pan-collagen, sparc, integrin β1, laminin, transforming growth factor beta (TGFβ), and fibronectin in control T-cell-treated or attIL12-T cell-treated sensitive (OS1 and OS2) and resistant (OS33 and OS34) PDX tumors. (D) Masson's trichrome staining of control T-cell-treated or attIL12-T cell-treated OS1 and OS33 tumor sections. Blue staining indicates fiber, and red staining indicates collagen. The graphs show the staining intensity (individual values±SEM) of fiber or collagen. Scale bar: 500 µm. (E) Immunofluorescence staining and whole-slide scanning images of α-smooth muscle actin and FAP colocalization on OS1 and OS2 tumors with the indicated treatments. Scale bar: 500 µm. Counterstain: Hoechst. **p<0.01; NS, not significant.
and OS2) but not in two resistant PDX models (OS33 and OS34) (figure 3C). Immunohistology staining also showed a significant decrease in the expression of these ECM markers in the sensitive OS1 and OS2 models, but little change in resistant OS33 and OS34 tumors (online supplemental figure S2).

In agreement with these findings, trichrome staining showed significantly lower fiber and collagen staining intensity in attIL12-T cell-treated sensitive OS1 tumor sections, but no change in OS33 resistant tumor sections (figure 3D). These observations validated the RNA-seq results showing that a tumor’s ECM regulation is associated with its sensitivity to the attIL12-T cell treatment. Furthermore, we performed whole-slide scanning following immunofluorescence staining of the established CAF markers FAP and αSMA on tumor sections after different indicated treatments (figure 3E). We included two additional controls (aIL12 and ttIL12) to determine whether the observed ECM reduction was specific to attIL2-T cell treatment. aIL12 is attIL2 without the tumor-targeted peptide, and ttIL12 is the soluble form of attIL12 without the transmembrane domain. We found that only attIL12-T cell treatment effectively destroyed the CAF barrier of osteosarcoma tumors (figure 3E).

**ECM destruction by attIL12-T cells—but not by either wild-type IL12-T cells or ttIL12-T cells—promotes T-cell infiltration into tumors**

Others have found that the ECM in tumors impedes T-cell penetration, compromising the efficacy of anticancer immunotherapy.\(^{15}\) To validate our hypothesis that attIL12-T cell treatment promotes T-cell infiltration via disrupting ECM and CAFs, we performed Mason’s trichrome staining of OS1, OS2, and OS33 tumor sections to compare ECM/CAF density after different treatments. As expected, rich collagen and fiber was found in osteosarcoma PDX tumors infused with control T cells, aIL12-T cells, wild-type IL12-T (wtIL12-T) cells, or ttIL12-T cells (figure 4A). Strikingly, attIL12-T cell treatment destroyed the ECM structure in sensitive OS1 and OS2 tumors, but not in resistant OS33 tumors (figure 4A).

To test our hypothesis that T-cell exclusion by the CAF barrier hinders the response to attIL12-T cell treatment, we examined T-cell infiltration in OS1, OS2, OS33, and OS34 tumors. T cells were absent in the tumors after control-T, wtIL12-T, and ttIL12-T cell infusions, but T-cell infiltration in the tumor center was much greater after attIL12-T cell treatment in sensitive OS1 and OS2 tumors (figure 4B). In contrast to the immune inflammation observed after attIL12-T cell treatment in both sensitive models (figure 4B), the resistant models OS33 and OS34 retained an immune-excluded phenotype even after attIL12-T cell treatment (figure 4B). In addition, T-cell infiltration and PDX tumor size were strongly inversely correlated (p=0.004; Pearson r=−0.74) (figure 4C). These results together suggest that tumor responses to attIL12-T cell treatment depend on T-cell infiltration status.

**CAFs undergo apoptosis after attIL12-T cell treatment**

Our next question was how attIL12-T cells eliminate CAFs from osteosarcoma PDX tumors. Compared with CAFs in control-T cell-treated OS1 tumors, CAFs in attIL12-T cell-treated tumors expressed much higher levels of cleaved caspase 3, suggesting that attIL12-T cell treatment triggered CAF apoptosis (figure 5A). To decipher the CAF regulation mechanism, we conducted in vitro coculture experiments. CAFs were isolated from osteosarcoma PDX tumors, and tumor tissues were digested with an enzyme cocktail before culturing (online supplemental figure S3A). Small spindle-shaped cells in colonies were harvested 6 days later and identified as FAP⁺ cells via flow cytometry (online supplemental figure S3B). To mimic the TME, we cocultured CAFs and T cells (1:3) in conditioned medium from coculture of the same T cells with osteosarcoma tumor cells (3:1) for 24 hours (online supplemental figure S4). After 2 days, the cell death of CAFs from sensitive models was dramatically increased (>70%) by attIL12-T cells, but only ~30% of resistant tumor derived CAFs were induced cell death by attIL12-T cells (figure 5B). This in vitro CAF regulation model was further tested with immunofluorescence chamber slide staining, which showed that only attIL12-T cells, not control-T, aIL12-T, wtIL12-T, or ttIL12-T cells, triggered CAF apoptosis as measured by active caspase 3 (figure 5C). This attIL12-T cell-specific CAF apoptosis was further validated in both the sensitive OS1 and OS2 models and the resistant OS33 model via flow cytometry (online supplemental figure S5). attIL12-T cells induced apoptosis in more than 50% of CAFs from sensitive tumor models, in contrast to less than 30% of CAFs from the resistant tumor model (figure 5C, online supplemental figure S5). This lower level of CAF apoptosis is insufficient to enhance T-cell infiltration.

Emerging evidence has demonstrated that TGFβ signaling is essential for CAF development; in a positive feedback loop, CAFs secrete excess TGFβ as a paracrine factor for ECM remodeling.\(^{30}\) In the osteosarcoma PDX models, TGFβ1 mRNA expression was correlated with expression of CAF hallmark gene sets (p=0.03; Pearson r=0.4135) (figure 5D). If attIL12-T cell treatment indeed induced CAF death, we expected that less TGFβ would be produced by CAFs. We therefore tested TGFβ expression by CAFs and found that it was significantly lower after attIL12-T cell coculture than after control T-cell coculture (figure 5E). However, much higher level of TGFβ in OS33 CAFs protected CAFs from apoptosis. attIL12T cells had limited effect in reducing the TGFβ producing OS33 CAFs (figure 5E).

CAFs function was assessed by its contractility via a collagen gel contraction assay. Control T-cell or attIL12-T cell-treated CAFs were suspended in collagen solution, and the gel was allowed to solidify. Control T-cell-treated CAFs started to contract the collagen gel 24 hours later, and the gel reached less than 60% of its original area by day 7. In contrast, attIL12-T cell treatment markedly decreased CAFs’ contractility, such that the collagen gel...
retained more than 80% of its original area (figure 5F).

Altogether, these results demonstrated that attIL12-T cells induced CAF apoptosis, abrogating active CAFs’ role in altering ECM architecture.

**CAF apoptosis through FAS upregulation**

Apoptosis can be induced by intrinsic stimulation, extrinsic signaling, or T-cell cytotoxicity.31 We ruled out intrinsic stimulation and T-cell cytotoxicity because no intrinsic stimuli, such as chemicals, hypoxia, or radiation, were present in our models, and CAFs express a low level of CSV (online supplemental figure S6), which stimulates low attIL12-T cell cytotoxicity. Therefore, we reasoned that attIL12-T cell-induced CAF apoptosis is most likely initiated by an extrinsic pathway characterized by FAS/FASL interaction. Activated T cells express c-Myc-driven FASL to control the apoptotic cell death of other cell types.32 We therefore investigated FAS expression on the cell surface of CAFs in our in vitro coculture.
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system as described in figure 5B. Our results showed that FAS was markedly upregulated in CAFs treated with attIL12-T cells in comparison to those treated with control T cells, but FAS expression on attIL12-T cell-treated bone marrow cells from healthy donors remained unchanged (online supplemental figure S7). Moreover, conditioned medium from attIL12-T cells cocultured with OSD tumor cells in the presence of a CSV-blocking antibody partially abolished FAS induction (figure 6A). Notably, CAF apoptosis after coculture with attIL12-T cells impaired collagen and fibronectin production in the supernatant, which also depends
Figure 6 Membrane-anchored and tumor-targeted IL-12 (attIL12)-T cell treatment upregulates FAS expression on the cell surface of cancer-associated fibroblasts (CAFs). (A–C) CAFs were cocultured with the indicated T cells (1:3 ratio) in OSD-conditioned medium (OSD CM) with or without the cell-surface vimentin (CSV)-blocking antibody 84–1 (10 µg/mL) for 48 hours (n=3). (A) FAS expression on OS1 and OS2 CAFs as determined by flow cytometry. Graphs show the percentages (mean±SEM) of FAS+ CAFs. (B) Violin plot showing the concentrations (mean±SEM) of interferon gamma (IFNγ) in supernatant as determined via ELISA. (C) Violin plot shows the transforming growth factor beta (TGFβ) level (mean±SEM) in supernatant as determined via ELISA. (D, E, F) CAFs were cocultured with T cells (1:3 ratio) in OSD CM with or without an IFNγ-blocking antibody (10 µg/mL) (n=3). (D) FAS expression on OS1 and OS2 CAFs as determined by flow cytometry. Graphs show the percentages (mean±SEM) of FAS+ CAFs. (E) Suspended T cells were removed from the coculture system after 48 hours. Immunoblots of FAS, cleaved caspase 3, total caspase 3, Bcl-xL, cleaved PARP, α-SMA, and GAPDH in OS1 and OS2 CAFs are shown. (F) Caspase 3/7 expression on OS1 and OS2 CAFs as determined by flow cytometry. Graphs show the percentages (mean±SEM) of cas3/7+ CAFs. (G) Immunofluorescence staining and whole-slide staining of OS1 and OS3 tumor sections stained with fibroblast activation protein (FAP) (red), α-smooth muscle actin (α-SMA) (yellow), Fas (green), and Hoechst (blue). Scale bar: 500 µm. **p<0.01; ***p<0.001; ****p<0.0001.
on the CSV-attIL12 interaction (online supplemental figure S8).

We learned from our previous study that blocking CSV during attIL12-T cell/OSD tumor cell coculture abrogates IFNγ production, and this finding was confirmed by the present study’s ELISA results (figure 6B), and TGFβ made by CAFs may also counteract IFNγ’s function. These findings were confirmed by the present study’s ELISA results (figure 6B and C). Therefore, attIL12-T cell treatment triggered a cytokine network balance shift from TGFβ to IFNγ in the TME from sensitive osteosarcoma tumors. When we neutralized IFNγ in the conditioned medium, FAS upregulation by attIL12-T cells was completely impaired (figure 6D), suggesting a substantial role for IFNγ in FAS induction in CAFs. Thus, our in vitro system revealed how the interaction between CSV and attIL12-T cells changed the cytokine profile dynamics in osteosarcoma tumors to favor T-cell activity.

That this CAF apoptosis was extrinsic and triggered by attIL12-T cell treatment was confirmed by immunoblotting results that showed increased apoptosis signaling (cleaved caspase 3, cleaved PARP, and Bcl-XL) and reduced fibroblast markers (FAS and αSMA) after attIL12-T cell coculture (figure 6E). We further confirmed that this increased CAF apoptosis was dependent on IFNγ upregulation because depletion of IFNγ completely abolished attIL12-T cell-induced CAF apoptosis (figure 6F). Immunofluorescence staining further demonstrated that attIL12-T cells, but not other IL12-T cells induced FAS upregulation that colocalized with CAFs from OS1 tumors so as to break down the physical barrier for T-cell infiltration of tumors, but attIL12-T cells failed to induce FAS on CAFs from OS33 model (figure 6G).

**DISCUSSION**

In this study, we have discovered that a novel therapy disrupts and destroys the CAFs/ECM that form a physical barrier to T-cell infiltration into tumors. Furthermore, our attIL12-T cell treatment, but not treatment with other types of IL12-armed T cells, such as wtIL12-T cells or tIL12-T cells, diminished the function of these CAFs in TME remodeling to dismantle the ECM structure and promote effector T-cell infiltration. We also found that this CAF abrogation was initiated by tumor-specific induction of IFNγ via the interaction between CSV on tumor cells and attIL12-T cells, which in turn induced FAS expression in CAFs to trigger apoptosis. These results revealed unexpected mechanisms behind a simple T-cell therapy for disrupting and destroying the ECM in tumors.

This newly discovered ECM-targeting T-cell therapy, attIL12-T cell therapy, is significant because solid tumors across the board have an ECM that blocks T-cell infiltration and promotes tumor progression and metastasis. Human osteosarcoma tumors are no exception. Our study using osteosarcoma patient tumor tissue arrays confirmed the presence of a high-density ECM. During osteosarcoma pathogenesis, the osteoid and ECM form a scaffold that supports tumor growth. The dysregulation of ECM remodeling further facilitates the metastatic dissemination of osteosarcoma cells. The lack of a competitive antosteosarcoma response in a current CAR-T cell therapy clinical trial may be partially caused by this mechanism. Evidence for this is provided by the clear association of attIL12-T cell-sensitive tumors with ECM destruction and alterations in expression of ECM-associated genes. Resistance to immunotherapy may also be attributable to robust ECM deposits, which create a protective shield around tumor cells to prevent immune-cell infiltration. In light of our finding that T-cell density was inversely correlated with ECM density, immunotherapy strategies targeting both tumor cells and ECM components may hold the key for improving treatment outcomes in osteosarcoma. Indeed, we demonstrated in a previous publication that attIL12-T cells specifically bind to CSV+ osteosarcoma tumor cells to exert cytotoxic activity. Furthermore, here our data indicate that attIL12-T cells also impair the ECM barrier and, more importantly, allow the recruited T cells to penetrate the tumor region.

Given that CAFs are the most common non-neoplastic cells in osteosarcoma, and that they are critical for ECM production and stiffness, we assessed CAF accumulation in osteosarcoma tumors. As expected, attIL12-T cell treatment-resistant tumors had much greater CAF density than did sensitive tumors. Surprisingly, though, CAFs were enriched surrounding sensitive tumors and markedly diminished by attIL12-T cell treatment, in contrast to the high CAF accumulation in stroma throughout the entirety of resistant tumors. On one hand, these CAFs secrete excessive TGFβ, which upregulates PD-1/CTLA-4 on regulatory T cells to suppress effector immune-cell function; on the other hand, they produce ECM components and remodel the ECM to build a barrier that excludes T cells. Under this indirect immune suppression, T cells often accumulate in the tumor edges, where they are blocked by the stiff matrix generated by CAFs and fail to infiltrate into tumors where they can eliminate tumor cells. These findings can explain the results from a recent cohort of patients with high-grade osteosarcoma. In that study, the patients were divided into three clusters based on increasing levels of immune infiltration. The patients in the high-immune-infiltration group saw no advantage in survival despite having significantly higher numbers of immune cells and levels of immunocytolytic activity in their tumors than the other two groups. In addition to the low levels of neoantigen and TCR clonality mentioned in that publication, it is very likely that immune cells’ access to these tumors was restricted by the ECM-rich tumor stroma. Our results suggested that attIL12-T cells break down the CAFs and the rigid matrix structure and in turn open the gate for T-cell infiltration into the tumor core. In this regard, this treatment may resensitize osteosarcoma tumors with an immune-exclusion phenotype to immunotherapy.
The cytokine network in the TME regulates the dynamics of CAFs, cancer cells, and immune cells. In general, cancer cells release soluble factors such as TGFβ that are involved in CAF activation and proliferation. The activated CAFs, in turn, secrete more TGFβ, enhancing the paracrine signaling that promotes tumor progression and immune resistance. In PDX models, we detected low levels of IFNγ and high levels of TGFβ, a favorable environment for activation of CAFs, especially myofibroblast-subtype CAFs. We reported previously that attIL12-T cells dramatically elevate IFNγ levels in response to their interaction with CSV+ tumor cells, but how these changes in the cytokine network affect the cellular components of the TME was not investigated at that time. In this study, we used an in vitro system in which the TME was simplified as conditioned medium from tumor cell—T cell coculture, CAFs, and T cells. Using this system, we demonstrated that CAFs upregulated FAS expression in a CSV-dependent and IFNγ-dependent manner. Several studies have reported IFNγ-induced FAS upregulation on tumor cells and fibroblasts, but to our knowledge, we are the first to show that increased IFNγ triggered FAS induction in CAFs to induce apoptosis of CAFs. Furthermore, CAF inhibition impaired collagen gel contraction, suggesting reduced stiffness as a result of downregulated integrin, fibronectin, and other ECM factors. Most importantly, CAF apoptosis abrogated TGFβ production, the central promoter of myofibroblast-subtype CAF differentiation and immune suppression. Taken together, attIL12-T cells shifted the cytokine network in the osteosarcoma TME, resulting in the tumor stroma’s switch from a TGFβ-dependent CAF-promoting phenotype to an IFNγ-induced immune-inflamed phenotype.

Since the failure of CAR-T cell therapy against solid tumors is partially attributable to CAF-induced tumor barriers, researchers have made efforts to eliminate CAFs by developing anti-FAP-CAR-T cells. Early preclinical study of FAP-CAR-T cells in mice yielded limited tumor inhibition but also serious cachexia and lethal bone toxicities due to the high FAP expression on multipotent bone marrow stromal cells. A different FAP-CAR-T cell generated by using the single-chain variable fragment (clone 73.3) exhibited impressive tumor control and minimal adverse effects. The anti-solid tumor efficacy of these FAP-CAR-T cells was further enhanced by combination with a vaccine or tumor-targeted T-cell therapy. Encouraged by these promising results, in-human trials applied FAP-CAR-T cells and a humanized FAP monoclonal antibody (sibrotuzumab) to treat pleural effusion mesothelioma (NCT01722149) and metastatic colorectal cancer, respectively, and showed good tolerability. However, the antitumor activity was not satisfactory, suggesting that anti-FAP treatment may be more effective in combination with another antitumor approach. In a recent study by Sakemura et al., CAR-T cells with dual targets of both malignant plasma cells and CAFs showed good antitumor efficacy against multiple myeloma, but there were some concerns about life-threatening cytokine release syndrome and neurotoxicity from these highly activated multitargeted CAR-T cells. Unlike other engineered T cells, our attIL12-T cells avoid the need for use of inhibitors, antibodies, or CARs that can cause unspecific targeting, severe toxicities, or crosstalk between signaling pathways. The attIL12-T cell therapy has also proven safe for bone marrow cells from cancer patients and eliminated the toxicities associated with T-cell or IL-12 treatment, such as cytokine release syndrome, by reducing IL-6 in peripheral tissues and tumor-specific induction of IFNγ and TNFα.

By targeting CSV+ tumor cells, attIL12-T cells also interfere with the cancer cell/CAF cytokine network feedback loop, thereby impairing TGFβ-mediated ECM deposition and leading to enhanced osteosarcoma tumor accessibility. Thus, attIL12-T cell transfer is a novel anti-ECM strategy that can be combined with tumor-targeting immunotherapy to render ECM-rich tumors such as osteosarcoma more susceptible to T cell-mediated tumor killing.

**MATERIALS AND METHODS**

**Study design**

The object of this study was to understand how attIL12-T cell transfer regulates CAFs and ECM structure in osteosarcoma. We took advantage of osteosarcoma PDX models developed by Dr. Gorlick’s lab. We compared tumor growth inhibition after control-T or attIL2-T cell treatment and identified three sensitive and three resistant models. To decipher the underlying mechanism of how osteosarcoma PDX tumors respond to attIL12-T cell transfer, we compared gene expression in sensitive and resistant PDX tumors via RNA-seq. Immunoblotting and immunofluorescence staining of the same tumor samples were performed to validate the RNA-seq results. Immunohistochemistry staining of human patient tissues was used to clinically validate the significance of our findings. To further investigate the molecular mechanisms, we used an in vitro coculture system of tumor cells, CAFs, and T cells to mimic the complex interactions in tumors and conducted flow cytometry, immunoblotting, immunocytochemistry, and ELISA analyses. All in vitro tissue samples were collected 4 days after the second T-cell infusions. All in vitro samples were collected 48 hours after coculture. Independent experiment numbers and replicates are included in the Materials and methods section or figure legends. Sample size was calculated for p=0.8 and α<0.05. Tumor-bearing mice for different treatment groups were selected randomly. Tumor transplantation and treatments were not done blindly. The directly measured outcomes were analyzed using a two-sided Student’s t-test to compare two treatment groups or one-way analysis of variance to compare more than two treatment groups. The statistical analyses were conducted using GraphPad Prism 8 software.
Animal studies and tumor models

Six-week-old to eight-week-old C.B-17SC scid−/− mice of both sexes were purchased from The Jackson Laboratory. To generate PDX tumors in mice, patient-derived OS1, OS2, OS31, OS9, OS33, and OS34 OS tumor cells (generously provided by Dr. Richard Gorlick, the Pediatric Preclinical Testing Consortium, The University of Texas MD Anderson Cancer Center) were implanted subcutaneously into C.B-17SC scid−/− mice. Mice were then preconditioned with cyclophosphamide (Baxter Healthcare) when tumors reached 6–8 mm in diameter, followed by two or three T-cell infusions (2.5×10⁶) 14 days apart. Tumors were measured with calipers twice a week after implantation. Tumor volume was calculated by the formula \( V = \left(\pi / 6\right) \times (a b^2) \), where \( V \) = tumor vol in cubic centimeters, \( a \) = maximum tumor diameter, and \( b \) = diameter at 90° to \( a \).

Cell culture

CCH.OS.D (OSD) human osteosarcoma cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) supplemented with antibiotics and non-essential amino acid solution, and maintained in an incubator at 5% CO₂ and 37°C. The tumor cell line was characterized by DNA fingerprinting at MD Anderson Cancer Center’s Characterized Cell Line Core Facility within 6 months of initiating the experiments and treated with a mycoplasma removal agent from Bio-Rad.

Human T cells

Buffy coats from deidentified healthy blood donors were purchased from the Gulf Coast Regional Blood Center. Peripheral blood mononuclear cells (PBMCs) were isolated from Buffy coat samples or bone marrow samples via centrifugation over Ficoll-Paque. Human T cells were enriched from PBMCs using an EasySep Human CD8C T-cell Isolation Kit (STEMCELL Technologies). Human T cells were cultured in 45% RPMI-1640 and 45% Click’s medium containing 10% FBS and supplemented with recombinant human (rh) IL-2 (50 U/mL), rhIL-7 (10 ng/mL), and rhIL-15 (5 ng/mL).

Plasmid constructs

Human IL-12 subunit P35 with and without a transmembrane domain and subunit P40 with and without a tumor-targeted peptide were synthesized by Genscript and cloned into a third-generation self-inactivating lentiviral expression vector (Takara Bio) under a murine stem cell virus and modified cytomegalovirus promoter.

Generation of lentivirus

High-titer replication-defective lentiviral vectors were produced and concentrated by the MD Anderson Functional Genomics Core Facility. Briefly, HEK293T human embryonic kidney cells were transfected with pSVS-G (a VSV glycoprotein expression plasmid), pCMV-Gag/Pol/Rev, and a transfer plasmid using Lipofectamine 2000 (Thermo Fisher Scientific). The viral supernatant was harvested 48 hours after transfection. Viral particles were concentrated using a Lenti-X Concentrator (Takara Bio).

Human T-cell lentiviral transduction

Human T cells were stimulated by CD3/CD28 Dynabeads (Thermo Fisher Scientific) according to the manufacturer’s instructions. On day 2, T cells were plated in non-tissue culture-coated 24-well plates, and polybrene (8 μg/mL) was added to the medium. The lentiviral supernatant was first centrifuged at 1500g for 2 hours on retronectin (Takara)-coated non-tissue culture-treated plates. T cells were then plated and centrifuged at 1000g for 20 min and incubated at 37°C. After 3 days, the medium was changed to 45% RPMI-1640 and 45% Click’s medium containing 10% FBS and supplemented with rhIL-2 (50 U/mL), rhIL-7 (10 ng/mL), and rhIL-15 (5 ng/mL).

Total RNA sequencing

The osteosarcoma PDX tumor samples were processed and analyzed by LC Sciences. Briefly, total RNA was extracted using Trizol reagent (Thermo Fisher) following the manufacturer’s procedure. The total RNA quantity and purity were analyzed using a Bioanalyzer 2100 system and RNA 6000 Nano LabChip, and high-quality RNA samples with RNA integrity number >7.0 were used to construct the sequencing library. After total RNA was extracted, mRNA was purified from 5 μg total RNA using Dynabeads Oligo (dT) (Thermo Fisher) with two rounds of purification. The 2×150bp paired-end sequencing (PE150) was performed on an Illumina Novaseq 6000 system following the vendor’s recommended protocol. All samples were aligned to the human reference genome using the HISAT2 (https://dewhankimlab.github.io/hisat2/, version:hisat2-2.2.0.4) package. Analysis of DEGs was performed by DESeq2 software for comparisons of two groups and by edgeR for comparisons of two samples. Genes with a false discovery rate below 0.05 and absolute fold change ≥2 were considered DEGs.

DEGs were then subjected to analysis of enrichment of GO functions and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. For the GO enrichment analysis, all DEGs were mapped to GO terms in the GO database (http://www.geneontology.org/), gene numbers were calculated for every term, and significantly enriched GO terms in DEGs compared with the reference genome were defined by hypergeometric test. KEGG pathway enrichment analysis identified significantly enriched metabolic pathways or signal transduction pathways in DEGs compared with the whole genome background (https://www.kegg.jp/kegg/).

cBioPortal for cancer genomics

CCN4, TGFB1, FAP, and FNI gene expression data were obtained from The Cancer Genome Atlas portal (https://www.cbioportal.org/). To identify relationships between the expression levels of these genes, Pearson correlation coefficients were calculated using the R statistical computing package.
**Immunoblotting**

Frozen tissue samples were smashed before being homogenized using a minibead beater with five to eight silicone beads (BioSpec Products) in 0.4 mL of ice-cold radioimmunoprecipitation assay lysis buffer. The homogenized tumor cells were then subjected to lysis with this buffer. The protein extracts were separated from the tissue residues by centrifugation at the maximum speed for 20 min at 4°C. Forty microgram samples of total protein were fractionated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes using a Trans-Blot Turbo transfer system (Bio-Rad). The membranes were blotted with different primary and secondary antibodies (see online supplemental file 1) to detect the proteins of interest.

**Collagen gel contraction assay**

As described by Liu et al., 5 mg/mL of collagen I (Thermo Fisher Scientific), DMEM, FBS, and 1 N NaOH solution were mixed on ice at a volume ratio of 1:1.65:0.3:0.05 to make a 1 mg/mL collagen solution. CAFs were pretreated with control-T or attIL12-T cells at a ratio of 1:3 for 24 hours. Next, 50,000 CAFs were resuspended in 500 μL of 1 mg/mL collagen solution and transferred into one well of a 24-well tissue culture plate. After a 30-min incubation, 1 mL of fresh medium was added on top of the gel for a 72-hours incubation. Afterward, the gels were detached from the wall of each well and allowed to contract. The size of the collagen gels was measured using Image J software.

**CAF isolation**

CAFs were isolated as previously described. In brief, tumors were harvested, and necrotic tissues were removed. Tumor tissues were minced into 0.2 cm³ pieces and digested in 5 mL trypsin solution at room temperature for 5 min, and then digestion was stopped by addition of 10 mL FBS. The suspended tissues were then washed with phosphate-buffered saline (PBS) and resuspended in 10 mg collagenase intravenous in 20 mL DMEM at 37°C for 1 hour with rotation. Tissues were then gently pipetted, washed with PBS, and resuspended in 10 mL complete DMEM. The mixture containing dissociated tumor cells was plated into 25 cm² cell culture flasks and cultured in an incubator for 2 days before passage.

**Immunohistochemistry and immunofluorescence staining**

Frozen tumor sections were sequentially fixed with cold acetone, acetone plus chloroform (1:1), and acetone. Paraffin-embedded sections were deparaffinized and heated in antigen retrieval buffer. Tissue sections were blocked with 3% H₂O₂ in distilled water for 20 min and then in blocking buffer (5% normal horse serum and 1% normal goat serum in PBS). Slides were incubated with primary antibodies (see online supplemental file 1) overnight at 4°C and secondary antibodies (see online supplemental file 1) for 1 hour at room temperature. For immunohistochemistry staining, the secondary antibody was biotin conjugated, the sections were treated with ABC reagent (Vector Labs), and the nuclei were counterstained with hematoxylin (Sigma-Aldrich). Tumor sections were mounted with Cytoseal mounting medium (Life Technologies). Quantifications of immunohistochemistry images were assessed by examining three randomly selected low-power fields per slide. For immunofluorescence staining, tumor sections were mounted in an antifade fluorescence mounting medium with 4',6-diamidino-2-phenylindole. Slides were visualized under a Nikon Eclipse Ti fluorescence microscope.

**ELISA**

Culture medium was collected at 1 mL medium/10⁶ T cells. The levels of IFNγ, TGFβ, collagen, and fibronectin were measured by using ELISA Ready-SET-Go! kits (eBioscience) or ELISA Kit Picokine (Boster Bio).

**Flow cytometry**

Cells were sequentially incubated with primary and secondary antibodies (see online supplemental file 1) for 30 min each at 4°C. Stained cells were analyzed using an Attune acoustic focusing cytometer (Applied Biosystems) or a BD LSR-Fortessa cell analyzer (BD Biosciences). Flow cytometry data were analyzed using the FlowJo software program (FlowJo).

**Tumor-ell dissociation**

Tumors were minced into 2 mm fragments, placed in 5 mL of collagenase buffer (RPMI-1640 medium with 100 U/mL collagenase type IV and 100 U/mL DNase I), and incubated at 37°C while shaking at 120 rpm for 30 min to 1 hour. The released cells were filtered with 70 μm strainers and centrifuged at 600 rpm for 5 min, followed by red blood cell lysis. Cells were then resuspended in fluorescence-activated cell sorting solution containing 2% FBS. Single-tumor-cell suspensions were obtained after CD45 deletion using an EasySep Human CD45 Depletion Kit (Stem Cell Technologies).

**Statistical analysis**

The directly measured outcomes were analyzed using a two-sided Student’s t-test to compare two treatment groups or one-way analysis of variance to compare more than two treatment groups. The statistical analyses were conducted using GraphPad Prism 8 software. All data values represent replicates and are shown as means±SEM. Significance was defined as *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. All experiments were repeated at least three times.

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Contributors JH: conceptualization, data curation, formal analysis, investigation, methodology, project administration, validation, visualization, writing original draft and editing. AJL, DI, W-LW, DR, KM: resources. WZ, RG: methodology, resources. ZJ: methodology. JW: formal analysis. XX: resources. SL: Guarantor, conceptualization, supervision, funding acquisition, writing-review and editing.

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Competing interests  No, there are no competing interests.

Patient consent for publication  Not applicable.

Ethics approval  The mouse care and handling procedures were approved by the Institutional Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center (IACUC 00001046-RN03). The use of human buffy coats was approved by the MD Anderson Institutional Review Board (IRB PA12-0604).

Provenance and peer review  Not commissioned; externally peer reviewed.

Data availability statement  All data needed to evaluate the conclusions in the paper are present in the paper and/or online supplemental materials. The TCGA datasets are publicly accessible at https://www.cbioportal.org/public-portal/. Additional data related to this paper may be requested with reasonable considerations.

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ORCID iDs

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REFERENCES


## Antibody list

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Fig S1. High density of ECM in OS patient samples. Trichrome staining of OS patient metastatic tumor samples.
Fig S2
Fig. S2. Immunohistochemistry staining of ECM markers on sensitive and resistant tumor models after control-T or attIL12-T cell treatment. Osteosarcoma PDX-bearing mice were subjected to control-T or attIL12-T cell treatment. Four days after the second T-cell infusion, tumors were collected and sectioned. (A-E) Immunohistochemistry staining of (A) sparc, (B) integrin β1, (C) fibronectin, (D) laminin, and (E) collagen. Scale bar: 500 µm. The bar graphs show the density (mean ± SEM) of ECM marker staining. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.
**Fig. S3.** Isolation of CAFs from OS tumors. (A) Schema of CAF isolation and images showing cell shape. (B) Flow cytometry confirmation of FAP expression on isolated CAFs.
Fig. S4. Schema of tumor cell-CAF in vitro coculture experiment. CM, conditioned medium.
Fig. S5. CAF apoptosis is induced specifically by attIL12-T cells. CAFs from OS1, OS2, and OS33 tumors were cocultured with control-T, attIL12-T, aIL12-T, wtIL12-T, or ttIL12-T cells at a 1:3 ratio in CM for 48 h. Cells were stained with ghost dye 510, anti-human CD3, FAP, and cas3/7 for flow cytometry analysis. The bar graphs show the percentages (mean ± SEM) of cas3^+\text{FAP}^+ cells. ****P < 0.0001.
**Fig. S6.** CSV expression on CAFs. CAFs from OS1, OS2, or OS33 tumors were stained with anti-CVS antibody (84-1) and AF405 anti-mouse IgG to detect the percentages of CAFs that express CVS.
**Fig S7.**

**Fig. S7. attIL12-T cells do not induce FAS expression on bone marrow cells.** Patient-derived BM cells were cocultured with control-T or attIL12-T cells at a 1:3 ratio for 48 h. Flow cytometry was used to determine FAS expression on CD33⁺ BM cells.
Fig. S8. Downregulation of collagen and fibronectin is dependent on CSV-attIL12-T cell coculture. OSD cells, CAFs, and T cells were cocultured at a 1:1:3 ratio for 48 h. Collagen I and fibronectin levels in supernatant were determined via ELISA. Violin plots represent the concentrations (mean± SEM) of collagen and fibronectin. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.